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Biochemical and Biophysical Research Communications 296 (2002) 106–110

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SHIP2 overexpression strongly reduces the proliferation rate of K562 erythroleukemia cell line

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Received 26 June 2002

Abstract

SHIP2 belongs to the inositol 5-phosphatase family and is characterized by a phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) 5-phosphatase activity. Evidence based on mice lacking the SHIP2 gene has demonstrated its predominant role in the control of insulin sensitivity. However, SHIP2 expression in both hematopoietic and non-hematopoietic cells suggests additional functions. SHIP2 was previously identified in chronic myelogenous progenitor cells, in which its constitutive tyrosine phosphorylation was reported by Wisniewski et al., [Blood 93 (1999) 2707–2720]. Here, we further investigated the function of SHIP2 in this hematopoietic and malignant context. A detailed analysis of the substrate specificity of SHIP2 indicated that this phosphatase is primarily directed towards $PI(3,4,5)P_3$ both in vitro and in K562 chronic myeloid leukemia cells. The SHIP2-mediated decrease in $PI(3,4,5)P_3$ levels and increase in phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$) was accompanied by a reduction of cell proliferation, characterized by an accumulation of the cells in the G2/M phase of the cell cycle. Thus, in addition to its role in the control of insulin sensitivity, SHIP2 may also play a role in cell proliferation, at least in chronic myelogenous progenitor cells.

Keywords: Phosphatidylinositol metabolism; SHIP2; Proliferation; Chronic myelogenous leukemia

SHIP2 belongs to the inositol 5-phosphatase family and is characterized by an SH2 domain in its N-terminal part [1]. Its central catalytic domain hydrolyzes the 5′-position phosphate from inositol(1,3,4,5)-tetrakisphosphate and phosphatidylinositol(3,4,5)-trisphosphate [2]. Its C-terminal region contains one NPXY motif, several proline-rich sequences and a sterile α motif (SAM). It shares significant sequence identity with the inositol 5-phosphatase SHIP1 whose negative role in cell proliferation has been largely studied in several hematopoietic cell types. SHIP1 is indeed a key negative regulator of signals derived from cytokine [3], antigen [4], and growth factor receptors [5]. SHIP1 also plays an important role in graft rejection and graft versus host disease by affecting the repertoire of NK cells [6].

In contrast to SHIP1, whose expression is restricted to the hematopoietic lineage, SHIP2 is widely expressed. Its critical negative role in insulin signaling has been highlighted by the generation of homozygous mice lacking SHIP2 [7]. The absence of SHIP2 resulted in an increased sensitivity to insulin, which is characterized by severe neonatal hypoglycaemia, deregulated expression of several genes involved in gluconeogenesis, and perinatal death. In contrast, absence of SHIP1 resulted in a myeloproliferative-like syndrome, characterized by the uncontrolled proliferation of myeloid cells in the lungs, leading to a decrease in the SHIP1minus/− mice survival [8].

The negative role of SHIP proteins in signaling could be attributed to inositol 5-phosphatase dependent or independent mechanisms [9]. Concerning the regulation of cell proliferation and survival, SHIP1 and SHIP2 may affect the two major MAP kinase and PI 3-kinase

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0006-291X/02/$ - see front matter
PII: S0006-291X(02)00787-8

pathways. Indeed, the two inositol 5-phosphatases are tyrosine phosphorylated in a number of activated cells [9]. Their tyrosine phosphorylation, together with the protein–protein interaction motifs (SH2, proline-rich sequences), serves as adaptor motifs used to form protein complexes preventing the activation of the MAP kinase pathway. In addition, their $PI(3,4,5)P_3$ 5-phosphatase activity places them as critical negative regulators of the PI 3-kinase/Akt pathway which is involved in proliferation and survival [10].

Glioblastoma brain tumor cells, characterized by mutation of the $PI(3,4,5)P_3$ 3-phosphatase tumor suppressor PTEN, display a potent cell cycle arrest when SHIP2 is overexpressed [11]. Thus, in this model, SHIP2 may act as a tumor suppressor by decreasing $PI(3,4,5)P_3$ levels. In the present paper, we have further investigated this SHIP2 potential function using K562 erythroleukemia cell line. These cells express the Bcr–Abl oncogene [12]. It has been reported that Bcr–Abl expression induces the repression of SHIP1 both at the protein and mRNA level [13]. Consistent with these results, SHIP1 was barely detectable [14] or even absent in K562 cells [15]. Our results demonstrate that, in contrast to SHIP1, SHIP2 overexpression in K562 cell lines strongly reduces the proliferation rate of the cells.

Materials and methods

Obtention of stable SHIP2 transfected K562 cell lines. K562 cells (5×10^6 cells) were suspended in 0.6 ml of RPMI medium 1640 supplemented with 10% FBS and 1.25% DMSO. They were placed in a 0.4 cm gap electroporation cuvette (Bio-Rad) and 25 μg plasmid DNA (in pcDNA3 His, Invitrogen) expressing SHIP2 full length or vector alone was added. The cells were transfected by electroporation by using a single electric pulse (960 μF, 250 V) in a Bio-Rad Gene Pulser electroporator and then replated into growth medium. Twenty-four hours after transfection, cells were resuspended in fresh media without DMSO and, after 24 h, 0.8 mg/ml G418 (Invitrogen) was added to the medium. Cells resistant to G418 were counted, diluted, and distributed in 96-well plates to have a cell per well. Clonal populations were then expanded and analyzed for SHIP2 expression by Western blotting [16].

Preparation of cell extracts. Wild-type K562 cells were grown in RPMI medium 1640, containing 10% fetal calf serum and 1% penicillin/streptomycin. Transfected K562 cells were grown in the same medium supplemented by 0.8 mg/ml of G418. Cell extracts were prepared by incubating 10^7 cells for 45 min at 4 °C in a lysis buffer containing 250 mM NaCl, 50 mM Hepes (pH 7), 0.1% NP40, 5 mM Na_3VO_4, and 10 μg/ml of leupeptin and aprotinin. Cell lysates were collected after centrifugation at 10,000 rpm for 10 min at 4 °C.

Gel electrophoresis and immunoblotting. Wild-type and SHIP2 transfected K562 extracts (100 μg of proteins) were denatured at 100 °C for 5 min in electrophoresis sample buffer. Proteins were separated by 7.5% SDS–PAGE and transferred onto a nitro-cellulose membrane. The blots were blocked for 1 h at room temperature with 1% (wt/vol) milk powder and 1% (wt/vol) bovine serum albumin in a TBST buffer containing 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.05% (wt/vol) Tween 20. Immunodetection was achieved using the anti-SHIP2 antibodies (1/1000).

Phosphatase assays. Phosphatase assay for immunoprecipitated His-tagged SHIP2 protein was carried out using synthetic fluorescent phosphoinositides (Echelon Research Laboratories). Briefly, immunoprecipitated SHIP2 [16] was incubated in 30 μl of reaction buffer containing 50 mM ammonium acetate (pH 7) and 2 mM dithiothreitol with 1.5 μg fluorescent di-C6-NBD6 phosphoinositide substrate for 30 min at 37 °C under shaking. The reaction products were separated on thin layer chromatography as previously described [17,18].

Lipid extraction and analysis. Wild-type, SHIP2 and vector transfected K562 cells (10^7 cells in 10 ml) were labeled with 150 μCi/ml of [^{32}P]orthophosphate (Amersham International) during 5 h in phosphate free RPMI medium 1640 supplemented by 5% fetal calf serum. Cells were then centrifuged at 1500 rpm for 5 min and washed once in PBS. Pellets were resuspended by 3 ml of methanol and lipids were extracted following a Bligh and Dyer modified procedure by addition of 4 ml chloroform and 3.75 ml of 2.4 N HCl. Lipids were first resolved by thin layer chromatography using chloroform/acetone/methanol/acetic acid/water (80/30/26/24/14, vol/vol). Radioactive spots were visualized by a PhosphorImager 445 SI (Molecular Dynamics). Spots corresponding to $PI(3,4,5)P_3$, phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), and $PI(3,4)P_2$ were scrapped off, deacylated by 20% methylamine, and analyzed by HPLC on a Whatman Partisphere 5 SAX column (Whatman International, United Kingdom) as described in [19].

Proliferation assay and cell cycle distribution. Wild-type, SHIP2, and vector transfected K562 cells were seeded at a density of 30×10^4 cells per plate. The living cells were estimated every day by counting the number of Trypan blue-negative cells over 6 days. Cell cycle distribution of wild-type and SHIP2 transfected cells were compared after 6 days in culture. Wild-type or SHIP2 transfected cells (5×10^5) were washed in PBS and fixed overnight in 70% ethanol at 4 °C. The ethanol was removed and cells were washed once in PBS. Cells were then resuspended and stained in PBS containing 0.5 μg/ml of RNAseA and 50 μg/ml of propidium iodide. After 30 min at room temperature, fluorescence data were collected and analyzed with a Becton Dickinson FACScan to determine DNA content and the distribution of cells in the different phases of the cell cycle.

Results and discussion

The two SHIP proteins, SHIP1 and SHIP2, were shown to be constitutively tyrosine phosphorylated in p210 Bcr–Abl expressing cells. The expression of the two proteins and their state of phosphorylation may lead to alteration of the normal balance of $PI(3,4,5)P_3$ and $PI(3,4)P_2$. This, in turn, would disrupt signaling events involved in growth and maturation of hematopoietic progenitor cells [14]. In this study, we attempted to generate SHIP2 transfected K562 cell lines and analyze their biochemical properties.

Two K562 clones, overexpressing SHIP2 at the same level, were obtained and referred to as S2 and S6 (Fig. 1). Cells transfected with the vector alone display the same low level of endogenous SHIP2 than wild-type control K562 cells (or untransfected cells). Since SHIP2 substrate specificity was not yet clearly defined, we first performed a detailed in vitro analysis of SHIP2 phosphatase activity using fluorescent phosphoinositides. The lipid preferentially degraded by SHIP2 was $PI(3,4,5)P_3$. $PI(4,5)P_2$ was hydrolyzed to a lesser extent and the degradation of the other phosphoinositides (in particular, PI(5)P and $PI(3,5)P_2$) was barely detectable

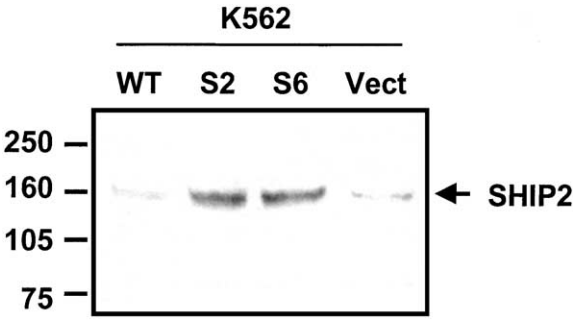

Fig. 1. SHIP2 expression in wild-type and transfected K562 cells. K562 cells were stably transfected with SHIP2 (S2 and S6 clones) or with the vector alone (Vect). SHIP2 expression in wild-type (WT) and transfected K562 cell extracts (100 μg of protein) was analyzed by Western blotting using SHIP2 antibodies. The migrations of SHIP2 and molecular weight standards are indicated on the right- and left-side, respectively.

under our assay conditions (Fig. 2A). $PI(3,4,5)P_3$ levels of SHIP2 transfected K562 cells were compared to vector transfected or wild-type cells. As shown in Fig. 2B, $PI(3,4,5)P_3$ was also hydrolyzed by SHIP2 in vivo. Its level was decreased threefold and concomitantly, $PI(3,4)P_2$ was 1.6-fold increased in SHIP2 transfected K562 cells as compared to control cells. There was no difference in phosphoinositide levels between untransfected (wild-type) cells and cells transfected with the vector alone (Fig. 2B). The data were identical for S2 and S6 clones and consistent with SHIP2 being primarily a $PI(3,4,5)P_3$ 5-phosphatase [14,16]. The $PI(3,4,5)P_3$ 5-phosphatase activity measured in vitro in SHIP2 transfected cell extracts was increased when SHIP2 was overexpressed but remained unchanged upon treatment of the cells with the Bcr/Abl tyrosine kinase inhibitor STI571 (10 μM) for 12 h although it prevented SHIP2 tyrosine phosphorylation (data not shown). This indicated that SHIP2 phosphatase activity was unaffected by its state of tyrosine phosphorylation as also suggested previously [11,20]. We next analyzed over 6 days the influence of SHIP2 overexpression on the proliferation rate of K562 cells. Whereas wild-type or vector transfected cells grew exponentially over 6 days, there was a clear reduction in the proliferation of SHIP2 transfected K562 cells (Fig. 3). The data of SHIP2 transfected K562 cells contrast with SHIP1 transfected cells: SHIP1 overexpression in K562 cells

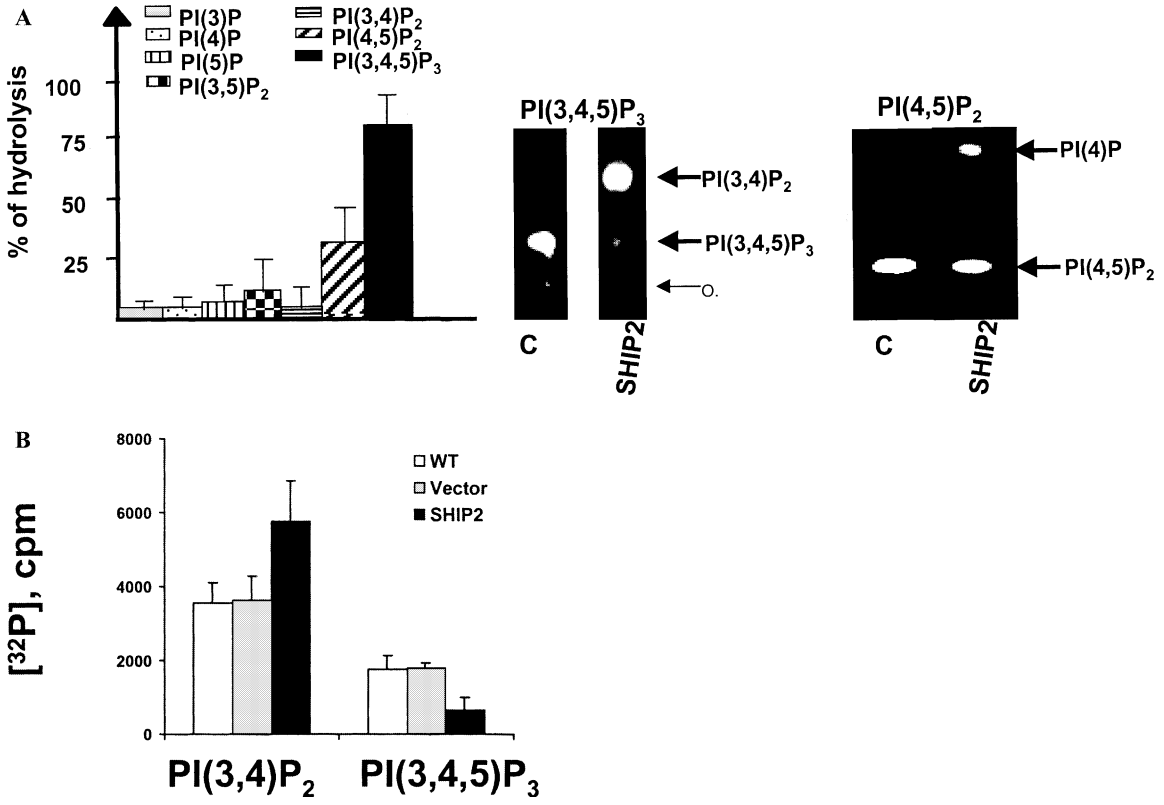

Fig. 2. SHIP2 hydrolyzes preferentially $PI(3,4,5)P_3$ in vitro and in vivo. (A) SHIP2 was expressed as His-tagged protein in COS-7 cells [16] and immunoprecipated with anti-His antibody. Phosphatase activity against the various fluorescent phosphoinositides was determined in vitro. The immunoprecipitated enzyme dephosphorylated efficiently $PI(3,4,5)P_3$ and to lesser extent $PI(4,5)P_2$. The data are means ± SD of three experiments (left panel) or are representative of three experiments (right panel). (B) $PI(3,4,5)P_3$ and $PI(3,4)P_2$ levels in wild-type and transfected K562 cells. Wild-type, vector, and SHIP2 transfected K562 cells were incubated with $[^{32}P]$orthophosphate. The levels of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ are shown by the cpm quantification of the corresponding HPLC peak. The data are means ± SD of three independent experiments.

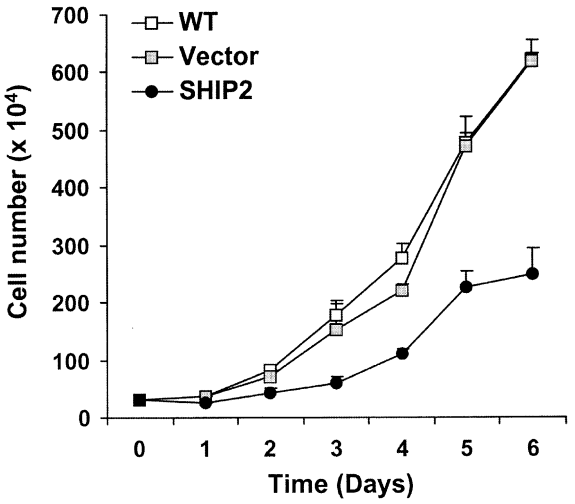

Fig. 3. Effect of SHIP2 overexpression on cell proliferation. Wild-type, vector, and SHIP2 transfected K562 cells were seeded at 30×10^4 cells per plate and maintained in media for 6 days. Determination of viability by Trypan blue exclusion was performed each day to count the number of alive cells. Representative results of five independent experiments ± SEM are shown.

has been described previously not to alter the doubling time of these cells but to impair the erythroid differentiation program [15]. This emphasizes the concept that the two SHIP proteins are not identical lipid phosphatases: e.g., SHIP1 and SHIP2 have a different hierarchy of binding SH3 containing proteins; only SHIP2 shows a typical SAM domain at the C-terminal end. A series of protein partners of SHIP2 have now been identified: $p130^{Cas}$ was shown to interact with SHIP2 SH2 domain and may regulate cellular adhesion and spreading [21]. The actin binding protein filamin has been described to associate with SHIP2 through its proline-rich sequences and thereby could regulate actin rearrangement [22]. Presumably, the localization of SHIP2, which is directly depending on interaction with protein partners, dictates its biological activity and possible effect on cell proliferation. It was previously reported that SHIP2 causes cell cycle arrest in G1 in glioblastoma cells [11]. We observed that SHIP2 overexpression in K562 cells causes, after 6 days, an accumulation of the cells in G2/M phase of the cell cycle (Fig. 4). Recently, Kisseleva et al. have reported that overexpression of the phosphoinositide polyphosphate 5-phosphatase IV leads to a G2/M cell cycle arrest in HEK 293. This phosphatase was able to degrade both $PI(3,4,5)P_3$ and $PI(4,5)P_2$. The G2/M arrest was attributed to cytoskeletal disturbance associated to a decrease in $PI(4,5)P_2$ [23]. We did not observe a significant variation in the $PI(4,5)P_2$ levels in the SHIP2 transfected K562 cells as compared to vector transfected cells (data not shown). This result is consistent with $PI(4,5)P_2$ being a poor substrate of SHIP2 (Fig. 2A).

In conclusion, we show that SHIP2 is primarily directed towards $PI(3,4,5)P_3$ as substrate both in vitro and in intact cells. SHIP1, SHIP2, PTEN, phosphoinositide 5-phosphatase type IV, and perhaps many other inositol polyphosphate 5-phosphatase have been shown to control the PI3-kinase/Akt pathway in cells, illustrating a redundancy of controlling this pathway. This may result from a decrease in $PI(3,4,5)P_3$ levels with different consequences depending on the cell type and catalytic specificity of each phosphatase. We have shown that in K562 cells, SHIP2 overexpression directly inhibits the proliferation of the cells as compared to untransfected cells. This effect could be attributed to the SHIP2 $PI(3,4,5)P_3$ 5-phosphatase activity and/or to SHIP2 participation in the MAP kinase pathway. Further investigations should clarify this point. Our results suggest that SHIP2 overexpression in K562 cells may lead to the recovery of the G2/M cell cycle checkpoint which was presumably abolished in the Bcr/Abl tumorigenesis process. SHIP2 involvement in the control of cell proliferation was previously described in

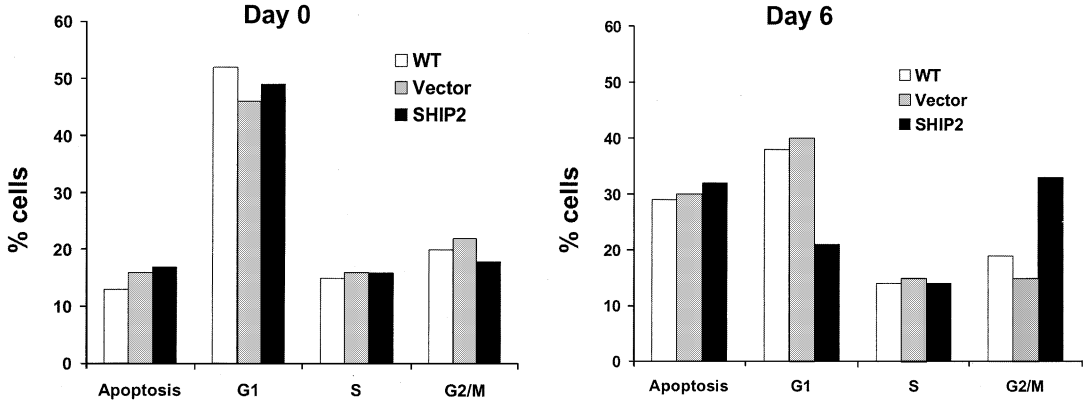

Fig. 4. SHIP2 overexpression provokes an accumulation of the K562 cells in G2/M cell cycle phase. Wild-type, vector, and SHIP2 transfected K562 cells were maintained in culture media for 6 days. The proportion of cells in the different phases of the cell cycle, at day 0 (Day 0) and after 6 days in culture (Day 6), was determined by flow cytometry using propidium iodide DNA staining. The percentage of cells found in subG1 (apoptotic cells), G1, S, and G2/M phases are indicated. These data are representative of one experiment out of three with very similar results.

glioblastoma cells [11]. Our data are consistent with SHIP2 harboring anti-proliferation properties and extend this new function to the hematopoietic lineage.

Acknowledgments

This work was supported by E.U. Biomed 2 programme PL962609, grants of the Fonds de la Recherche Scientifique Médicale, Action de Recherche Concertée of the Communauté Française de Belgique, and a joint project between INSERM and CFB. Daniel Blero is chargé de recherches FNRS. We thank Dr. S. Schurmans and Dr. J.E. Dumont for reading the manuscript.

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