The SH2 domain containing inositol 5-phosphatase SHIP2 is recruited to the EGF receptor and dephosphorylates phosphatidylinositol 3,4,5-trisphosphate in EGF stimulated COS-7 cells


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Abstract

The lipid phosphatase SHIP2 (SH2 inositol 5-phosphatase 2) has been shown to be expressed in non-haemopoietic and haemopoietic cells. It has been implicated in signalling events initiated by several extracellular signals such as epidermal growth factor (EGF) and insulin. In COS-7 cells, SHIP2 was tyrosine phosphorylated at least, at two separated tyrosine phosphorylation sites, in response to EGF. SHIP2 was coimmunoprecipitated with the EGF receptor (EGFR) and also with the adaptor protein Shc. A C-terminal truncated form of SHIP2 that lacks the 366 last amino acids, referred to as tSHIP2, was also precipitated with the EGFR when transfected in COS-7 cells. The SH2 domain of SHIP2 was unable to precipitate the EGFR in EGF stimulated cells. Moreover, when transfected in COS-7 cells, it could not be detected in immunoprecipitates of the EGFR. When the His-tagged full length enzyme was expressed in COS-7 cells and stained with anti-6xHis monoclonal antibody, a signal was observed at plasma membranes in EGF stimulated cells that colocalize with the EGFR by double staining. Upon stimulation by EGF, PIP3 and protein kinase B (PKB) activity were decreased in SHIP2 transfected COS-7 cells as compared to the vector alone. SHIP2 appears therefore in a tyrosine phosphorylated complex with at least two other proteins, the EGFR and Shc.
Introduction

Protein tyrosine phosphorylation plays a central role in the regulation of protein-protein interactions and modulation of enzyme activities (1). Key events in receptor signalling are the interactions of proteins such as the adaptor protein Shc with other phosphorylated proteins. In epidermal growth factor (EGF) signalling, the activation of the EGF receptor (EGFR) could mediate the phosphorylation of a series of proteins such as the p85 subunit of the phosphoinositide 3-kinase (PI 3-kinase) (2). This phosphorylation has been linked to the activation of PI 3-kinase which plays an important role in signalling. All mammalian cell types express at least class IA PI 3-kinase isoform and stimulation of almost every receptor that induces Tyr kinase activity also leads to class Ia PI 3-kinase activation (3, 4). PI 3-kinase IA substrates in vitro are phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI(4)P) and phosphatidylinositol 4,5-bisphosphate (PI 4,5-P₂) whereas the preferred substrate in vivo is PI 4,5-P₂ (2). Phosphatidylinositol 3,4,5-trisphosphate (PIP3) and/or phosphatidylinositol 3,4-bisphosphate (PI 3,4-P₂) could activate protein kinase B (PKB) through the binding to the PH domain of 3'-phosphoinositide-dependent kinase-1 (PDK1) phosphorylating protein kinase B (5, 6, 7).

The control of the levels of the second messenger PIP3 depends on the activity of both PI 3-kinase and PIP3 phosphatases, members of inositol and phosphatidylinositol 5-phosphatase family and 3-phosphatases such as PTEN (8). SHIP1 and SHIP2 belong to the inositol and phosphatidylinositol 5-phosphatase family (9-11). Both phosphatases contain different protein-protein interaction domains such as a N-terminal specific SH2 domain, a potential phosphotyrosine binding (PTB) domain binding site NPAY and C-terminal proline-rich sequences allowing to bind SH3 domain. SHIP2 contains also a sterile alpha motif (SAM) at the C-terminal end that was not present in SHIP1 (12). cDNAs encoding SHIP2 have been reported in human, mouse and rat (12-15). In human, the protein contains 1258 amino acids whereas a splice variant which lacks exon 28 and the carboxy terminal SAM motif has been isolated in rat skeletal muscle (13). SHIP2 has been shown to be expressed in non-haemopoietic and haemopoietic cells as shown by Western blotting (16, 17). When tested in vitro on recombinant SHIP2
produced in COS-7 cells, we could demonstrate a PIP3 5-phosphatase activity. In another study, PIP3 5-phosphatase activity was also demonstrated in K562 cells after immunoprecipitation with anti-SHIP2 antibodies (18).

Growth factors (EGF, platelet derived growth factor) and insulin stimulate tyrosine phosphorylation of SHIP2 in various cell models such SH-SY5Y cells (19). SHIP2 was also constitutively tyrosine phosphorylated in chronic myelogenous leukemia progenitor cells (18). In B cells, SHIP2 was also maximally tyrosine phosphorylated and associated to Shc after BCR and FcγRIIB crosslinking but not after stimulation of BCR alone (16, 20). SHIP2 has been shown to control insulin sensitivity in a model of SHIP2 deficient mice (21). A role of SHIP2 in cellular adhesion and spreading has also been recently proposed (22).

Previous data obtained in B cells have suggested that PIP3 initiates a PLCγ2-dependent inositol trisphosphate production through its ability to activate Tec kinases. Moreover FcγRIIB1, an inhibitory receptor which recruits SHIP1 eliminates BCR-induced PIP3 accumulation (23-25). The data implicate PIP3 as a crucial regulator of calcium signalling through its ability to initiate Tec kinase activation. It also stressed the importance of SHIP1 as a PIP3 5-phosphatase in an intact cell model (26).

Given the potential role of SHIP2 in regulation of PI 3-kinase signalling by growth factors and insulin (19), we aimed to measure PIP3 levels in intact cells. In the course of these studies, we observed that SHIP2 was coimmunoprecipitated with the EGF receptor (EGFR) and also with the adaptor protein Shc. The EGFR was also present in SHIP2 immunoprecipitates. We have observed a colocalization of SHIP2 and the EGFR in COS-7 cells stimulated by EGF. Our data could be interpreted as the recruitment of a complex of at least three proteins, SHIP2, Shc and the EGFR in EGF stimulated cells.
Experimental Procedures

Materials
Vector pcDNA3-His and Hyperfilm-MP were from Amersham. Superfect was from Qiagen. Protein A-Sepharose CL4B was obtained from Amersham Pharmacia. Anti-6xHis monoclonal antibody was from Clontech. Anti-phosphotyrosine monoclonal antibody 4G10, anti-Shc and anti-PKB antibodies were purchased from Upstate Biotechnology; goat polyclonal anti-EGFR antibodies for Western blotting, immunofluorescence and monoclonal antibody to EGFR for immunoprecipitation were from Santa Cruz. Rabbit polyclonal antibodies against Shc were obtained from Affinity. Fluorescein-labeled mouse secondary antibodies were were from Jackson Immuno-research Lab. SlowFade kit was purchased from Molecular Probes. PI 4,5-P₂, phosphatidylserine and Triton X-100 were from Sigma. TLC plates (20 X 20 cm) were from Merck. Recombinant bovine brain PI 3-kinase was prepared as described (28) and kindly provided by Dr. Bart Vanhaesebroeck (Ludwig Institute for Cancer Research, London). Protease inhibitors cocktail was from Boehringer. Quickchange site directed mutagenesis kit was from Stratagene. Phosphocellulose P81 paper was obtained from Whatman. The peptide RPRAATF was synthesized at the laboratoire de Chimie Biologique et de la Nutrition (ULB). The peptide GGDGpYYDLSPPL was kindly provided by Drs. Rüdiger Woscholski and Peter Parker (ICRF). It was coupled to Actigel ALD (Sterogene) as described by the manufactor.

Methods

Subcloning of SHIP2 in pcDNA3-His and site directed mutagenesis.
A truncated form of SHIP2 (tSHIP2) that lacks 366 amino acids at the C-terminus (12, 27) was partialy digested for 5 min by NcoI to obtain a 2.5 kb fragment. This was further digested with BamHI and resulted in an insert of 2.4 kb. The partial SHIP2 clone (Clone 7 in (12)) was partialy digested by NcoI and XhoI to obtain a
2 kb fragment. Both fragments were subcloned into pcDNA3-His vector digested with BamHI and XhoI.

A construct corresponding to the SH2 domain of SHIP2 was obtained by PCR using the tSHIP2 (27) as template and a 5′-primer containing a BamHI restriction site (underlined) 5′-GTGCCGATCCATGGCCCCCTCCTGGTA-3′ and a 3′-primer containing an XhoI restriction site (underlined) 5′-CCGCTCGAGTCACTCTACAGGAAGAAGC-3′. The PCR product was subcloned into pcDNA3-His C vector. The same construct was also subcloned in pTrc-His vector to produce the SH2 domain in bacteria as His-tagged construct. Production and purification on ProBond resin was performed as reported before (27). The catalytic domain of SHIP2 was obtained by PCR using tSHIP2 as template and a 5′-primer containing a BamHI restriction site (underlined) 5′-CGCGCAGATCCATGAAGGACCAGACTCAGCGCAA-3′ and the 3′-primer containing an XhoI restriction site (underlined) 5′-CGCTCGAGTCACGTGACTGGATCATGGAT-3′. The PCR product was subcloned into pcDNA3-His C vector. A SHIP2 construct which does not have SHIP2 SH2 domain (SH_2-SHIP2) was prepared with SHIP2 as template and a 5′-primer containing an EcoRI restriction site (underlined) 5′-CGGAATTCCATGTCAGATGGGGAGGATGAG-3′ and a 3′-primer containing an XhoI site (underlined) 5′-CCGCTCGAGTCACGTGACTGGCATGGAT-3′. The PCR product was subcloned in pcDNA3-HisC vector. The catalytic mutant of SHIP2 in which cysteine 689 has been replaced by a serine was generated by PCR-based mutagenesis using SHIP2 subcloned into pBlueScript as template according to the manufacturer’s instructions.

Transfection of COS-7 cells

COS-7 cells (platted at 1.5 × 10⁶ cells/dish the previous day) were transfected in 10 cm dishes using the Superfect method of transfection according to the manufacturer’s instructions. Cells were stimulated with 50 ng/ml EGF at 37°C for different times. The cells were washed with sterile phosphate buffered saline (PBS) and recovered in 1 ml of buffer A containing 50 nM Tris/HCl pH 7.5, NaCl 100 mM, EDTA 5 mM, Brij 1%, Na₃VO₄ 2 mM, PMSF 2 mM, leupeptine 10 µg/ml and aprotinin 10 µg/ml. After shaking the lysates during 20 min at 4 °C and
centrifugation at 10,000 x g, the supernatants were recovered and immediately subjected to immunoprecipitation.

**Immunoprecipitation and Western blotting analysis**

The following antibodies were used for immunoprecipitations: anti-SHIP2 (12, 16), anti-Shc (Upstate), anti-EGFR (Santa-Cruz) anti-6xHis (Clontech) and antiphosphotyrosine 4G10 (Upstate). The supernatants were precleared for 5 min at 4°C with 150 µl of 10% (w/v) protein A-Sepharose CL4B. This was centrifuged at 12000 x g for 20 min at 4°C. The soluble fraction was collected and incubated with the adequate antibodies and protein A-Sepharose for 2 h at 4°C (10 µl of serum for anti-SHIP2, 10 µl for anti-Shc, 5 µl for anti-6xHis and 25 µl for anti-EGFR). The immune complexes were recovered by centrifugation and washed four times in lysis buffer. The last wash was made without protease and phosphatase inhibitors. The immunoprecipitates were applied on SDS gels followed by Western blotting. The blots were analysed by enhanced chemoluminescence detection. Affinity adsorption of SHIP2 and tSHIP2 was performed using coupled GGDGpYYDLSPL peptide to Actigel ALD. After addition of 30 µl of peptide conjugated resin to 1 ml COS-7 lysate, the complex was recovered by centrifugation and washed as described above.

**Enzymatic activity**

The [3-32P]PIP3 was prepared as described (27) using recombinant PI 3-kinase. TLC-purified [32P]PIP3 was evaporated under nitrogen and resuspended with 100 µg phosphatidylserine into vesicles. The [32P]PIP3 5-phosphatase activity was measured as described (28-29). Briefly, a total of 20,000 cpm/sample of [32P]PIP3 was resuspended in 50 mM Tris/HCl pH 7.5 and 100 µg phosphatidylserine. After sonication, the reaction was started by adding immunoprecipitated SHIP2 from transfected COS-7 cells and 5 mM MgCl2. The assay was stopped and lipids were extracted. PIP3 and PI 3,4-P2 were separated by thin layer chromatography in chloroform/acetone/methanol/acetic acid/water (50:30:26:24:14, v/v/v/v/v). The corresponding spots were analysed by PhosphoImager and autoradiography.

**PIP3 levels determined in COS-7 transfected cells**

Two days after the transfection of 1.2 10⁶ cells in 10 cm diameter dishes (vector alone, tSHIP2 or full-length SHIP2 cDNAs), COS-7 cells were labelled with [32P]-
orthophosphate 5 h at 37°C. Cells were treated with 50 ng/ml EGF for different time and reactions were stopped by addition of chloroform/methanol (1:1, v/v). After lipid extraction, phospholipid were separated on TLC by chloroform/acetone/methanol/acetic acid/water (50:30:24:14, v/v/v/v/v) and PIP3-PI 3,4-P$_2$ spots were scrapped and deacylated. Lipids were resolved by HPLC (strong anion-exchange column partisphere SAX) and eluted by a linear gradient [(NH$_4$)$_2$HPO$_4$, 0-1 M] as described in (30). PIP3 and PI 3,4-P$_2$ were identified with internal standards.

**Confocal immunofluorescence microscopy**

Immunofluorescence using anti-6xHis and anti-EGFR antibodies was performed on transfected COS-7 cells. 1.2 10$^5$ transfected COS-7 cells were grown on uncoated glass coverslips in 3 cm diameter dishes. Cells were stimulated or not with EGF, rinsed in Tris-buffered saline (TBS) and then fixed in 4% of paraformaldehyde solution in 0.1 M phosphate-buffered saline pH 7.4 for 10 min. The cells were washed three times for 10 min in TBS, permeabilized with 0.15% Triton X-100 in TBS for 10 min and washed again with TBS. The fixed cells were incubated for 1 h at room temperature with 1/20 normal serum in TBS (goat or horse serum depending on the origin of the secondary antiserum). Incubation with immune serum was performed overnight at room temperature in the presence of blocking serum diluted 1/20 in TBS. The anti-6xHis antibody was used at a 1/1000 dilution and the anti-EGFR antibody at 1/250. After being rinsed with TBS, cells were incubated for 60 min in the dark with a fluorescein-labeled goat anti-mouse secondary antibody (direct immunofluorescence). For the colocalization experiments, a Texas Red-labeled donkey anti-mouse secondary antibody at a 1/200 dilution and a fluorescein donkey anti-goat secondary antibody at a 1/250 dilution were used. The cells were then washed three times with TBS for 10 min and mounted with SlowFade light antifade kit following the manufacturer’s instructions. Cells were observed under a Nikon Optiphot fluorescence microscope and images were obtained using a laser-scanning confocal microscope (MRC 1000, Bio-Rad) equipped with argon-krypton laser and COSMOS software (Bio-Rad).
**Akt/PKB kinase assay**

After transfection and stimulation of $1.2 \times 10^6$ cells in 10 cm diameter dishes COS-7 cells, cells were lysed in 800 µl of ice-cold lysis buffer (80 mM TrisHCl pH 7.5, 20 mM EDTA, 200 mM NaCl, 0.75% Triton X-100, 80 mM sodium pyrophosphate, 4 mM sodium orthovanadate, 200 mM NaF, protease inhibitors cocktail). After 20 min agitation at 4°C, the different cell supernatants were immunoprecipitated with 4µg antibody to PKB coupled to 25 µl of protein A-Sepharose in a total volume of 400 µl buffer H (80 mM TrisHCl pH 7.5, 20 mM EDTA, 1 mM EGTA, 200 mM NaCl, 0.2% Triton X-100, 0.1% β-mercaptoethanol, protease inhibitors cocktail). PKB activity was determined as described before (31).
Results

**EGF induces tyrosine phosphorylation of SHIP2 in COS-7 cells**

The molecular mass of SHIP2 was approximately 160 kDa in B cells (16, 20). A similar value was determined in COS-7 cells. EGF was particularly potent in stimulating the tyrosine phosphorylation of SHIP2 in COS-7 cells: Fig 1A shows a time course study. SHIP2 tyrosine phosphorylation could be seen at 0.5 min up to 120 min. When the blot was stripped and reprobed with SHIP2 antibodies, SHIP2 was recovered in the presence and absence of EGF confirming that the immunoprecipitation was efficient in both cases (Fig. 1A, bottom). Similar results were obtained in SHIP2 transfected cells although a higher basal phosphorylation of SHIP2 could be observed depending on the transfection (data not shown). In similar transfection experiments, immunoprecipitations were performed with an anti-phosphotyrosine and the blot was probed with SHIP2 antibodies; we observed that a 160 kDa band corresponding to SHIP2 was increased when cells had been stimulated for 5 min with EGF (Fig. 1B). The high basal level seen in unstimulated cells results either from the migration of other tyrosine phosphorylated proteins at the same molecular weight that could recruit SHIP2 or of a basal SHIP2 phosphorylation seen in transfected cells.

**SHIP2 associates the EGFR and Shc in transfected COS-7 cells**

The association between the adaptor protein Shc and SHIP2 has been reported by others in EGF, platelet derived growth factor stimulated cells and also in K562 cells or in B cells (17, 19, 20). This was also observed in our model of transfected COS-7 cells. COS-7 cells were transfected with SHIP2 and immunoprecipitated with anti-Shc antibodies. Fig. 1C shows that when probed with SHIP2, a 160 kDa protein band was detected in EGF-stimulated but not in control cells. We also tested whether SHIP2 could associate to the EGFR. When COS-7 cells were subjected to immunoprecipitation of the EGFR, SHIP2 was immunodetected in the immunoprecipitate provided the cells had been stimulated by EGF. This result was obtained in untransfected or SHIP2 transfected cells (Fig. 1D). The bottom part of
Fig. 1D shows the presence of the EGFR by immunodetection of the same blot. Moreover, when the cell lysates were immunoprecipitated with SHIP2 and probed with EGFR antibodies, a protein of 170 kDa was detected in EGF-stimulated cells (Fig. 1E). This band was not detected in control cells. Fig 1E (bottom part) shows that the same amounts of SHIP2 were immunoprecipitated in control and EGF-stimulated cells. Immunoprecipitation of the EGFR in SHIP2 transfected cells also shows the presence of Shc in immunoprecipitates (data not shown). Our data therefore indicate the presence of two proteins that co-precipitated with the EGFR: SHIP2 and Shc in EGF stimulated cells.

**SHIP2 tyrosine phosphorylation at two sites**

The phosphorylation of SHIP2 in response to EGF prompted us to test whether it was phosphorylated at its NPAY site at the C-terminal part of SHIP2 (Fig. 2). This site was indeed proposed to account for Shc binding through its PTB domain of SHIP2 (18). We have tested an antibody made against a tyrosine phosphorylated peptide KNSFNNPApYYVLEGV that surrounded SHIP2 NPAY site (Fig. 3A). When SHIP2 was transfected in COS-7 cells, the antibody recognized SHIP2 in EGF stimulated cells particularly upon stimulation. It does not recognize the truncated form of SHIP2 or tSHIP2 that does not have the NPAY site (Figs 2 and 3A). Antibodies to SHIP2 phosphorylated peptide cross-reacted with the EGFR at 170 kDa that was strongly phosphorylated in response to EGF (confirmed by reprobing experiments with EGFR antibodies, data not shown). The presence of tyrosine phosphorylated NPXY sites in autophosphorylated EGFR could perhaps explain the cross-reactivity. The data are consistent with the tyrosine phosphorylation of SHIP2 at NPAY site i.e. Tyr 986 in response to EGF.

We have prepared a construct SH2 -SHIP2 which does not have SHIP2 SH2 domain. This construct was much less phosphorylated in response to EGF as compared to wild type SHIP2 (Fig. 3A). The bottom part of the figure shows the expression of the constructs as detected by immunoblotting with anti-SHIP2 antibodies (SHIP2, SH2 -SHIP2 and tSHIP2 in Fig. 3B).

In previous studies, it was proposed that the optimal ligand for SHIP1 SH2 domain was Y(Y/D)X(L/I/V) consistent with SHIP1-binding to immunoreceptors (32). SHIP2 could be isolated by the same peptides by affinity chromatography (Erneux, unpublished). We used this procedure to isolate transfected SHIP2 and
tSHIP2. Western blot analysis shows that the two constructs were tyrosine phosphorylated particularly in EGF stimulated cells (Fig. 4). The bottom part of the figure shows the expression of both constructs by anti-His immunobloting.

SHIP2 colocalisation with the EGFR in EGF-stimulated COS-7 cells

His-tagged SHIP2 was expressed in COS-7 cells and its cellular localisation was revealed by anti-His antibody and confocal analysis. Cells were stimulated or not by EGF. Cells transfected with the vector alone did not show any signal (Fig. 5A,B), in contrast to cells transfected with SHIP2 (Fig. 5C, D). SHIP2 transfected cells showed a cytoplasmic localisation in the absence of EGF (Fig. 5C). In EGF stimulated cells, a relocation of SHIP2 could be seen at plasma membranes as shown in Fig. 5D. The colocalisation of SHIP2 and the EGFR in the same membranes by double staining as pointed out by an arrow is shown in Figs. 5E,F,G.

SHIP2 SH2 domain does not interact with the EGFR

Since we have shown that SHIP2 was precipitated by the EGFR, we addressed the question whether this was mediated by SHIP2 SH2 domain, directly. His tagged recombinant SHIP2 SH2 domain could be affinity trapped with a tyrosine phosphorylated peptide immobilized on a resin suggesting that it is properly folded. It does not interact with unphosphorylated peptide (data not shown). When added to a COS-7 cell lysate SHIP2 SH2 domain was not able to precipitate the EGFR particularly in cells, which had been stimulated by EGF. Fig. 6 A shows in a total lysate the phosphorylation of the EGFR in the presence of EGF (blot pTyr in lanes 1 and 2). The presence of the SH2 construct (His tagged) after adsorption on Pro_bound resin could be seen in lanes 5 and 6. Lanes 3 and 4 are control lanes without addition of any construct but the resin alone. No signal was detected in anti-EGFR immunoblots (Fig. 6B, lanes 5 and 6). Fig. 6C shows the detection of Shc in EGF stimulated cells upon interaction with SHIP2 SH2 construct (in lane 6 of the same blot probed with anti-Shc antibodies). In another series of experiments we transfected our SHIP2 constructs in COS-7 cells (see Fig. 3 for the various His tagged constructs). The EGFR was immunoprecipitated and the various constructs were immunodetected by anti-6xHis antibody. Neither the SH2 domain (Fig. 7D), nor SHIP2 catalytic domain (Fig. 7A) were precipitated with the EGFR. In
contrast, SHIP2, tSHIP2 and SH2 -SHIP2 were precipitated by the EGFR (Fig. 7A). The presence of the SHIP2 constructs in total lysates was checked by Western blotting and anti-6xHis immunoblotting (total lysates in Fig. 7C and D). The precipitation of the EGFR could be seen in Fig. 7B. It was verified that Shc was immunodetected in all transfected cells after precipitation of the EGFR (data not shown).

**PIP3 levels and PKB activity in SHIP2 transfected COS-7 cells**

PIP3 phosphatase activity has been measured in transfected COS-7 cells after immunoprecipitation with anti-SHIP2 antibodies. SHIP2 transfected cells showed PIP3 phosphatase activity after immunoprecipitation. When cells were stimulated with EGF for 2, 5 and 120 min no change in activity was detected as compared to unstimulated cells (Fig. 8A). No activity could be detected in the presence of the antigenic peptide added in the presence of SHIP2 antibodies in transfected COS-7 cells (Fig. 8A). COS-7 cells were transfected with SHIP2, tSHIP2 or with the vector as control. In the absence of EGF, no PIP3 could be detected in vector or SHIP2 transfected cells (data not shown). In the presence of EGF, PIP3 was produced in the cells with a maximal value at 0.5 min (data not shown). EGF induced production of PIP3 was decreased in SHIP2 transfected cells as compared to vector or control transfected cells (Fig. 8B). Similar results were obtained with tSHIP2. The data therefore indicate that, in intact cells, SHIP2 is acting as a PIP3 5-phosphatase.

We also measured PKB activity in COS-7 cells transfected with SHIP2. COS-7 cells were transfected by vector alone or SHIP2. After stimulation of the cells with EGF, PKB was immunoprecipitated and its activity was determined. SHIP2 overexpression led to an important decrease in PKB activation upon EGF stimulation (Fig. 9). This modulation was about 40% as compared to cells transfected with the vector alone and varied from 20 to 50% (three experiments). The decrease in PKB activity was reversed when a catalytic mutant SHIP2C689S was transfected in COS-7 cells (Fig. 9).
Discussion

SHIP1 is expressed exclusively in haematopoetic tissue and developing spermatogonia (33-36). It has been identified as a crucial regulator of BCR signalling with a potential role in proliferation and apoptosis (37, 38). The inhibitory receptor FcγRIIB1 recruitment of SHIP1 results in blocked Tec kinase-dependent calcium signalling (23). SHIP2 has a much wider distribution in both non-haemopoietic and haemopoietic cells (12, 16). Both SHIP1 and SHIP2 have an SH2 domain that could interact with ITIM motifs in Fc receptors (16, 20). Both proteins could be phosphorylated on tyrosine and could bind Shc (18, 33). Proline rich sequences found at their carboxyl terminus resulted in the recruitment of other proteins i.e. Grb2 or Abl (18). The absence of SHIP1 in mice resulted in a myeloproliferative-like syndrome and consolidation of the lungs by infiltration of macrophages (39). In contrast, recent data obtained on SHIP2 deficient mice indicated that loss of SHIP2 leads to increased sensitivity to insulin indicating therefore that in this model, SHIP2 is involved in the insulin-signalling pathway \textit{in vivo} (21).

The association of the PI 3-kinase pathway with activated growth factor receptors and insulin signalling has been reported (40-41). A series of growth factors (EGF, platelet derived growth factor, NGF) and insulin stimulated tyrosine phosphorylation of a 145 kDa protein referred to as 51C/SHIP2 (19). Based on the data presented here and before by Western blot analysis, SHIP2 runs in our experiments as a 160 kDa protein (discussed in 13-18) and EGF stimulates the tyrosine phosphorylation of a 160 kDa that was recognized by our SHIP2 antibodies. The identity of 160 kDa SHIP2 in cells was also confirmed at the protein level by mass spectrometry (Erneux and Communi, unpublished data).

EGF being the most potent extracellular signal to phosphorylate SHIP2, this effect was further characterized in this study. In our model of COS-7 transfected cells, SHIP2 tyrosine phosphorylation was prolonged over 120 min of stimulation by EGF. This is different in SH-SY5Y cells where Habib et al. only found a transient phosphorylation after 5 min EGF stimulation. The reasons for this are not understood but could result from the use of two different cell models. Since SHP-2, an SH2 domain tyrosine phosphatase was shown to interact with growth factors
receptors (EGFR, c-KIT and the erythropoietin receptor, 42-44), we addressed the question whether such interactions could also be observed between SHIP2 and the EGFR.

Our data indicate that immunoprecipitation of the EGFR shows the presence of SHIP2 in untransfected and SHIP2 transfected COS-7 cells. We could do the reciprocal immunoprecipitation experiment in SHIP2 transfected cells: the EGFR was clearly detected in anti SHIP2 immunoprecipitates. SHIP1 SH2 domain had been shown before to interact with a series of receptors (25, 38, 45). This was not observed with SHIP2 SH2 domain and the EGFR. Neither in transfection experiments nor in direct pull down experiments, we were able to detect the EGFR directly bound to SHIP2 SH2 domain. We could however detect the presence of Shc in pull down experiments suggesting that SHIP2 SH2 domain could interact with this adaptor protein as shown before for SHIP1 SH2 domain (46). By immunoprecipitating the EGFR, tSHIP2 and SH2-SHIP2 could be detected in anti-His immunoblots. No interaction was detected in cells overexpressing SHIP2 SH2 domain, nor SHIP2 catalytic domain. The data indicate that the interaction does not require the last 366 amino acids of SHIP2, nor SHIP2 catalytic domain. We could however not rule out that SHIP2 SH2 domain is not participating in the interaction particularly, if the interaction is indirect. Recent data indicated that SHIP2 SH2 domain was able to interact with the p130Cas adaptor protein (22). We did not observe the presence of this protein in SHIP2 immunoprecipitates of COS-7 cells stimulated by EGF. The interaction observed with SHIP2 in COS-7 cells is quite different from the SH2-dependent recruitment of PLC-γ1 to the EGFR (47), SHIP1 to the erythropoietin receptor (48) or to c-Met (45).

We have clearly shown in immunoprecipitates of the EGFR the presence of Shc and SHIP2 suggesting the formation of a complex of at least three proteins SHIP2, Shc and the EGFR. The formation of ternary complexes between SHIP1, Grb2 and Shc in BCR stimulated cells has been reported in B lymphocytes (49). CD22 is a transmembrane protein that is expressed on the surface of mature B cells. The 140 amino acid cytoplasmic domain of CD22 contains six tyrosines localized within immunoreceptor tyrosine-based inhibitory motifs and immunoreceptor tyrosine-based activation sequences. It is proposed that SHIP1 binds CD22 indirectly through the formation of a complex that includes Shc, Grb2 and SHIP1 (50). We propose a similar type of interaction between SHIP2 and the EGFR.
When the His-tagged SHIP2 was expressed in COS-7 cells and stained with anti-6xHis antibody, a signal was observed at cell membranes upon EGF stimulation and was colocalized with the EGFR. In the absence of EGF, SHIP2 was mainly in the cytoplasm. It is important to note that the relocation of SHIP1 at the vicinity of the membrane seems to be a general phenomenon observed in stimulated platelets (29), B cells (51) and in this study in COS-7 cells stimulated by EGF and transfected by SHIP2.

We have tested an antibody made against a tyrosine phosphorylated peptide that surrounded SHIP2 single NPAY site (i.e. Tyr-986). The data we have obtained in COS-7 cells and CHO-IR (31) suggest that SHIP2 is phosphorylated at that site. The antibody recognized SHIP2 in EGF stimulated cells and essentially upon stimulation. It does not recognized tSHIP2 which does not have the C-terminal Tyr-986 residue. It also poorly recognize SH2 -SHIP2 in response to EGF suggesting the involvement of SHIP2 SH2 domain in SHIP2 phosphorylation and/or localization to the active tyrosine kinase. Similar phosphorylation data have been obtained in primary astrocytes stimulated by PDGF and a mutant of SHIP2 SH2 domain (53). In HeLa cells, SHIP2 was predominantly found in focal contacts formed in early spreading cells. The SH2-defective SHIP2 mutant did not localize to focal contacts arguing in favour of a role of SHIP2 SH2 domain in localization (22).

We could isolate SHIP2 and tSHIP2 by affinity chromatography. Both constructs were tyrosine phosphorylated upon EGF stimulation. Since tSHIP2 is phosphorylated on tyrosine (in a EGF dependent manner), we concluded for the first time that SHIP2 must be phosphorylated at least at two sites: one at the C-terminal end (presumably Tyr-986) and another tyrosine residue present in tSHIP2. These two sites could generate docking sites for SH2 containing proteins and take part of the complex between SHIP2, Shc and the EGFR. Interestingly, tSHIP2 was still an active PIP3 phosphatase when tested in vitro (27) and in intact cells in COS-7 transfected cells (this study).

We do not observe any change of PIP3 5-phosphatase activity upon EGF stimulation. Similar data have been shown for SHIP1 in B cells after FcγRIIB coligation (25, 51, 52). SHIP1 has been shown to be relocated to the actin cytoskeleton (29) upon thrombin stimulation in human platelets. It is well known that EGF could stimulate the activation of PI 3-kinase (54, 55). PKB is an enzyme
activated downstream the PI 3-kinase activation and is involved in protection of apoptosis (56, 57). We show that when SHIP2 is transfected in COS-7 cells, it provokes a decrease of PIP3 formation in response to EGF. The effect (30-50 % of its control value i.e. cells transfected with the vector alone) is certainly underestimated since all COS-7 cells were not transfected by SHIP2. Taken together with the relocation of SHIP2 in membranes, the data therefore indicate that SHIP2 is acting as a PIP3 phosphatase upon EGF stimulation. The drop in PI 3-kinase lipid products in COS-7 cells in response to EGF, platelet derived growth factor or lysophosphatidic acid after an initial rise of PIP3 could be related to SHIP2 activation (58). We also see a decrease in PKB activity when SHIP2 is transfected suggesting therefore that the drop in PIP3 may be sufficient to inhibit PKB activity. Similar data on PIP3 and PKB were also obtained in Rat1 fibroblasts overexpressing SHIP2 (13), in glioblastoma cells, insulin stimulated 3T3 adipocytes (19) and in CHO-IR (31).

In swiss 3T3 cells, PKBγ activation triggered by EGF was transient and weaker than with IGF1 (59) an effect that could result from differential activation of PIP3 dephosphorylation and perhaps SHIP2 present in these cells. In conclusion, the importance of tyrosine protein phosphatases (SHP-1 and -2) and SHIP1 and SHIP2 has been related to negative signaling triggered by immunoreceptors and growth factors (26). SHIP2 appears to be widely expressed and phosphorylated. The formation of a ternary complex of SHIP2 with the EGFR and Shc involving probably other proteins not yet identified occurs at cell membranes. This relocation mechanism could be determinant in the function of SHIP2 in vivo (21) as shown before in vitro for SHIP1 in B cell models (52, 60).
Legends to the figures

Figure 1 Western blot analysis of SHIP2 in COS-7 cells

(A) COS-7 cells (3.6 10^6 cells/condition) were incubated with 50 ng/ml EGF for the indicated time. SHIP2 was immunoprecipitated with anti-SHIP2 serum and the phosphorylation of SHIP2 was analysed by immunoblot analysis probed with anti-phosphotyrosine antibody. The membrane was then stripped and reprobed with anti-SHIP2 antibodies (lower panel). (B) COS-7 cells (1.2 10^6 cells/condition) were transfected with SHIP2 and stimulated for 5 min with 50 ng/ml EGF. Cells were lysed and immunoprecipitated with an anti-phosphotyrosine antibody followed by anti-SHIP2 Western blotting. (C) COS-7 cells (1.2 10^6 cells/condition) were transfected with SHIP2. Incubation was for 5 min with 50 ng/ml EGF. After lysis, immunoprecipitation was performed with antibodies against Shc. The blot was probed with anti-SHIP2 antibodies. (D) SHIP2 untransfected or transfected COS-7 cells (1.2 10^6 cells/condition) were incubated with 50 ng/ml EGF for 5 min. Immunoprecipitation was performed with an antibody to the EGFR followed by SHIP2 Western blotting. The same blot was stripped and probed with the anti-EGFR antibody (lower panel). (E) SHIP2 transfected COS-7 cells (1.2 10^6 cells/condition) were immunoprecipitated with anti-SHIP2 antibodies followed by EGFR immunodetection. The membrane was then stripped and reprobed with SHIP2 antibodies (lower panel). The data are representative of one experiment out of two.

Figure 2 Structure of SHIP2 and deletion mutants

All constructs were His tagged. SHIP2 contains an SH2 domain, a catalytic domain, a single NPAY site (i.e. Tyr-986), a proline rich sequence and a SAM domain. A catalytic mutant SHIP2_{C689S} was obtained by mutation of cysteine 689 by a serine residue.

Figure 3 Western blot analysis of tyrosine phosphorylated SHIP2 in response to EGF

(A) COS-7 cells (1.2 10^6 cells/condition) were transfected with SHIP2, tSHIP2 or SH2 -SHIP2 encoding cDNA and stimulated or not with 50 ng/ml EGF for 5 min.
After lysis, crude lysates were probed with phosphorylated SHIP2 peptide antibodies (pSHIP2). (B) A sample of each lysate was analysed by Western blotting using SHIP2 antibodies. The migration of SHIP2 (160 kDa), SH2 -SHIP2 (150 kDa) and tSHIP2 at 105 kDa in (A) and (B) are indicated by arrows. The data are representative of one experiment out of three.

Figure 4 EGF dependent phosphorylation of SHIP2 and tSHIP2
COS-7 cells (1.2 \(10^6\) cells/condition) were transfected with vector alone, SHIP2 or tSHIP2 and stimulated with 50 ng/ml EGF for 5 min. After lysis, crude lysates were mixed with immobilized tyrosine phosphorylated peptide coupled to Actigel. After extensive washing, the beads were subjected to Western blotting probed with anti-phosphotyrosine antibody (top panel). The blot was stripped and probed with anti-6xHis antibody to verify that equivalent amounts of protein were precipitated (lower panel). The data are representative of one experiment out of two.

Figure 5 Immunofluorescent localization of SHIP2 in COS-7 cells upon EGF stimulation.
COS-7 cells transfected with vector alone (A and B) or with SHIP2 (C and D) were unstimulated (left pictures) or stimulated with 50 ng/ml EGF for 3 min (right pictures) as indicated. Cells were stained with anti-6xHis antibody and fluorescein-labelled mouse antibody. In the lower panel, COS-7 cells were transfected with SHIP2 and stimulated with 50 ng/ml EGF for 3 min. The staining in green (E) is for the EGFR. The staining in red (F) is for SHIP2. The pictures G shows the overlaid of the red and green signals. The arrow shows the same membrane in the three pictures (E, F, G).

Figure 6 EGFR does not associate with SHIP2 SH2 domain
(A) COS-7 cells (2.4 \(10^6\) cells/condition) were stimulated for 5 min (lanes 2, 4 and 6) or not (lanes 1, 3 and 5) with 50 ng/ml EGF. Lysates were mixed with 100 µl of Probond resin alone (lanes 3 and 4) or the resin with the added His-tagged SH2 construct (lanes 5 and 6). Samples were analysed by immunoblotting probed with anti-phosphotyrosine antibody (lanes 1 and 2) or anti-6xHis antibody (lanes 3-6).
After stripping, the blots were probed with anti-EGFR antibody (B) and anti-Shc antibody (C). The data are representative of one experiment out of two.

**Figure 7 Co-immunoprecipitation of the EGFR and SHIP2 upon EGF stimulation.**
Transfected COS-7 cells (2.4 \(10^6\) cells/condition) with the indicated constructs (vector, SH2-SHIP2, SHIP2, catal and tSHIP2) were unstimulated (-) or stimulated 3 min (+) by 50 ng/ml EGF. After lysis, cells were immunoprecipitated with anti-EGFR antibody. The samples were analyzed by Western blotting probed with anti-6xHis antibody (A and D). The same membranes were stripped and probed with anti EGFR antibody (B). The amounts of the different overexpressed proteins were analysed in total lysates by immunodetection with an anti-6xHis antibody (C and D). Samples of the transfected SH2 construct (14 kDa) crude and after immunoprecipitation were subjected to SDS/PAGE on a 15 % gel (D). The data are representative of one experiment out of two.

**Figure 8 PIP3 phosphatase activity of COS-7 cells transfected with SHIP2**
(A) Immunoprecipitated SHIP2 of unstimulated (0) or EGF stimulated COS-7 cells transfected with SHIP2 were analyzed for PIP3 phosphatase activity. A negative control is obtained by addition of the antigenic peptide in the presence of SHIP2 serum. The assay is representative of two independent experiments. (B) COS-7 cells were transfected with vector alone, tSHIP2 or SHIP2 and labelled with \(^{32}\)P-orthophosphate as described in the methods section. Analysis of PIP3 and PI 3,4-P\(_2\) was performed after stimulation with 50 ng/ml for 0.5 and 2 min. The data are representative of five different experiments and expressed as a percentage of control (i.e. vector) transfected cells (mean ± SEM). The inhibition of PIP3 is underestimated since only 40 % of the cells were actually transfected.

**Figure 9 SHIP2-dependent inhibition of PKB activity after EGF stimulation.**
COS-7 cells (1.2 \(10^6\) cells/condition) transfected with SHIP2 constructs were unstimulated (-) or stimulated (+) with 50 ng/ml EGF for 10 min. After lysis, cells were immunoprecipitated with an anti-PKB antibody. PKB kinase assays were performed as described in the methods section. Data are mean value ± SEM of a typical experiment out of five.
REFERENCES


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Footnotes

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Abbreviations used: EGFR, EGF receptor; PI 3-kinase, phosphoinositide 3-kinase; SHIP, SH2 domain containing inositol 5-phosphatase; PI 3,4-P₂, phosphatidylinositol 3,4-bisphosphate; PI 4,5-P₂, phosphatidylinositol 4,5-bisphosphate; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; EGF, epidermal growth factor; PKB, protein kinase B; SAM, sterile alpha motif; SH2 domain, Src homology domain; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography.
Figure 1

A

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B

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SHIP2

Mr

Min

220
97
66

Blot pTyr

Blot SHIP2
Figure 1

C  IP  α Shc  M_r
0  5 min  - 220
  - 97
  - 66
  - 46

Blot SHIP2

D  IP  α EGFR  M_r
transfection  -  -  +  +
EGF  -  +  -  +

Blot SHIP2

E  IP  α SHIP2
0  5 min  M_r
- 220
  - EGFR
  - 97
  - 66

Blot EGFR

Blot SHIP2
Figure 2

SH2

catal

ΔSH2-SHIP2

SHIP2

SHIP2_C689S

18

18

18

18

PSWCDRIL

PSWCDRIL

PSWCDRIL

PSWSDRIL

Catalytic

Catalytic

Catalytic

Catalytic

NPAY

NPAY

NPAY

NPAY

SAM

SAM

SAM

SAM

Δ

Δ

Δ

Δ

1258

1258

1258

1258

120

120

18

18

18

18

SH2

SH2

SH2

SH2

118

892

382

847

847
Figure 4

Y(Y/D)X(L/I/V) affinity adsorption

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<td>-</td>
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<tr>
<td>+</td>
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EGF

- +

Blot pTyr

Mr

250

SHIP2

tSHIP2

75

50

35

Blot 6xHis

SHIP2

tSHIP2
Figure 5

0

EGF

A

B

Vector

C

D

SHIP2

E

F

G

EGFR

SHIP2

Overlaid
Figure 6

A

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EGFR

Blot pTyr 6xHis

Control resin Resin + SH2

M_r

250

160

75

50

35

25

15

SH2

B

Blot

EGFR

C

Blot

Shc
Figure 7

A  IP  \( \alpha \) EGFR

\[
\begin{array}{cccccc}
\text{EGF} & \text{vector} & \text{ΔSH2-SHIP2} & \text{SHIP2} & \text{catal} & \text{tSHIP2} \\
- & + & - & + & - & + \\
\end{array}
\]

B  Blot  6xHis

C  Total lysates

D  SH2

\[
\begin{array}{cccccc}
\text{EGF} & \text{IP} & \alpha \text{EGFR} & \text{Total lysates} \\
- & + & - & + \\
\end{array}
\]
Figure 8

A

0.5 min EGF

B

0.5 min EGF

Vector

tSHIP2

SHIP2

% of control

PI 3,4-P_2

PIP3

origin

2 min EGF

Vector

tSHIP2

SHIP2

% of control

PI 3,4-P_2

PIP3
Figure 9

A

\[ \alpha \text{ PKB} \]

\[ \text{IP} \]

\[ \text{vector} \quad \text{SHIP2} \quad \text{SHIP2}_{C689S} \]

EGF  \(-\)  \(+\)

\[ \text{cpm} \]

\[ 0 \quad 1000 \quad 2000 \quad 3000 \]

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The SH2 domain containing inositol 5-phosphatase SHIP2 is recruited to the EGF receptor and dephosphorylates phosphatidylinositol 3,4,5-trisphosphate in EGF stimulated COS-7 cells
Xavier Pesesse, Valerie Dewaste, Florence De Smedt, Muriel Laffargue, Sylvie Giuriato, Colette Moreau, Bernard Payrastre and Christophe Erneux

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