The SH2 domain containing inositol 5-phosphatase SHIP2 displays phosphatidylinositol 3,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate 5-phosphatase activity

Xavier Pesesse[a,*], Colette Moreau[a], A. Lyndsay Drayer[a], Rüdiger Woscholski[b,c], Peter Parker[a], Christophe Erneux[a]

[a] *Interdisciplinary Research Institute (IRIBHN), Université Libre de Bruxelles, Campus Erasme, Bldg. C, 808 Route de Lennik, 1070 Brussels, Belgium*
[b] *Protein Phosphorylation Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK*
[c] *Imperial College of Science, Technology and Medicine, Institute of Biochemistry, London SW7 2AZ, UK*

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Abstract Distinct forms of inositol and phosphatidylinositol polyphosphate 5-phosphatases selectively remove the phosphate from the 5-position of the inositol ring from both soluble and lipid substrates. SHIP1 is the 145-kDa SH2 domain-containing inositol 5-phosphatase expressed in haematopoietic cells. SHIP2 is a related but distinct gene product. We report here that SHIP2 can be expressed in an active form both in *Escherichia coli* and in COS-7 cells. A truncated 103-kDa recombinant protein could be purified from bacteria that display both inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) and phosphatidylinositol 3,4,5-trisphosphate ($PtdIns(3,4,5)P_3$) phosphatase activities. COS-7 cell lysates transfected with SHIP2 had increased $PtdIns(3,4,5)P_3$ phosphatase activity as compared to the vector alone.

Key words: Phosphatidylinositol metabolism; Signal transduction

1. Introduction

The family of inositol and phosphatidylinositol polyphosphate 5-phosphatases has been implicated in signal transduction events initiated by several extracellular signals and is responsible for the degradation of different second messengers such as inositol 1,4,5-trisphosphate ($InsP_3$), inositol 1,3,4,5-tetrakisphosphate ($InsP_4$), phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$) and/or phosphatidylinositol 3,4,5-trisphosphate ($PtdIns(3,4,5)P_3$) [1–3]. In mammalian cells, this family contains distinct genes and splice variants that can be classified into Type I and Type II 5-phosphatases [4]. Type II 5-phosphatases can use both $PtdIns(4,5)P_2$ and $PtdIns(3,4,5)P_3$ as substrates. We have used PCR to identify putative 5-phosphatase sequences by designing degenerate primers coding for conserved amino acid sequence regions within the catalytic domain of known 5-phosphatases. This resulted in the cloning of two SH2 domain containing inositol 5-phosphatases SHIP1 and SHIP2 [5,6]. SHIP1 and SHIP2 contain N-terminal specific SH2 domains, potential PTB domain-binding sites (NPXY) and C-terminal proline-rich regions with consensus sites for SH3 domain interactions [5–9]. When expressed in COS-7 cells, SHIP1 displays 5-phosphatase activity specifically with both $PtdIns(3,4,5)P_3$ and $InsP_4$ as substrates [5,7–9]. SHIP2 has not been characterised at the enzymological level in similar experiments [6]. In this report, SHIP2 5-phosphatase activity has been demonstrated: both $InsP_4$ and $PtdIns(3,4,5)P_3$ 5-phosphatase activities could be measured after expression in *Escherichia coli* and COS-7 cells identifying SHIP2 as a functional member of the 5-phosphatase family.

*Corresponding author. Fax: (32) (2) 555 46 55.
E-mail: xpesesse@ulb.ac.be

Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate; $PtdIns(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; $PtdIns(3,4,5)P_3$, phosphatidylinositol 3,4,5-trisphosphate; $PtdIns(3,4)P_2$, phosphatidylinositol 3,4-bisphosphate; rSHIP2, recombinant SHIP2

2. Materials and methods

2.1. Subcloning of SHIP1 in pcDNA3

The catalytic part of human SHIP1 [5] starting from amino acid 170 to 888 was amplified by PCR using the 5′-primer containing an *Eco*RV restriction site (underlined) 5′-GCTAGATATCGACTG-CAAAGCATGGAC-3′ and the 3′-primer containing an *Xba*I restriction site (underlined) 5′-GTGCTCTAGATCAGTCGTGGCTGGT-GAGGCT-3′. The PCR product was subcloned into pcDNA3 (Invitrogen).

2.2. Expression of His-tagged recombinant SHIP2 (rSHIP2) in bacteria and in COS-7 cells

A construct (rSHIP2) of 874 amino acids was generated by PCR amplification of SHIP2 (i.e. clone 5 in [6]) using the 5′-primer containing a *Bam*HI restriction site (underlined) 5′-GTGCGGATC-CATGGCCCCCTCCTGGTA-3′ and the 3′-primer containing an *Xho*I restriction site (underlined) 5′-GCGTCTCGAGTCAGG-CAGGGTTATTGAAGC-3′. The PCR product was subcloned into pTrc-His A and pcDNA3-His C (Invitrogen). The resulting pTrc-His-construct was used to transform DH5αF′ bacteria. An overnight 5-ml culture was used to inoculate 4 l of Luria-Bertani medium with 50 μg/ml ampicillin. After an overnight culture at 37°C, it was diluted to an A_{600} of 0.6 and 0.5 M of isopropyl-β-galactopyranoside was added for another 6 h at 30°C. The cells were harvested by centrifugation at 5000×*g* for 15 min, resuspended in 200 ml of buffer A, pH 8 (18.6 mM Na_2HPO_4, 1.36 mM NaH_2PO_4, 300 mM NaCl, 12 mM β-mercaptoethanol, 50 μg/ml Pefabloc, 5 μM leupeptin) and lysed using lysozyme (100 μg/ml) for 15 min at 4°C. The crude lysate was applied onto 10 ml of ProBond Ni^{2+}-agarose column equilibrated in the same buffer. The column was washed with buffer B at pH 6 (18.6 mM Na_2HPO_4, 1.36 mM NaH_2PO_4, 300 mM NaCl, 12 mM β-mercaptoethanol, 50 μg/ml Pefabloc, 5 μM leupeptin and 10% glycerol). The protein was eluted with a linear gradient starting from 0 to 200 mM imidazole in buffer B (total volume: 240 ml).

SHIP1, SHIP2 and Type I 5-phosphatase constructs were transfected into COS-7 cells as reported before [10]. $InsP_3$ and $InsP_4$ 5-phosphatase activities were determined as previously reported with $[^{32}P]InsP_4$ as substrate to assess the specificity of dephosphorylation [11,12]. $PtdIns(3,4,5)P_3$ 5-phosphatase activity was measured as described [13]. Briefly, a total of 15000 cpm/sample of $[3\text{-}^{32}P]PtdIns(3,4,5)P_3$ (approximately 60 μM) was resuspended in 100 mM Tris-HCl, pH 7.5, and 1% cholate. The reaction was started

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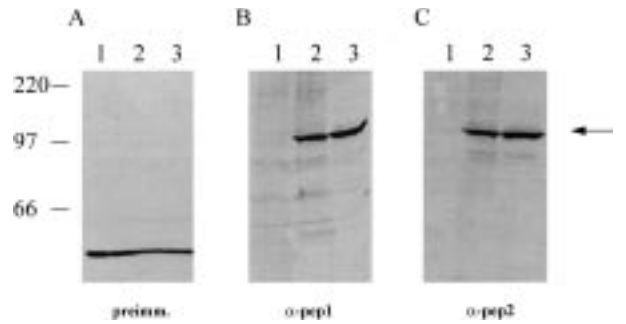

Fig. 1. Western blot analysis of rSHIP2 expressed in bacteria. Extracts of bacteria transformed with the vector pTrc-His (5 μg in lane 1 of each blot), SHIP2 in crude extracts (5 μg in lane 2 of each blot) or after one purification step (300 mM imidazole elution, 0.6 μg in lane 3) were analysed by Western blotting. Immunodetections were performed with preimmune serum diluted 250-fold (A), anti-peptide 1 serum diluted 250-fold (B), anti-peptide 2 diluted 250-fold (C). Immunodetection was performed using the chemoluminescence ECL method.

by adding 10 μl of enzyme, 5 mM $MgCl_2$, 0.5 mM EGTA and 0.5% cholate for 5 min. PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 were separated by thin layer chromatography using 1-propanol/2 M acetic acid (1:1). The corresponding spots were analysed by PhosphoImager and autoradiography. To follow the expression of rSHIP2 on Western blots, two polyclonal rabbit antisera were raised against synthetic peptides (peptide 1 and 2) of the SHIP2 sequence APSWYHRDLSRAAAEEL derived from amino acid 18–35 and SEEEISFPPTYRYERGSRDT from 651 to 670.

3. Results and discussion

3.1. Expression of recombinant rSHIP2 in Escherichia coli

A construct containing the SH2 domain and the putative catalytic part of SHIP2 (predicted molecular mass of 103 kDa) was subcloned in the bacterial expression vector pTrc-His. Crude extracts from bacteria transformed by the SHIP2 construct or by the vector alone have been prepared. The molecular mass of rSHIP2 was estimated to be approx. 103 kDa by Western blotting (Fig. 1). No signal could be seen in lysates of bacteria transformed with the vector alone (Fig. 1, lane 1 of each blot) or by the use of preimmune serum (Fig. 1A). The crude lysates were adsorbed to Ni^{2+}-agarose resin. rSHIP2 was eluted in 300 mM imidazole-containing buffer B (Section 2). The 103-kDa protein was present in these imidazole-containing fractions (Fig. 1B,C, lane 3). A peak of $InsP_4$ 5-phosphatase activity could be measured in the same fractions from rSHIP2 (Fig. 2). rSHIP2 has been purified 11-fold as detected in our $InsP_4$ 5-phosphatase assay. In contrast, it did not show any $InsP_3$ 5-phosphatase activity (Table 1).

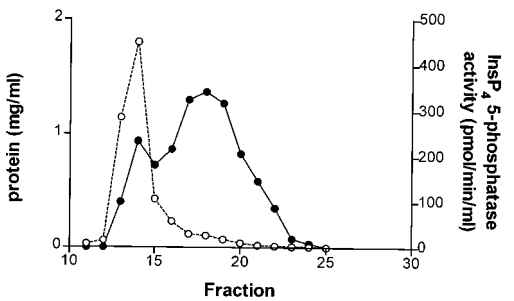

Fig. 2. Chromatography profile of bacterial rSHIP2 on Ni^{2+}-agarose column. An extract of a 4-l culture of rSHIP2 was applied to a Ni^{2+}-agarose column and eluted with 240 ml of a linear gradient (0–200 mM imidazole). 20-ml fractions were collected. Enzyme activity (closed circles) was estimated using $InsP_4$ (1 μM) as substrate and protein content was measured in each fraction (open circles).

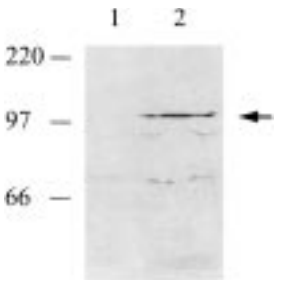

Fig. 3. Western blot analysis of rSHIP2 expressed in COS-7 cells. Crude homogenate of COS-7 cells transfected with the vector pcDNA3-His (lane 1) or SHIP2 (lane 2) were loaded on SDS-PAGE. Immunodetection was performed with anti-peptide 2 serum diluted 250-fold.

3.2. Expression of rSHIP2 in COS-7 cells

The SHIP2 construct containing the SH2 domain and catalytic part has been subcloned in pcDNA3-His. A single immunoreactive band of approx. 103 kDa could be seen in cells transfected with SHIP2 (Fig. 3, lane 2). No signal could be seen in COS-7 cells transfected with the vector alone (Fig. 3, lane 1). Total $InsP_4$ 5-phosphatase activity was slightly in-

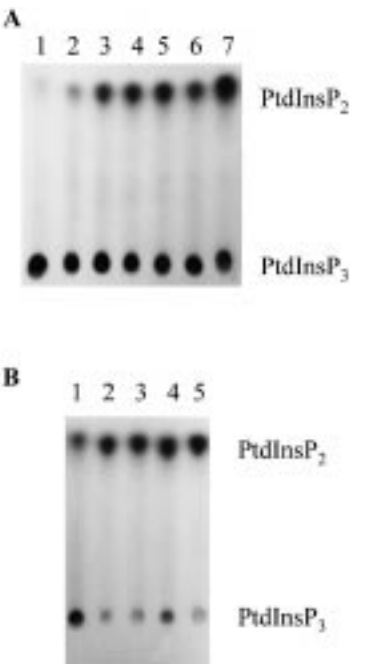

Fig. 4. rSHIP2 shows a PtdIns(3,4,5)P_3 phosphatase activity. A: Recombinant Type I 5-phosphatase activity (lane 2), fractions 17–20 of rSHIP2 expressed in bacteria and purified on Ni^{2+}-agarose column (lanes 3–6), crude extract of rSHIP2 (lane 7) were incubated with 60 μM [^{32}P]PtdIns(3,4,5)P_3 for 6 min at 37°C and the products were separated by TLC. Lane 1 is the negative control where water was added in place of enzyme. B: Homogenates of COS-7 cells transfected with pcDNA3-His vector alone (lane 1), SHIP1 (lanes 2 and 3) or SHIP2 (lanes 4 and 5) were assayed for PtdIns(3,4,5)P_3 phosphatase activity at 60 μM PtdIns(3,4,5)P_3. Spots corresponding to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are indicated.

Table 1
Purification of rSHIP2 expressed in *Escherichia coli*

Step	Volume (ml)	Protein (mg/ml)	$InsP_3$ 5-phosphatase activity (pmol/ml/min)	$InsP_4$ 5-phosphatase activity	
				(pmol/ml/min)	(pmol/mg/min)
Crude extract	200	3.9	N.D.	962	247
Ni^{2+} affinity	10	0.41	N.D.	1079	2632

$InsP_3$ and $InsP_4$ 5-phosphatase activity were assayed at 1 μM substrate level. The data are from one representative experiment out of three. N.D.: non-detectable.

creased in homogenates of SHIP2 transfected cells. Data from a typical transfection experiment were 0.39 ± 0.05 and 0.65 ± 0.07 nmol/min/ml for vector and SHIP2 transfected cells, respectively (as determined at 1 μM $InsP_4$ substrate level).

3.3. rSHIP2 demonstrates PtdIns(3,4,5)P_3 phosphatase activity

Since rSHIP2 expressed in bacteria demonstrates $InsP_4$ 5-phosphatase activity, we determined whether this construct had PtdIns(3,4,5)P_3 phosphatase activity. In these assays, synaptojanin and the Type I enzyme were used as positive and negative controls, respectively [5]. rSHIP2 expressed in bacteria had PtdIns(3,4,5)P_3 phosphatase activity (Fig. 4A). PtdIns(3,4,5)P_3 phosphatase activity was also detected in crude homogenates of SHIP1 and SHIP2 expressed in COS-7 cells (Fig. 4B).

The data presented here show that when expressed in *Escherichia coli* or in COS-7 cells, rSHIP2 displays enzymatic activity particularly with PtdIns(3,4,5)P_3 as substrate. A 103-kDa SHIP2 construct could be expressed in *Escherichia coli*. We have purified mg amounts of this protein that displays both $InsP_4$ and PtdIns(3,4,5)P_3 phosphatase activity. The specific activity of rSHIP2 expressed in bacteria and measured at 1 μM $InsP_4$ is about 4000 times lower as compared to the Type I specific activity. Therefore the catalytic efficiency of both proteins for $InsP_4$ as substrate appears rather different. If expressed at the same levels, $InsP_4$ would be preferentially hydrolysed by Type I 5-phosphatase.

During the preparation of this manuscript, it was reported that growth factors and insulin stimulate tyrosine phosphorylation of 51C/SHIP2 from SH-SY5Y cells [14]. Antibodies raised against 51C/SHIP2 precipitated a protein along with an activity which hydrolysed PtdIns(3,4,5)P_3. $InsP_3$, $InsP_4$ were not tested as potential substrates nor was the recombinant protein directly checked as an active enzyme. The studies here define SHIP2 as having PtdIns(3,4,5)P_3 and Ins(1,3,4,5)P_4 5-phosphatase activities.

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