SH2-containing inositol 5-phosphatases 1 and 2 in blood platelets: their interactions and roles in the control of phosphatidylinositol 3,4,5-trisphosphate levels

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Src homology domain 2-containing inositol 5-phosphatases 1 and 2 (SHIP1 and SHIP2) are capable of dephosphorylating the second messenger $\text{PtdIns}(3,4,5)\text{P}_3$ (phosphatidylinositol 3,4,5-trisphosphate) and interacting with several signalling proteins. SHIP1 is essentially expressed in haematopoietic cells, whereas SHIP2, a closely related enzyme, is ubiquitous. In the present study, we show that SHIP1 and SHIP2 are expressed as functional $\text{PtdIns}(3,4,5)\text{P}_3$ 5-phosphatases in human blood platelets and are capable of interacting when these two lipid phosphatases are co-expressed, either naturally (platelets and A20 B lymphoma cells) or artificially (COS-7 cells). Using COS-7 cells transfected with deletion mutants of SHIP2, we demonstrate that the Src homology

domain 2 of SHIP2 is the minimal and sufficient protein motif responsible for the interaction between the two phosphatases. These results prompted us to investigate the relative importance of SHIP1 and SHIP2 in the control of $\text{PtdIns}(3,4,5)\text{P}_3$ levels in platelets using homozygous or heterozygous SHIP1- or SHIP2-deficient mice. Our results strongly suggest that SHIP1, rather than SHIP2, plays a major role in controlling $\text{PtdIns}(3,4,5)\text{P}_3$ levels in response to thrombin or collagen activation of mouse blood platelets.

Key words: haematopoietic cell, platelet, $\text{PtdIns}(3,4,5)\text{P}_3$, Src homology domain 2 (SH2)-containing inositol 5-phosphatase.

INTRODUCTION

SHIP1 (Src homology domain 2-containing inositol 5-phosphatase) and SHIP2 proteins constitute a subgroup of the inositol polyphosphate 5-phosphatase family, characterized by an SH2 domain (Src homology domain 2) at their N-terminal ends. These two proteins share a high percentage of amino acid identity and contain multiple protein–protein interaction domains, including an SH2 domain, proline-rich sequences and NPXY motifs. Both of them are capable of hydrolysing $\text{PtdIns}(3,4,5)\text{P}_3$ (phosphatidylinositol 3,4,5-trisphosphate) at position 5 of the inositol ring to produce $\text{PtdIns}(3,4)\text{P}_2$ [1]. SHIP1 is also capable of dephosphorylating $\text{Ins}(1,3,4,5)\text{P}_4$, whereas SHIP2 is not [2,3]. In addition, it has been shown that SHIP1 and SHIP2 can participate in signal-transduction mechanisms as docking proteins [4]. Their common ability to hydrolyse $\text{PtdIns}(3,4,5)\text{P}_3$ place them as important signalling proteins involved in the regulation of phosphoinositide 3-kinase-dependent processes [4]. SHIP1 plays an important role as a negative regulator in the activation of B lymphoid cells [5], myeloid cells [6] and mast cells [7]. Its Smad-dependent expression in haematopoietic cells and its involvement in the apoptotic signalling pathway induced by members of the activin/transforming growth factor β family has been reported previously [8]. Mice bearing a targeted disruption of both SHIP1 alleles have been generated. The absence of SHIP1 results in a myeloproliferative-like syndrome and consolidation of the lungs

by infiltration of macrophages [9]. It is believed that SHIP1 deficiency in blood cells results in hyper-responsiveness to growth factor stimulation [10]. The major role of SHIP1 in the down-regulation of the growth of immune cells has been further demonstrated by the identification of the first dominant-negative mutation of the SHIP1 gene in one example of human acute myeloid leukaemia [11]. Besides its central role in the control of cell proliferation, SHIP1 has also been implicated in other processes such as cell migration [12], adhesion [13] and dispersion [14]. Recently, Wang et al. [15] have demonstrated that SHIP1 can influence the natural killer cells repertoire and contribute, in this way, to the success of allogeneic marrow transplantation. Finally, SHIP1 may play an important role in the negative regulation of osteoclast formation and function [16].

The cDNA of a closely related but distinct enzyme, SHIP2, has been cloned recently [3]. SHIP2 tissue distribution is quite ubiquitous [17]. It has been involved in epidermal growth factor-, insulin-, insulin-like growth factor-1- and nerve growth factor-mediated signal transduction [18]. Its involvement in the control of cell proliferation has been reported in glioblastoma cells where it can decrease cell growth by relaying the defective $\text{PtdIns}(3,4,5)\text{P}_3$ hydrolysis observed in these cells [19]. The SHIP2 anti-proliferative properties were also reported when over-expressed in K562 erythroleukaemia cells [20]. Another study describes the association between SHIP2 and the p130Cas adapter protein, suggesting its participation in the control of cell adhesion

Abbreviations used: $\text{PtdIns}(3,4,5)\text{P}_3$, phosphatidylinositol 3,4,5-trisphosphate; SH2 domain, Src homology domain 2; SHIP, SH2-containing inositol 5-phosphatase.

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and spreading [21]. The association of SHIP2 with the actin-binding protein filamin might play a role in the control of actin reorganization [22]. Homozygous disruption of SHIP2 causes severe hypoglycemia and death within a few hours after birth. Heterozygous disruption of this gene leads to hypersensitivity to insulin as demonstrated by the increased glycogen synthesis in skeletal muscles in response to insulin. Injection of D-glucose resulted in a more rapid glucose clearance in SHIP2^{+/-} than in wild-type mice [23].

SHIP1 is expressed in human blood platelets and becomes strongly tyrosine-phosphorylated after thrombin stimulation in an aggregation- and integrin-engagement-dependent manner [24,25]. Moreover, the striking correlation observed between the tyrosine phosphorylation of SHIP1, its relocation to the actin cytoskeleton and the production of PtdIns(3,4)P₂ after thrombin stimulation suggest a role for SHIP1 in the aggregation-dependent accumulation of this phosphoinositide, which may be involved in the irreversibility of platelet aggregation [26]. Altogether, these results suggest an essential role of SHIP1 in the regulation of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ levels after platelet activation. In agreement with these results, Pasquet et al. [27] have shown an accumulation of PtdIns(3,4,5)P₃, a decrease in PtdIns(3,4)P₂ production and an increase in Ca²⁺ entry in SHIP1-deficient mouse blood platelets stimulated by the collagen-related peptide [27]. This entry of Ca²⁺ seemed to be regulated by PtdIns(3,4,5)P₃ and the Bruton tyrosine kinase, but the molecular basis of this mechanism is still unknown.

SHIP1 and SHIP2 have been found to be co-expressed in certain haematopoietic cells including B and T lymphocytes and some p210 Bcr-Abl transformed cell lines [17,28,29]. They are capable of interacting with the same immunoreceptor tyrosine-based inhibition motif on the intracellular part of the Fcγ RIIB receptor in B-cells [30,31]. The co-expression of SHIP1 and SHIP2 in certain cells raises the possible question of co-regulation and redundancy of these two important lipid phosphatases. In this respect, it is noteworthy that lipopolysaccharide-activated B-cells obtained from SHIP1-deficient mice are still significantly inhibited by signals mediated through Fcγ RIIB. This effect was attributed to the expression of SHIP2 [32]. SHIP2 expression increases in SHIP1^{-/-} mast cells, suggesting that in the absence of SHIP1, SHIP2 could provide a compensatory PtdIns(3,4,5)P₃ 5-phosphatase activity in this model [33].

In the present study, we have investigated the relative importance of SHIP1 and SHIP2 in the control of PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ levels in platelets. Although both phosphatases are expressed and can interact in platelets, SHIP1 seems to be the major enzyme involved in the regulation of the levels of phosphoinositide 3-kinase products during activation.

MATERIALS AND METHODS

Materials

The cDNAs encoding human SHIP1 and SHIP2 have been reported previously [2,3]. Antipeptide antiserum to human SHIP1 and SHIP2 were raised in rabbits against the two synthetic peptides HGKHRPEEGPPGGLRTAMQ and DPAHKRLLL-DTLQLSK. These peptides were taken in the C-terminal end of human SHIP1 [2] and SHIP2 [3] respectively. Antibodies were characterized previously [2,17]. For Western-blot analysis, the serum was used at a dilution of 1:1000 and 1:100 for immunoprecipitation analysis. The mouse anti-phosphotyrosine 4G10 antibody was purchased from Euromedex (Strasbourg, France) and the mouse monoclonal anti-His antibody from Clontech (Erembodegem, Belgium). The enhanced chemiluminescence

(ECL[®]) Western-blotting reagents were obtained from Amersham Biosciences (Piscataway, NJ, U.S.A.). All other reagents were purchased from Sigma unless otherwise indicated.

Animals

Mutant mice deficient in SHIP1 or SHIP2 were generated as described previously [6,23] and both wild-type and mutant mice were of C57BL/6 genetic background. For all experiments, 6–8-week-old mice were used. Since SHIP2^{-/-} mice die shortly after birth, we could not isolate and study their platelets. Animals were housed in the IFR-Toulouse Purpan *vivarium* according to approved methods.

Preparation and activation of human and mouse blood platelets

Human blood platelet concentrates were obtained from the local blood bank (Centre Régional de Transfusion Sanguine, Toulouse, France) and prepared as described previously [34]. For all experiments, human blood platelets were used at 2×10^9 cells/ml and stimulated for different times with 1 i.u./ml thrombin at 37 °C with gentle shaking (150 strokes/min), unless otherwise indicated. Mouse blood was taken by intracardiac puncture of anesthetized mice (Imalgen/Rompun mix). Blood samples were recovered in plastic syringes containing 100 μl of heparin at 1000 i.u./ml. Pooled blood samples (5–15 ml) were diluted in 1 vol. of modified Tyrode's-Hepes buffer (134 mM NaCl/2.9 mM KCl/12 mM NaHCO₃/0.34 mM Na₂HPO₄/1 mM MgCl₂/1 mM EGTA/5 mM glucose/20 mM Hepes, pH 7.3) and centrifuged at 300 g for 8 min at 22 °C. Platelet-rich plasma was removed and centrifuged at 1570 g for 5 min at 22 °C. The platelet pellet was washed once in Tyrode's-Hepes buffer and finally resuspended at a density of 7.5×10^8 platelets/ml in the same buffer without EGTA. Before centrifugations and resuspensions, 500 nM prostaglandin I₂ was added to the platelets. They were then kept at 37 °C for 30 min before stimulating with 0.5 i.u./ml thrombin.

PtdIns(3,4,5)P₃ and Ins(1,3,4,5)P₄ phosphatase activities

As described previously [24], TLC-purified [³²P]PtdIns(3,4,5)P₃ and [³H]Ins(1,3,4,5)P₄ (DuPont-NEN-PerkinElmer, Zaventem, Belgium) were used as substrates to measure respectively phosphoinositide and inositol phosphatase activities of immunoprecipitated SHIP2.

Preparation of the cytoskeleton and Triton X-100-soluble fraction

The reactions were stopped by the addition of 1 vol. of ice-cold $2 \times$ CSK buffer [100 mM Tris/HCl (pH 7.4), 20 mM EGTA, 2 mM Na₃VO₄, 4 μg/ml each of aprotinin and leupeptin, 2 mM PMSF and 2 % (v/v) Triton X-100]. After 10 min at 4 °C, the cytoskeleton was recovered by centrifugation (at 12 000 g for 10 min at 4 °C) and the supernatant corresponded to the Triton X-100-soluble fraction. The cytoskeleton was washed once in $1 \times$ CSK buffer with 0.5 % (v/v) Triton X-100 and once with the same buffer but without Triton X-100. The cytoskeleton was immediately resuspended in the electrophoresis sample buffer.

Mouse blood platelet labelling and phosphoinositide assays

Blood samples were prepared as described previously except that platelets were resuspended in phosphate-free Tyrode's-Hepes buffer. Platelets at 7.5×10^8 /ml were labelled with 0.6 mCi/ml

[32 P] P_i for 50 min at 37 °C. They were then centrifuged at 1570 g for 8 min, resuspended in Tyrode's-Hepes buffer and left for 15 min at 37 °C before stimulation with 0.5 i.u./ml thrombin. Reactions were stopped by the addition of chloroform/methanol in the ratio 1:1, containing 1 M HCl. Lipids were immediately extracted by the modified procedure of Bligh and Dyer [35,36], and PtdIns(3,4,5) P_3 , PtdIns(4,5) P_2 and PtdIns(3,4) P_2 were resolved by TLC using chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7, by vol.). Spots corresponding to these lipids were then scraped off, deacylated and analysed by HPLC on a Whatman Partisphere 5 SAX column (Whatman International, Maidstone, U.K.).

Generation of truncated mutants

A construct corresponding to the SH2 domain of SHIP2 was obtained by PCR using tSHIP2 [37] as a template and a 5'-primer containing a *Bam*HI restriction site (underlined) 5'-GTGC-GGATCCATGGCCCCCTCTGGTA-3' and the 3'-primer containing an *Xho*I restriction site (underlined) 5'-CCGCTCGAGTCACTCTACAGGAAGAAGC-3'. The PCR product of 306 bp (residues 18–118) was subcloned into pcDNA3-His C vector and sequenced. A construct deprived of the SH2 domain was obtained by PCR using the SHIP2 full-length cDNA as a template and a 5'-primer containing an *Eco*RI restriction site (underlined) 5'-CGGAATTCATGTCAGATGGGGAGGATGAG-3' and a 3'-primer containing the *Xho*I restriction site (underlined) 5'-CCGCTCGAGTCACTTGCTGAGCTGC-3'. The PCR product of 3384 bp (residues 132–1258) was subcloned into pcDNA3-His C vector and sequenced.

Transfection in COS-7 cells and preparation of cell extracts

COS-7 cells were grown and transfected as described previously [37,38]. Cell extracts were prepared with the same lysis buffer as described below.

Immunoprecipitations

Reactions were stopped by the addition of ice-cold lysis buffer. Composition and final concentrations of the lysis buffer were 50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1 % (v/v) Brij (polyoxyethylene 9 lauryl ether), 2 mM Na_3VO_4 , 1 mM PMSF and 10 μ g/ml each of aprotinin and leupeptin. After gentle shaking for 20 min at 4 °C and centrifugation (at 12 000 g for 10 min at 4 °C), the soluble fraction was collected and pre-cleared for 30 min at 4 °C with Protein A–Sepharose CL4B. The pre-cleared suspensions were then incubated for 1 h at 4 °C with adequate antibodies and the immune complexes were precipitated by the addition of 50 μ l of 10 % (w/v) Protein A–Sepharose CL4B for 1 h. After centrifugation (at 6000 g for 5 min at 4 °C), the immunoprecipitates were washed four times in a buffer containing 50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 2 mM Na_3VO_4 , 0.1 % Brij and 5 μ g/ml each of aprotinin and leupeptin. Western-blot analysis was performed as reported previously.

B-cell activation, lysis and immunoprecipitation

A20 B lymphoma cell line activation was performed at 37 °C with 60 μ g/ml intact rabbit IgG raised against mouse IgG (bio-Merieux INDUSTRY, Hazelwood, MO, U.S.A.). Resting and stimulated cells (10^8) were lysed and submitted to anti-SHIP1 immunoprecipitation as described previously [17].

Figure 1 Expression of p155 SHIP2 in human blood platelets

(A) Control empty pcDNA 3 transfected COS-7 cells ('COS') or pcDNA 3 human SHIP2 cDNA transfected COS-7 cells (COS + SHIP2) were homogenized and the proteins were separated by SDS/PAGE (7.5 % gel). SHIP2 expression was revealed by immunoblotting with the anti-SHIP2 antibody (α -SHIP2). (B) SHIP2 was immunoprecipitated (IP) from resting platelets with the anti-SHIP2 antibody. Immunoprecipitation with normal rabbit serum was performed as a negative control (C). Immunoprecipitated proteins were separated by SDS/PAGE (7.5 % gel). The blot was probed with the anti-SHIP2 antibody (α -SHIP2). A platelet homogenate (60 μ g of protein; 'H') was also analysed. The migration positions of molecular-mass standards are indicated on the left of each panel. The position of SHIP2 is indicated by arrows. Results are representative of three independent experiments.

RESULTS

Expression and activity of 155 kDa SHIP2 in human blood platelets

SHIP2 antiserum was tested on COS-7 cell lysates, transfected or not with the cDNA encoding human SHIP2. A slight signal at 155 kDa was detected in non-transfected COS-7 cells corresponding to endogenous SHIP2. As expected, this signal was strongly increased after transfection of SHIP2 cDNA (Figure 1A). After transfection of SHIP1, no increase in SHIP2 signal was detected by anti-SHIP2 Western-blot analysis, indicating the specificity of our SHIP2 antibody (results not shown). The same antibody detected a protein of 155 kDa in human blood platelet lysates. This protein was also found in the anti-SHIP2 immunoprecipitate, but not in the preimmune immunoprecipitate (Figure 1B). Moreover, this 155 kDa immunoprecipitated protein was capable of dephosphorylating PtdIns(3,4,5) P_3 into PtdIns(3,4) P_2 in an *in vitro* phosphatase assay. The same material had no Ins(1,3,4,5) P_4 5-phosphatase activity (results not shown). These features are characteristic of SHIP2 activity *in vitro* [29,37]. As observed for SHIP1 [24], PtdIns(3,4,5) P_3 5-phosphatase activity of SHIP2 was not increased after thrombin stimulation (results not shown). Altogether, these results indicate that, in addition to SHIP1 [24,25,27], SHIP2 is also expressed in human blood platelets as a functional PtdIns(3,4,5) P_3 5-phosphatase.

Cytoskeleton association and tyrosine phosphorylation of SHIP2 after thrombin stimulation

To characterize further SHIP2 in human blood platelets, we investigated its subcellular localization in resting and in thrombin-activated platelets. As shown in Table 1, in resting platelets, SHIP2 was mainly present in the Triton X-100-soluble fraction and approx. 20 % of the enzyme content co-sedimented with the actin cytoskeleton. After thrombin stimulation, SHIP2 further relocated to the cytoskeleton in an aggregation-dependent manner (Figure 2C). Interestingly, when compared with SHIP1, SHIP2 exhibited a higher affinity for the cytoskeleton network, even in resting platelets (Table 1). The interaction of SHIP2 with p130Cas [21] and the presence of a C-terminal proline-rich domain capable

Table 1 Relative subcellular localization of SHIP2 and SHIP1 in resting and thrombin-stimulated human blood platelets

Cytoskeletons and Triton X-100-soluble fractions were isolated from resting platelets or platelets stimulated with 0.5 i.u./ml thrombin for 5 min as described in the Materials and methods section. The relative amounts of SHIP2 and SHIP1 in these fractions were assessed by immunoblotting using specific antibodies and quantified by densitometric analysis. Data are expressed as a percentage of total SHIP1 and SHIP2 and are means $\pm$ S.E.M. from three independent experiments.

	Cytoskeleton (%)		Triton X-100-soluble (%)	
	Resting	Activated	Resting	Activated
SHIP1	< 1	9 $\pm$ 3	> 99	91 $\pm$ 3
SHIP2	20 $\pm$ 5	40 $\pm$ 7	80 $\pm$ 5	60 $\pm$ 7

of interacting with filamin in SHIP2 [22] may support these differences.

Next, we examined whether SHIP2 undergoes tyrosine phosphorylation after thrombin stimulation, as observed previously for SHIP1. The phosphatase was immunoprecipitated from resting or thrombin-stimulated platelets, followed by immunoblotting with an anti-phosphotyrosine antibody (Figure 2A). The position of SHIP2 was identified by stripping and reprobing the same membrane with anti-SHIP2 antibodies (Figure 2B). SHIP2 was not tyrosine-phosphorylated in resting platelets, but its tyrosine phosphorylation was clearly detectable after 1 and 3 min of thrombin stimulation. As described previously for SHIP1 [24], SHIP2 tyrosine phosphorylation was impaired when stimulation occurred without shaking and, consequently, without aggregation (Figure 2A). Interestingly, another tyrosine-phosphorylated protein of mass 145 kDa co-immunoprecipitated with SHIP2 in both resting and thrombin-stimulated platelets.

Interaction of SHIP2 with SHIP1 in cells co-expressing the two phosphatases

We have shown previously that SHIP1 is a major 145 kDa tyrosine-phosphorylated protein in thrombin-activated platelets [24]. Therefore we checked whether the phosphotyrosyl protein co-immunoprecipitated with SHIP2 could actually be SHIP1. This issue was tested by immunoprecipitation of SHIP1 from resting and thrombin-activated platelets, followed by SHIP2 Western-blot analysis (Figure 3A). At 155 kDa, a clear signal was revealed under both conditions, suggesting a constitutive interaction between the two phosphatases. To confirm this observation, we investigated whether this association could also be observed in A20 B-cells, a cell type known to co-express SHIP1 and SHIP2 [17]. A20 B-cells were either stimulated or not, as described in the Materials and methods section. Again, the 155 kDa SHIP2 protein was co-immunoprecipitated with the 145 kDa SHIP1 isoform in both resting and activated A20 B-cells (Figure 3B), indicating that the association between SHIP1 and SHIP2 is not restricted to platelets and could be found in other haematopoietic cells.

SHIP2 SH2 domain is necessary and sufficient for the formation of the SHIP1/SHIP2 complex

To characterize further this interaction, COS-7 cells were used to co-transfect SHIP1 cDNA with different SHIP2 His-tagged truncated cDNAs (Figure 4A). The resulting SHIP1 and SHIP2 His-tagged proteins were expressed at the same level (results not shown). SHIP2 was immunoprecipitated and its association with SHIP1 was analysed by Western-blot analysis. The co-immunoprecipitation of two full-length SHIP proteins with the anti-His antibody (Figure 4B, lane 1) definitely ruled out the possibility of an antibody cross-reactivity in platelets or A20 B-cells. Furthermore, using different SHIP2 deletion mutants, we were able

Figure 2 Tyrosine phosphorylation and cytoskeleton relocation of SHIP2 after thrombin stimulation

(A) Human platelets stimulated for the indicated time with 1 i.u./ml thrombin, with or without shaking were lysed. NS, no shaking. SHIP2 was immunoprecipitated (IP α -SHIP2). As a negative control, immunoprecipitation with preimmune serum was performed (C). Proteins were separated by SDS/PAGE (7.5% gel), transferred to a nitrocellulose membrane and probed with the anti-phosphotyrosine antibody (α -P-Tyr). (B) The same nitrocellulose membrane was stripped and reprobed with the anti-SHIP2 antibody (α -SHIP2). (C) Cytoskeleton was isolated from human platelets stimulated for the indicated time with 1 i.u./ml thrombin, with or without shaking. NS, no shaking. Cytoskeletal proteins were then separated by SDS/PAGE (7.5% gel), blotted on to a nitrocellulose membrane and probed with the anti-SHIP2 antibody (α -SHIP2). Molecular-mass standards are indicated on the left of each panel. The positions of SHIP2 and the major tyrosine-phosphorylated protein co-immunoprecipitated with this phosphatase are shown. Results are representative of five independent experiments with similar results.

Figure 3 Co-immunoprecipitation of SHIP1 with SHIP2 in human blood platelets and A20 B-cells

(A) SHIP1 was immunoprecipitated (IP α -SHIP1) from resting (R) and thrombin-activated (A) platelets. Samples were analysed by SDS/PAGE (7.5 % gel), followed by SHIP2 Western-blot analysis (α -SHIP2). (B) SHIP1 immunoprecipitations were performed in resting and activated A20 B-cells as described in the Materials and methods section. After separation by SDS/PAGE (7.5 % gel), proteins were transferred to a nitrocellulose membrane, which was successively submitted to anti-phosphotyrosine, SHIP1 and SHIP2 immunodetections (Blot α -P-Tyr, α -SHIP1 and α -SHIP2 respectively). SHIP1 and SHIP2 migration positions are indicated. Results are representative of three independent experiments with similar results.

Figure 4 Requirement of the SHIP2 SH2 domain to promote the interaction with SHIP1

(A) Schematic representation of His-tagged SHIP2 protein constructs. Catalytic and protein-protein interaction domains are indicated on the upper part of the Figure. Each construction is identified by a letter for simplification; a, full-length SHIP2 protein (His-SHIP2); b, SH2 domain-deleted SHIP2 protein (His- Δ SH2 SHIP2); c, C-terminus-truncated SHIP2 protein (His-tSHIP2); d, SH2 domain of SHIP2 alone (His-SH2). (B) COS-7 cells were co-transfected with SHIP1 cDNA and one of a-d SHIP2 cDNA constructions, as described in (A). Each SHIP2 protein was immunoprecipitated with anti-His antibodies (IP α -His) (lanes 1-4), and the association with SHIP1 was checked by Western-blot analysis (Blot α -SHIP1). As a control, immunoprecipitation of SHIP1 was performed (lane 5). (C) COS-7 cells transfected or not with SHIP1 cDNA were lysed and the expression of SHIP1 was followed by Western-blot analysis (left panel). Resting COS-7 cells transfected with SHIP1 cDNA were lysed and submitted to SHIP1 immunoprecipitation (IP α -SHIP1), followed by anti-phosphotyrosine Western-blot analysis (Blot α -P-Tyr) (right panel). The position of SHIP1 is indicated by the arrow. Results are representative of four experiments with similar results.

Figure 5 Intracellular levels of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ in resting platelets from wild-type, SHIP1^{-/-}, SHIP1^{+/-} and SHIP2^{+/-} mice

The [³²P]PtdIns(3,4,5)P₃ (A) and [³²P]PtdIns(3,4)P₂ (B) in resting ³²P-labelled platelets obtained from the various mice were quantified by the HPLC technique as described in the Materials and methods section. For comparative quantification of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ in wild-type (WT), SHIP1^{+/-}, SHIP2^{+/-} and SHIP1^{-/-} platelets, HPLC values were normalized according to the labelling of neutral lipids (i.e. phosphatidylcholine and phosphatidylethanolamine). Results are means ± S.D. from three independent experiments. Results for platelets from WT, SHIP1^{+/-}, SHIP2^{+/-} and SHIP1^{-/-} mice are shown (open, grey, hatched and black bars respectively).

to identify the SH2 domain of SHIP2 as the minimal motif necessary and sufficient for the association between the two 5-phosphatases (Figure 4B, lanes 2 and 4). This result suggests that a basal level of SHIP1 tyrosine phosphorylation would allow the interaction with the SH2 domain of SHIP2. In agreement, SHIP1 was clearly tyrosine-phosphorylated in resting COS-7 cells (Figure 4C). Moreover, a weak but reproducible tyrosine phosphorylation of SHIP1 in resting human blood platelets and A20 B-cells was also observed ([24] and Figure 3B). Although we could not exclude the intervention of other partners in the complex formation, these results indicate that the SH2 domain of SHIP2 can support an association with SHIP1.

Roles of SHIP1 and SHIP2 in the control of PtdIns(3,4,5)P₃ levels

The co-expression of and association between SHIP1 and SHIP2 in human blood platelets prompted us to investigate their respective roles in the control of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ levels. This was investigated using genetically modified mouse blood platelets. As SHIP2 knockout mice die early after birth [23], we performed comparative studies using platelets isolated from wild-type, heterozygous SHIP1^{+/-} and SHIP2^{+/-} and homozygous SHIP1^{-/-} mice, which had all reached the adult age. Both SHIP1 and SHIP2 were immunodetected by Western-blot analysis in wild-type mouse blood platelets, and their expression was decreased in platelets from heterozygous animals (results not shown).

In previous studies, PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ were weakly detected in resting platelets and were found to be up-regulated in response to various stimuli [39]. As shown in Figure 5(A), we detected a weak but significant increase (2.4 ± 0.75 -fold, $P < 0.04$, $n = 3$, Student's *t* test) in the PtdIns(3,4,5)P₃ level in resting SHIP1^{-/-} platelets when compared with wild-type platelets. The levels of PtdIns(3,4,5)P₃ were not significantly modified in resting SHIP2^{+/-} and SHIP1^{+/-} platelets (Figure 5A). In resting cells, the levels of PtdIns(3,4)P₂ were not significantly different (Figure 5B). After thrombin stimulation, a strong increase in PtdIns(3,4,5)P₃ and a dramatic decrease in PtdIns(3,4)P₂ productions were observed in the SHIP1^{-/-} platelets when

compared with the wild-type platelets (Figures 6 and 7). Although much less pronounced, similar results were observed in SHIP1^{+/-} platelets stimulated with thrombin (Figure 7). After 1 min stimulation, a (2 ± 0.8)-fold decrease ($P < 0.02$, $n = 3$, Student's *t* test) in PtdIns(3,4)P₂ production was detected in SHIP1^{+/-} platelets compared with wild-type platelets. In SHIP2^{+/-} platelets, only a weak and insignificant decrease in thrombin-mediated PtdIns(3,4)P₂ production was observed.

Moreover, in agreement with Pasquet et al. [27], we also observed a strong accumulation of PtdIns(3,4,5)P₃ and a weak production of PtdIns(3,4)P₂ after collagen stimulation (results not shown) in SHIP1^{-/-} platelets.

DISCUSSION

The phosphatidylinositol 5-phosphatases SHIP1 and SHIP2 play a fundamental role in the acute regulation of the levels of the two second messengers PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ in various cells. We have shown previously [24] that SHIP1 was expressed in human blood platelets and was involved in thrombin-induced signal-transduction mechanisms. In the present study, we show that SHIP2 is also expressed as a 155 kDa functional PtdIns(3,4,5)P₃ 5-phosphatase in human blood platelets. This observation is consistent with the fact that SHIP2 has also been detected in other haematopoietic cells as a 155 or 135 or 125 kDa protein, revealing the potential existence of SHIP2 isoforms and/or degradation products in these cells [40,41].

Although SHIP1 and SHIP2 have some common features in human blood platelets, several differences in their behaviour were observed. Both enzymes are capable of hydrolysing PtdIns(3,4,5)P₃ into PtdIns(3,4)P₂. However, in contrast with SHIP1, SHIP2 does not exhibit Ins(1,3,4,5)P₄ phosphatase activity *in vitro*. Both phosphatases are tyrosine-phosphorylated after thrombin stimulation. However, in contrast with SHIP2, SHIP1 is often found weakly tyrosine-phosphorylated in resting platelets [24]. Both enzymes relocate to the actin cytoskeleton after platelet activation, but the affinity of SHIP2 for the cytoskeleton is higher than that of SHIP1, even under resting conditions where approx. 20% of SHIP2 is recovered in the cytoskeleton. During the preparation of this paper, SHIP2 was reported to be expressed in human blood platelets [42]. The authors emphasize the localization of SHIP2 within a protein complex (filamin, actin and GPIb-IX-V). Our results highlight an unexpected interaction between SHIP1 and SHIP2. This association is detected not only in human blood platelets, but also in A20 B-cells and in a model of COS-7 cells co-transfected with SHIP1 and SHIP2. In a previous study, Wisniewski et al. [29] have mentioned an association of these two proteins in haematopoietic progenitor cells isolated from chronic myelogenous leukaemia patients. They attributed their results to a possible artefactual cross-reactivity of their polyclonal antibodies. To rule out such a potential artifact, we used COS-7 cells co-transfected with SHIP1 and His-tagged SHIP2 constructs to immunoprecipitate SHIP2 by an anti-His antibody. Under these conditions, we confirmed this interaction and identified the SH2 domain of SHIP2 as the minimal and necessary domain involved in the association. This domain allows a recognition of the tyrosine-phosphorylated residue in the consensus sequence phospho-Tyr (Tyr/Asp)X(Leu/Ile/Val) [43]. The sequence Tyr-Asp-Phe-Val (residues 864–867) is present in human SHIP1 (SWISS-PROT/TrEMBL bank accession no. Q92835) and may be involved in the interaction. The constitutive association between SHIP1 and SHIP2 in resting human blood platelets, A20 B-cells and COS-7 cells suggests that this motif is already phosphorylated under basal conditions. Indeed, a basal level of

Figure 6 Accumulation of PtdIns(3,4,5)P₃ and lack of PtdIns(3,4)P₂ production in SHIP1-deficient platelets stimulated by thrombin

³²P-labelled WT or SHIP1^{-/-} platelets were either stimulated (B, D) or not stimulated (A, C) with 0.5 i.u./ml thrombin for 3 min. ³²P-labelled intracellular polyphosphoinositides were analysed by the HPLC technique as in Figure 5. The position of authentic standards is indicated. Results shown are representative of three independent experiments.

tyrosine phosphorylation is classically observed for SHIP1, e.g. in platelets, A20 B-cells and SHIP1-transfected COS-7 cells.

In thrombin-stimulated platelets, the increase in tyrosine phosphorylation of SHIP1 and SHIP2 did not significantly modify their interaction. This suggests that the increase in tyrosine phosphorylation does not unmask additional interaction motifs or that interaction with other protein partners is probably privileged. Moreover, approx. 20% of SHIP2 was recovered in the cytoskeleton of resting platelets and SHIP1 was barely detected in the same cellular fraction, suggesting that only a subpopulation of these two enzymes actually interact.

Although the physiological relevance of the association between SHIP1 and SHIP2 is difficult to predict at this stage, it is suggested that this mechanism will concentrate a lipid phosphatase activity, contributing to the control of PtdIns(3,4,5)P₃ levels. In this context, it is noteworthy that PTEN (phosphatase and tensin homologue), another PtdIns(3,4,5)P₃ phosphatase, was not detected in human blood platelets (results not shown). Other common partners of SHIP1 and SHIP2 may also regulate

the complex formation. For instance, both enzymes have been shown to interact with Src-homology collagen [29,44] or Fcγ immunoreceptors [30]. The association of the protein isoforms involved in phosphoinositide metabolism is not unique: type I and II β phosphatidylinositol monophosphate kinases have been shown recently to interact in pig aortic endothelial cells, HeLa cells and platelets [45]. The interaction of SHIP1 and SHIP2 in platelets raises the question of their roles in the control of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ levels. Using resting and activated wild-type, SHIP1^{-/-}, SHIP1^{+/-} and SHIP2^{+/-} mouse blood platelets, we observed a prominent role of SHIP1 in the control of PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ levels. Indeed, SHIP1^{-/-} platelets present an increase in PtdIns(3,4,5)P₃ levels under resting conditions and a dramatic accumulation of this lipid after activation. Concomitantly, PtdIns(3,4)P₂ production was strongly impaired in stimulated SHIP1^{-/-} platelets. The residual production of PtdIns(3,4)P₂ suggests either a modest participation of both SHIP2 and the other platelet PtdIns(3,4,5)P₃ 5-phosphatase described by Mitchell and co-workers [46] or the

Figure 7 Impact of SHIP1 or SHIP2 deficiency on PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ production in thrombin-stimulated platelets

The amount of [³²P]PtdIns(3,4)P₂ (A) and [³²P]PtdIns(3,4,5)P₃ (B) in ³²P-labelled platelets stimulated with thrombin (0.5 i.u./ml) for 1 or 3 min was quantified by the HPLC technique as in Figures 5 and 6. Results are means ± S.D. from three independent experiments. Results for platelets from WT, SHIP1^{+/-}, SHIP2^{+/-} and SHIP1^{-/-} mice are shown (open, grey, hatched and black bars respectively).

existence of another pathway involving, e.g. a PtdIns(3)P 4-kinase, as proposed previously [47]. The comparison between platelets from SHIP1^{+/-} and SHIP2^{+/-} confirmed the major role of SHIP1 and the absence of compensation by SHIP2 in this model. However, as we have demonstrated an association between SHIP1 and SHIP2, one could not exclude the possibility that SHIP2 activity is impaired in the absence of SHIP1. Thus, in SHIP1^{-/-} platelets, the lack of SHIP2 compensation in PtdIns(3,4,5)P₃ hydrolysis may be attributed to a defect in its activation.

In conclusion, the present study demonstrates that SHIP1 and SHIP2 are expressed in platelets. Their interaction, which we initially observed in human blood platelets, also occurs in several cell types where both enzymes are expressed. At least in a transfectant cell model, the association requires the SH2 domain of SHIP2. The present study is the first to compare, in the same model (i.e. mouse platelets), the roles of the two lipid 5-phosphatases SHIP1 and SHIP2 in PtdIns(3,4,5)P₃ metabolism. SHIP1, rather

than SHIP2, seems to play a predominant role in regulating the levels of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ in platelets stimulated by thrombin or collagen. The present study also suggests that despite the similarity in their catalytic activity and in their possible interaction and, therefore, co-localization, the two SHIPs play specific and distinct roles in cell signalling *in vivo*.

We thank M. Plantavid, M. P. Gratacap and H. Chap for stimulating discussions and F. De Smedt and D. Blero for a critical reading of the paper. This work was supported by the CEE BIOMED 2 contract no. PL962609, by grants from Action de Recherche Concertée (Communauté française de Belgique Wallonie, Bruxelles, Belgium), Fonds de la Recherche Scientifique Médicale, Association pour la Recherche sur le Cancer (Villejuif, France; contract no. 4794 and ARECA-Toulouse) and the INSERM–Communauté française de Belgique exchange contract.

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Received 17 April 2003/23 July 2003; accepted 29 July 2003

Published as BJ Immediate Publication 29 July 2003, DOI 10.1042/BJ20030581