The lipid phosphatase SHIP2 (Src homology 2 domain containing inositol 5-phosphatase 2) has been shown to be expressed in nonhemopoietic and hemopoietic cells. It has been implicated in signaling 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 Src homology 2 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-His6 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, phosphatidylinositol 3,4,5-trisphosphate and protein kinase B activity were decreased in SHIP2-transfected COS-7 cells as compared with the vector alone. SHIP2 ap-

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The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PI 3-kinase, phosphoinositide 3-kinase; PI 3,4-P2, phosphatidylinositol 3,4-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B; SH, Src homology; tSHIP2, truncated SHIP2; PIP3, phosphoinositide chain reaction; TBS, Tris-buffered saline; SHP, SH2 domain containing protein tyrosine phosphatase.

EGF receptor; PI 3-kinase, phosphoinositide 3-kinase; PI 3,4-P2, phosphatidylinositol 3,4-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B; SH, Src homology; tSHIP2, truncated SHIP2; PIP3, phosphoinositide chain reaction; TBS, Tris-buffered saline; SHP, SH2 domain containing protein tyrosine phosphatase.
cell models, such as SH-SY5Y cells (19). SHIP2 was also con-
stitutively tyrosine-phosphorylated in chronic myelogenous
leukemia progenitor cells (18). In B cells, SHIP2 was also
maximally tyrosine-phosphorylated and associated to Shc after
BRCA1 and FcγRIIB cross-linking but not after stimulation of
BRCA1 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 re-
cently 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 FcγRIIB, an
inhibitory receptor that recruits SHIP1 eliminates BCR-
induced PIP3 accumulation (23–25). The data implicate PIP3 as
a crucial regulator of calcium signaling through its ability to
initiate Tec kinase activation. The data also stressed the im-
portance 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
signaling by growth factors and insulin (19), we aimed at mea-
suring PIP3 levels in intact cells. In the course of these studies,
we observed that SHIP2 was coimmunoprecipitated with the
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 stimu-
lated by EGF. Our data could be interpreted as the recruit-
ment of a complex of at least three proteins (SHIP2, Shc, and
the EGFR in EGF-stimulated cells.

EXPERIMENTAL PROCEDURES

Materials

Vector pcDNA3-His and Hypermil-MP were from Amersham Phar-
cmacia Biotech. Superfect was from Qiagen. Protein A-Sepharose CL4B
was obtained from Amersham Pharmacia Biotech. Anti-His, mono-
clonal antibody was from CLONTECH. Anti-phosphotyrosine mono-
clonal antibody 4G10, anti-Shc, and anti-PI3K antibodies were pur-
chased from Molecular Probes. Phosphatidylinositol 4,5-bisphosphate,
from Jackson ImmunoResearch Laboratories. SlowFade kit was pur-
chased from Molecular Probes. Anti-EGFR antibody for immunoprecipita-
tion was from Santa Cruz Biotechnologies. Anti-phosphotyrosine mono-
clonal antibody was from CLONTECH. Anti-phosphotyrosine 4G10 (Upstate
Biotechnology) and anti-EGFR (Santa Cruz Biotechnology) were used for
immunoprecipitations: anti-SHIP2 (12, 16), anti-EGFR (Santa Cruz Biotech-
nologies), anti-His, (CLONTECH), and anti-phosphotyrosine 4G10 (Upstate
Biotechnology). The supernatants were precleared for 5 min at 4 °C
with 150 μl of 10% (w/v) protein A-Sepharose CL4B. This was centri-
gufed at 12,000 × 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-His, 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 analyzed by enhanced chemiluminescence de-
tection. Affinity adsorption of SHIP2 and tSHIP2 was performed using
coupled GDPgYDLSPL peptide to Actigel ALD. After addition of 30 μl
of peptide to 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. Tissue-purified [32P]PIP3 was evaporated
under nitrogen and resuspended with 100 μg of phosphatidylserine into
vesicles. The [32P]PIP3 5-phosphatase activity was measured as de-
scribed (26, 29). Briefly, a total of 20,000 cpm/sample of [32P]PIP3 was
added to 50 μl Tris, pH 7.5, and 0.5 μl of 10,000 × g supernatant. After
incubation, the reaction was stopped, and lipids were extracted. PIP3 and
3,4-P2 were separated by thin layer chromatography in chloroform/acetone/metha-
non/acetic acid/water (50:30:26:24:14, v/v/v/v/v). The corresponding
bands were recovered by centrifugation and washed as described above.

Subcloning of SHIP2 in pcDNA3-His and Site-directed Mutagenese—A truncated form of SHIP2 (tSHIP2) that lacks 366 amino acids at
the C terminus (12, 27) was partially digested for 5 min by NcoI to
obtain a 2.5-kilobase pair fragment. This was further digested with
BamHI and XhoI and resulted in an insert of 2.4 kilobase pairs. The partial
SHIP2 clone (Clone 7 in Ref. 12) was partially digested with NcoI and
XhoI to obtain a 2-kilobase pair fragment. Both fragments were sub-
cloned 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′-GTGCGATCCATGGCC
CCCTCTGGTA-3′, and a 3′-primer containing an XhoI restriction
site (underlined), 5′-CCGCTCGAGTCATCTACAGGAAAGAC-3′.

The PCR product was subcloned into pcDNA3-His C vector. The same
construct was also subcloned in pTRE-His vector to produce the SH2
domain in bacteria as His-tagged construct. Production and purifica-
tion on Prepro 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′-CGCG
GATCCATGGAAGGCAGCTACGGCAAA-3′, and a 3′-primer con-
taining an XhoI restriction site (underlined), 5′-CCTCTGAGTC
CCCTCTGGTA-3′. The PCR product was subcloned into pcDNA3-His C vector. A SHIP2 construct that does not have SHIP2
SH2 domain (ASH2-SHIP2) was prepared with SHIP2 as template and a
5′-primer containing an EcoRI restriction site (underlined), 5′-CG
gATCCATGGAAGGCAGCTACGGCAAA-3′, and a 3′-primer con-
taining an XhoI restriction site (underlined), 5′-CCTCTGAGTC
CCCTCTGGTA-3′. The PCR product was subcloned into pcDNA3-His C vector. The catalytic mutant of SHIP2 in which cysteine 689 was
replaced by a serine was generated by PCR-based mutagenesis using SHIP2 sub-
cloned into pBlueScript as a template according to the manufacturer’s
instructions.

Transfection of COS-7 Cells—COS-7 cells (plated at 1.5 × 106 cells/dish
the previous day) were transected 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 and recovered in 1 ml of Buffer A containing 50 mM Tris/HCl, pH
7.5, 100 mM NaCl, 5 mM EDTA, 1% Brij, 2 mM Na3VO4, 2 μM phos-
phoinositides, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. After
washing the lysate at 4 °C, cells were homogenized at 10,000 × g
for 2 min at 4 °C 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-His, and 25 μl for anti-EGFR). The immune complexes were
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last wash was made without protease and phosphatase inhibitors.

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scribed (26, 29). Briefly, a total of 20,000 cpm/sample of [32P]PIP3 was
added to 50 μM Tris, pH 7.5, and 0.5 μl of 10,000 × g supernatant. After
incubation, the reaction was started by adding immuno-
precipitated SHIP2 from transfect COS-7 cells and 5 μM MgCl2.

The assay was stopped, and lipids were extracted. PIP3 and 3,4-P2 were
separated by thin layer chromatography in chloroform/acetone/metha-
non/acetic acid/water (50:30:26:24:14, v/v/v/v/v). The corresponding
spots were analyzed by PhosphorImager and autoradiography.
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-His_6 antibody was used at a 1:1000 dilution, and the anti-EGFR antibody was used at a 1:250 dilution. 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 was used. The cells were then washed three times with TBS for 10 min and mounted with the 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 1.2×10^6 COS-7 cells in 10-cm-diameter dishes, cells were lysed in 800 μl of ice-cold lysis buffer (80 mM Tris/HCl, 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 mixture). After 20 min of agitation at 4 °C, the different cell supernatants were immunoprecipitated with 4 μl of antibody to PKB coupled to 25 μl of protein A-Sepharose in a total volume of 400 μl Tris/HCl, pH 7.5, 20 mM EDTA, 1 mM EGTA, 200 mM NaCl, 0.2% Triton X-100, 0.1% β-mercaptoethanol, protease inhibitors mixture). PKB activity was determined as described previously (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 from 0.5 min 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 panel). 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 EGFR-stimulated and 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). Fig. 1D, bottom panel, 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 panel, 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 did not recognize tSHIP2, which 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, ΔSH2-SHIP2, that does not have SHIP2 SH2 domain. This construct was much less phosphorylated in response to EGF as compared with wild type SHIP2 (Fig. 2A, Fig. 3A). Fig. 3B shows the expression of the constructs as detected by immunoblotting with anti-SHIP2 antibodies (SHIP2, ASH2-SHIP2, and tSHIP2).

In previous studies, it was proposed that the optimal ligand for SHIP1 SH2 domain was Y(D/I/V)L(V), consistent with SHP1 binding to immunoreceptors (32). SHIP2 could be isolated by the same peptides by affinity chromatography.2 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). Fig. 4, bottom panel, shows the expression of both constructs by anti-His immunoblotting.

SHIP2 Colocalization with the EGFR in EGF-stimulated COS-7 Cells—His-tagged SHIP2 was expressed in COS-7 cells, and its cellular localization 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. 5, A and B), in contrast to cells transfected with SHIP2 (Fig. 5, C and D). SHIP2-transfected cells showed a cytoplasmic localization 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 colocalization of SHIP2 and the EGFR in the same membranes by double staining is shown in Fig. 5, E–G, arrows.

SHIP2 SH2 Domain Does Not Interact with the EGFR—Because we have shown that SHIP2 was precipitated by the EGFR, we addressed the question whether this was mediated

FIG. 2. Structure of SHIP2 and deletion mutants. All constructs were His-tagged. SHIP2 contains an SH2 domain, a catalytic domain (catal), a single NPAY site (NPAY), a proline-rich sequence, and a sterile alpha motif (SAM) domain. A catalytic mutant SHIP2C689S was obtained by mutation of cysteine 689 to a serine residue.

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, the SHIP2 SH2 domain was not able to precipitate the EGFR, particularly in cells that had been stimulated by EGF. Fig. 6A shows in a total lysate the phosphorylation of the EGFR in the presence of EGF (lanes 1 and 2). The presence of the SH2 construct (His-tagged) after adsorption on Probond resin could

FIG. 3. Western blot analysis of tyrosine-phosphorylated SHIP2 in response to EGF. A, COS-7 cells (1.2 x 10⁶ cells/condition) were transfected with SHIP2, tSHIP2, or ΔSH2-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 analyzed by Western blotting using SHIP2 antibodies. The migration of SHIP2 (160 kDa), ΔSH2-SHIP2 (150 kDa), and tSHIP2 (105 kDa) in A and B are indicated by arrows. The data are representative of one experiment out of three.

FIG. 4. EGF-dependent phosphorylation of SHIP2 and tSHIP2. COS-7 cells (1.2 x 10⁶ 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 and probed with anti-phosphotyrosine antibody (top panel). The blot was stripped and probed with anti-Hisα antibody to verify that equivalent amounts of protein were precipitated (bottom panel). The data are representative of one experiment out of two.
Cells were stained with anti-His 6 antibody and fluorescein-labeled right panels or stimulated with 50 ng/ml EGF for 3 min (total lysates in Fig. 7, checked by Western blotting and anti-His6 immunoblotting). The presence of the SHIP2 constructs in total lysates was tSHIP2, and A nor the SHIP2 catalytic domain (Fig. 7D) was precipitated with the EGFR. In contrast, SHIP2, 7A) was precipitated with the EGFR. In contrast, SHIP2, tSHIP2, and ΔSHIP2 were precipitated by the EGFR (Fig. 7A). The presence of the SHIP2 constructs in total lysates was checked by Western blotting and anti-His6 immunoblotting (total lysates in Fig. 7, C 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).

FIG. 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 panels) or stimulated with 50 ng/ml EGF for 3 min (right panels) as indicated. Cells were stained with anti-His6 antibody and fluorescein-labeled mouse antibody. In the bottom panels, COS-7 cells were transfected with SHIP2 and stimulated with 50 ng/ml EGF for 3 min. The staining in green (E) indicates the EGFR. The staining in red (F) indicates SHIP2. G shows the overlay of the red and green signals. The arrow indicates the same membrane in E, F, and G.

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 with 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 or tSHIP2 or with the vector as control. In the absence of EGF, no PIP3 could be detected in vector-transfected 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 with vector-transfected (control) 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 with 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, SHIP2(C689S), was transfected in COS-7 cells (Fig. 9).

FIG. 6. EGFR does not associate with SHIP2 SH2 domain. A, COS-7 cells (2.4 x 10⁶ 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 analyzed by immunoblotting and probed with anti-phosphotyrosine antibody (lanes 1 and 2) or anti-His6 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.

DISCUSSION

SHIP1 is expressed exclusively in hematopoietic tissue and developing spermatogonia (33–36). It has been identified as a crucial regulator of BCR signaling, with a potential role in proliferation and apoptosis (37, 38). The inhibitory receptor FcyRIIB1 recruitment of SHPI results in blocked Tec kinase-dependent calcium signaling (23). SHIP2 has a much wider distribution in both nonhemopoietic and hemopoietic 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 C termini 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-signaling pathway in vivo (21).

The association of the PI 3-kinase pathway with activated growth factor receptors and insulin signaling has been reported (40, 41). A series of growth factors (EGF, platelet-derived growth factor, and nerve growth factor) and insulin stimulated tyrosine phosphorylation of a 145-kDa protein referred to as 51C/SHIP2 (19). Based on the data presented here and previously by Western blot analysis, SHIP2 runs in our experiments as a 160-kDa protein (discussed in Refs. 13–18), and EGF stimulates the tyrosine phosphorylation of a 160-kDa protein 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.3

EGF being the most potent extracellular signal to phospho-
yrlylate 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, in which Habib et al. (19) only found a transient phosphorylation after 5 min of EGF stimulation. The reasons for this are not understood, but it could result from the use of two different cell models. Because 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 of 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 immuno-
precipitation 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 were we 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 or the SHIP2 catalytic domain. The data indicate that the interaction does not require the last 366 amino acids of SHIP2 or the SHIP2 catalytic domain. We could not, however, rule out the possibility 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

3 Erneux, C., and Communi, unpublished data.
in COS-7 cells is quite different from the SH2-dependent recruitment of PLCγ1 to the EGFR (47), SHIP1 to the erythropoietin receptor (48), or SHIP1 to c-Met (45).

We have clearly shown in immunoprecipitates of the EGFR that 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-His 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 the single SHIP2 NPAY site (i.e. Tyr-986). The data that we have obtained in COS-7 cells and Chinese hamster ovary cells overexpressing the insulin receptor (31) suggest that SHIP2 is phosphorylated at that site. The antibody recognized SHIP2 in EGF-stimulated cells and essentially upon stimulation. It does not recognize tSHIP2, which does not have the C-terminal Tyr-986 residue. It also poorly recognizes ΔSHIP2 in response to EGF, suggesting the involvement of the 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 platelet-derived growth factor 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 favor of a role of the SHIP2 SH2 domain in localization (22).

We could isolate SHIP2 and tSHIP2 by affinity chromatography. Both constructs were tyrosine-phosphorylated upon EGF stimulation. Because tSHIP2 is phosphorylated on tyrosine (in an 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 gen-
erate docking sites for SH2-containing proteins and form part of the complex between SHIP2, Src, 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 did not observe any change in PIP3 5-phosphatase activity upon EGFR stimulation. Similar data have been shown for SHP1 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 EGFR could stimulate the activation of PI 3-kinase and is involved in protection of apoptosis (5, 6). We show that when SHIP2 is transfected in COS-7 cells, it provokes a decrease in PIP3 formation in response to EGFR. The effect (30–50% of its control value, i.e. cells transfected with the vector alone) is certainly underestimated because not all COS-7 cells were 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 EGFR stimulation. The drop in PI 3-kinase lipid products in COS-7 cells in the presence of PIP3 may be sufficient to inhibit PKB activity. Similar data on PIP3 and PKB were also obtained in Rat1 fibroblasts when SHIP2 was transfected, suggesting, therefore, that the drop in PIP3 and PKB activity may be sufficient to inhibit PKB activity. Similar

In Swiss 3T3 cells, PKB activation triggered by EGFR was transient and weaker than with IGF1 (57), an effect that could result from differential activation of PIP3 dephosphorylation and perhaps SHIP2 present in these cells. In conclusion, the importance of protein-tyrosine phosphatases (SHIP-1 and SHP-2), 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, probably involving other proteins not yet identified, occurs at cell membranes. This relocation mechanism could be a determinant in the function of SHIP2 in vitro (21), as shown before in vitro for SHIP1 in B cell models (52, 56).

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