Regulation of EphA2 Receptor Endocytosis by SHIP2 Lipid Phosphatase via Phosphatidylinositol 3-Kinase-dependent Rac1 Activation*

Received for publication, September 5, 2006, and in revised form, November 28, 2006. Published, JBC Papers in Press, November 29, 2006, DOI 10.1074/jbc.M608509200

Guangli Zhuang†, Sonja Hunter†, Yoonha Hwang§, and Jin Chen†§
From the †Department of Cancer Biology, ‡Department of Medicine, Division of Rheumatology and Immunology, §Department of Cell and Developmental Biology, and †Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Endocytosis of Eph receptors is critical for a number of biological processes, including modulating axon growth cone collapse response and regulating cell surface levels of receptor in epithelial cells. In particular, ephrin-A ligand stimulation of tumor cells induces EphA2 receptor internalization and degradation, a process that has been explored as a means to reduce tumor malignancy. However, the mechanism and regulation of ligand-induced Eph receptor internalization are not well understood. Here we show that SHIP2 (Src homology 2 domain-containing phosphoinositide 5-phosphatase 2) is recruited to activated EphA2 via a heterotypic sterile α motif (SAM)-SAM domain interaction, leading to regulation of EphA2 internalization. Overexpression of SHIP2 inhibits EphA2 receptor endocytosis, whereas suppression of SHIP2 expression by small interfering RNA-mediated gene silencing promotes ligand-induced EphA2 internalization and degradation. SHIP2 regulates EphA2 endocytosis via phosphatidylinositol 3-kinase-dependent Rac1 activation. Phosphatidylinositol 3,4,5-trisphosphate levels are significantly elevated in SHIP2 knockdown cells, phosphatidylinositol 3-kinase inhibitor decreases phosphatidylinositol 3,4,5-trisphosphate levels and suppresses increased EphA2 endocytosis. Ephrin-A1 stimulation activates Rac1 GTPase, and the Rac1-GTP levels are further increased in SHIP2 knockdown cells. A dominant negative Rac1 GTPase effectively inhibited ephrin-A1-induced EphA2 endocytosis. Together, our findings provide evidence that recruitment of SHIP2 to EphA2 attenuates a positive signal to receptor endocytosis mediated by phosphatidylinositol 3-kinase and Rac1 GTPase.

The Eph family of receptor tyrosine kinases (Ephs) and their ligands, the ephrins, regulate a diverse array of biological responses in development and disease (reviewed in Refs. 1–4). These receptors represent the largest family of receptor tyrosine kinases in the genome, consisting of at least 16 receptors that interact with nine membrane-bound ephrin ligands (reviewed in Refs. 1 and 2). They can be further divided into two groups, class A and class B, based on sequence homology and binding affinity (5). Class A Eph receptors interact with multiple ligands of the ephrin-A family, a group of glycosyl-phosphatidylinositol-linked membrane proteins, whereas class B Eph receptors bind to ephrin-B ligands, a family of transmembrane proteins. Binding of Eph receptors to their ligands induces receptor clustering, receptor transphosphorylation, and activation of kinase activity, followed by activation of signaling cascades that mediate multiple cellular responses (reviewed in Refs. 1 and 4).

Major advances have been made in recent years to dissect the molecular mechanisms by which Ephs/ephrins regulate biological processes. In particular, ligand-induced receptor endocytosis has been studied in a number of biological systems. Upon juxtacrine interaction of cell surface ephrin-B ligand and EphB receptor, ligand–receptor complexes are internalized bidirectionally (6–8). This bidirectional endocytosis of ephrin-B and EphB complexes appears to be sufficient to promote cell detachment in axon withdrawal during growth cone collapse (6, 7). Endocytosis of EphA receptor also appears to be critical in converting an initial adhesive interaction into a repulsive event in growth cone collapse response (9). Outside of the nervous system, ligand-induced phosphorylation of the Eph receptors in tumor cells has been shown to result in receptor endocytosis and down-regulation of surface receptors (10, 11). Enhanced EphA2 endocytosis and subsequent degradation are associated with decreased malignant cell behavior. Activating EphA2 monoclonal antibodies (12), ephrin-mimetic peptides (13), or adenovirus-expressing ephrin-A1 ligand (14) have been developed to induce receptor endocytosis as a means to reduce EphA2 activity. Despite the important roles that Eph receptor endocytosis plays in biological responses, relatively little is known about how this pathway is regulated.

We have previously shown that activation of the EphA2 receptor regulates Rac1 GTPase activity through a PI2 3-kinase-dependent pathway (15). Through a yeast two-hybrid screen, we identified two proteins that interact with the activated

* This work was supported by National Institutes of Health Grants CA95004 and CA114301 (to J. C.) and National Institutes of Health Postdoctoral Fellowship GM072461 (to S. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Medicine, Vanderbilt University School of Medicine, A-4323 MCN, 1161 21st Ave. S., Nashville, TN 37232-2363. Tel.: 615-343-3819; Fax: 615-343-7392; E-mail: jin.chen@vanderbilt.edu.

‡ The abbreviations used are: PI, phosphatidylinositol; PIP3, phosphatidylinositol 3,4,5-trisphosphate; SH2, Src 2; GST, glutathione S-transferase; MBP, maltose-binding protein; ELISA, enzyme-linked immunosorbent assay; SAM, sterile α motif; EGF, epidermal growth factor; EGFR, EGF receptor; E3, ubiquitin-protein isopeptide ligase; siRNA, small interfering RNA.
SHIP2 Regulates EphA2 Endocytosis

EphA2 receptor and are capable of regulating the level of phosphatidylinositol 3,4,5-trisphosphate (PIP3); the p85 subunit of PI 3-kinase and SHIP2 (SH2-containing phosphoinositide 5-phosphatase 2). SHIP2 belongs to the SHIP family of lipid phosphatases that dephosphorylates PIP3 produced by PI 3-kinase (16). PIP3 has been shown to interact with pleckstrin homology domain-containing proteins, such as the Ras and Rho family guanine nucleotide exchange factors, leading to the membrane recruitment and activation of these proteins (17). Although expression of SHIP1 is restricted to the hematopoietic cell lineage, SHIP2 is expressed in many cell types (18). These data suggest that SHIP2, together with PI 3-kinase, may function downstream of the EphA2 receptor to regulate Rho family GTPases.

In this study, we investigate the molecular mechanisms that regulate ephrin-Eph endocytosis. We show that ligand stimulation induces EphA2 receptor internalization in MDA-MB-231 mammary carcinoma cells. In response to ephrin binding to EphA2, SHIP2 is recruited to the sterile α motif (SAM) domain of EphA2. Overexpression of SHIP2 inhibited ligand-induced receptor endocytosis. Conversely, suppression of SHIP2 expression by small interfering RNA (siRNA)-mediated gene silencing increased EphA2 endocytosis and subsequent degradation. The mechanism of regulation of EphA2 endocytosis by SHIP2 involves down-regulation of cellular PIP3 levels and inhibition of Rac1 GTPase activity. These findings suggest that SHIP2 plays a central role in regulation of EphA2 receptor endocytosis.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—The mouse EphA2 cytoplasmic domain was cloned into pBridge-LexA (BD Biosciences) (pSGS2) as a bait to screen a human placenta library consisting of 3.5 × 10^6 independent clones (Clontech) as described (19). Briefly, yeast strain L40 (MATa his3D200 trp1–901 leu2–3112 ade2 lys2–(4LexAop-HIS3) ura3–(8LexAop-lacZ) gal4) was transformed with pSGS2 and the placenta cDNA library. The resulting transformants were screened for histidine prototrophy and expression of LacZ. The His^+ /LacZ^+ clones that did not interact with lamin C were subjected to PCR analyses to eliminate duplicate clones. Among 14 unique His^+ /LacZ^+ clones, six overlapping clones encompassing the SAM domain of the SHIP2 gene were identified.

Antibodies—Antibodies used for immunoblot or immunocytochemistry include anti-Myc (1:500; BD Biosciences), anti-FLAG (1:1000; Sigma), anti-EphA2 (1:1000; Santa Cruz Biotechnology), anti-phosphotyrosine (1:400; Santa Cruz Biotechnology), anti-tubulin (1:1000; Sigma), anti-Rac1 and anti-Cdc42 antibodies (1:250; PharMingen), anti-PIP3 (1:250; Echelon), and anti-EEA1 (1:1000; BD Biosciences). For immunoprecipitation, 1.5 μg of polyclonal rabbit anti-SHIP2 antibody (Santa Cruz Biotechnology) was used. Anti-SHIP2 polyclonal chick antibodies were made by Zymed Laboratories Inc., using the purified GST-SAM domain of the SHIP2 protein as antigen, and used in Western blot analysis.

In Vitro Binding Assay—MBP-EphA2-SAM, the fusion of the intracellular portion of mouse EphA2 SAM domain and maltose-binding protein, was created from pMAl-c2X (New England Biolabs) and purified on amylose resin according to the manufacturer’s instructions. Escherichia coli lysate containing GST-SHIP2-SAM domains was incubated with amylose-bound MBP-EphA2-SAM or control MBP alone. After extensive washing, bound proteins were eluted and subjected to Western blot analyses using anti-EphA2 and anti-SHIP2 antibodies.

Co-immunoprecipitation and Western Blot Analyses—COS7 cells were co-transfected with 1 μg each of FLAG-tagged SHIP2 and EphA2 or Myc-tagged EphA2 mutants using Lipofectamine 2000. Cells were lysed in 1% Nonidet P-40 buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 plus 50 mM protease inhibitors). Anti-FLAG antibody (Sigma) was used to immunoprecipitate SHIP2. The resulting proteins were resolved on SDS-PAGE and subjected to Western blot using anti-EphA2 and anti-SHIP2 antibodies.

siRNA-mediated Silencing of SHIP2 in MDA-MB-231 Cells—SHIP2 knockdown was achieved by siRNA-mediated stable silencing of SHIP2 via retroviral transduction, as described (20). Briefly, human SHIP2 siRNAs and control siRNA were designed using Invitrogen software according to the manufacturer’s instructions. A 64-base pair oligonucleotide linker containing SHIP2-specific sense and corresponding antisense sequences, flanking a 6-base hairpin, was generated, PAGE-purified, and subcloned into retroviral vector pRS (a gift of R. Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands). pRS SHIP2 siRNA or control siRNA retrovirus were produced in the Phoenix cell packaging line. For siRNA expression, MDA-MB-231 cells were infected with pRS SHIP2 or control siRNA retroviruses and selected in the presence of 5 μg/ml puromycin. Pooled clones of MDA-MB-231, number 1, 2, 3, and 4, or single clone 2A expressing either SHIP2 or control siRNAs were analyzed. The level of SHIP2 knockdown in pooled clones was assessed by quantifying band intensity of SHIP2 over tubulin using Scion Image software.

Confocal Microscopy Analysis—Control or SHIP2 knockdown cells were plated on coverslips in 6-well dishes and cultured to 50% confluence. Growth medium was replaced with 1 ml of starvation medium (Dulbecco’s modified Eagle’s medium plus 1% bovine serum albumin or Opti-MEM) per well. For immunofluorescence assays, cells were stimulated with 1 μg/ml ephrin-A1 for 30 min and fixed with 4% paraformaldehyde. Following fixation, cells were incubated with primary antibodies (anti-EphA2, 1:1000 (Santa Cruz Biotechnology); monoclonal anti-EEA1, 1:1000 (BD Biosciences); monoclonal anti-Myc, 1:1000 (Cell Signaling); monoclonal anti-FLAG, 1:1000 (Sigma)) for 2 h at 25 °C or overnight at 4 °C, followed by secondary antibodies (Alexa594-conjugated goat anti-mouse (1:3000) or Alexa 488-conjugated goat anti-rabbit (1:3000) from Molecular Probes). Images were recorded by confocal microscopy. Internalization was quantified by drawing an area under the cell membrane (within the cell but excluding cell membrane) of each cell on the confocal image, and pixels of
The SHIP2 SAM domain interacts with the cytoplasmic domain of EphA2 in yeast. The binding of SHIP2 to EphA2 receptor is quite specific, since it binds to neither EphB1 receptor nor other EphA receptors (data not shown).

The observation that the SHIP2 SAM domain interacts with the cytoplasmic domain of EphA2 in yeast raised the possibility that SHIP2 and EphA2 interact in mammalian cells. To test this hypothesis, we transfected COS7 cells with a FLAG-tagged full-length SHIP2 expression construct and immunoprecipitated cell lysates with an anti-FLAG antibody. As shown in Fig. 1A, the EphA2 was readily detected in anti-FLAG immunoprecipitates. The co-immunoprecipitation of EphA2 with the anti-FLAG antibody was dependent on the expression of SHIP2. Since EphA2 was undetectable in the unstimulated samples and in immunoprecipitates in which a control vector was expressed. In the reverse direction, SHIP2 was easily detected in anti-EphA2 immunoprecipitates from cells transfected with SHIP2 and stimulated with ephrin-A1 (Fig. 1B). However, SHIP2 does not bind to EphA3 or EphA4 (Fig. 1E), demonstrating the binding specificity of SHIP2 to EphA2.

To determine whether endogenous EphA2 receptor can bind to SHIP2, MDA-MB-231 cells were stimulated with ephrin-A1, and cell lysates were subjected to a GST-SHIP2-SAM pull-down assay. As shown in Fig. 1C, GST-SHIP2, but not control GST, binds to endogenously expressed EphA2 in response to ephrin-A1 ligand stimulation. In addition, EphA2 was detected in anti-SHIP2 immunoprecipitates upon ephrin-A1 stimulation in MDA-MB-231 cells (Fig. 1D). These findings indicate that SHIP2 is recruited to activated EphA2 receptors in breast cancer cells.

**Mapping of Interaction Domains between EphA2 and SHIP2**

To identify the domains within the EphA2 receptor that mediate the interaction with SHIP2, a panel of EphA2 constructs was generated in which portions of EphA2 were deleted. The resulting EphA2 mutants were expressed at comparable levels in the yeast two-hybrid assay and tested for their interaction with SHIP2 (Fig. 2A). EphA2 receptors lacking the juxtamembrane domain, kinase domain, or PDZ binding motif were capable of binding to SHIP2, whereas the EphA2 SAM domain alone binds to SHIP2 as well as the wild-type receptor, indicating that the SAM-SAM interaction is required for binding to SHIP2. The original six independent and overlapping yeast two-hybrid interacting clones of SHIP2 all contained the SAM domain. As shown in Fig. 2B, the SAM domain of SHIP2 alone can bind to the EphA2 cytoplasmic domain, whereas the SHIP2 SH2 domain fails to bind to EphA2.

The heterotypic interaction between the EphA2 SAM and the SHIP2 SAM domains was verified in mammalian cells. As predicted from the yeast two-hybrid assay, binding of EphA2 to SHIP2 is independent of EphA2 receptor phosphorylation, since a kinase-dead (D738N) mutant or three Tyr to Phe mutations (Y921F, Y929F, and Y959F) in the EphA2 SAM domain did not affect binding significantly (Fig. 2C); nor did the deletion of the SH2 domain in SHIP2 protein (Fig. 2E). In contrast, deletion of the EphA2 SAM domain abolished the ability to bind to SHIP2, whereas the EphA2 SAM domain alone was
SHIP2 Regulates EphA2 Endocytosis

A capable of interacting with SHIP2 (Fig. 2D). Conversely, deletion of the SHIP2 SAM domain also inhibited binding to the EphA2 receptor (Fig. 2E), confirming the heterotypic interaction between the two SAM domains.

To test for a direct interaction between EphA2 and SHIP2, the SAM domains of SHIP2 and EphA2 were expressed as GST and MBP fusion proteins, respectively. As shown in Fig. 2F, GST-SHIP2-SAM bound to MBP-EphA2-SAM that was linked to amyllose beads. After extensive washing, only GST-SHIP2-SAM and MBP-EphA2-SAM were eluted from the column. Although GST-SHIP2-SAM bound to MBP-EphA2-SAM, it failed to interact with MBP alone, indicating that the binding is specific to the EphA2-SAM in vitro and independent of phosphorylation. Taken together, these results suggest that the EphA2 receptor binds to the SHIP2 phosphatase through a SAM-SAM heterotypic interaction.

Overexpression of SHIP2 Inhibits Ligand-induced EphA2 Receptor Endocytosis—As a first step to determine the functional link between SHIP2 and EphA2 endocytosis, we analyzed the kinetics of ligand-induced EphA2 receptor internalization in MDA-MB-231 cells by confocal microscopy. As shown in Fig. 3A, upon stimulation with soluble ephrin-A1, EphA2 receptor (green) clusters rapidly and localizes in large patches, followed by internalization of the receptor. These internalized vesicles were stained with EEA1 (red), an early endosomal marker (21), suggesting that EphA2 receptor is internalized by endocytosis. Next, we overexpressed wild-type Myc-tagged SHIP2 in COS7 cells and examined ephrin-A1-induced receptor endocytosis. Anti-Myc antibodies detected cells expressing exogenous SHIP2 (red), whereas subcellular localization of the endogenous EphA2 receptor was detected by anti-EphA2 antibodies (green). Internalization of EphA2 receptor (green) was significantly inhibited in cells overexpressing SHIP2 (red) but not SHIP2/ΔSAM (Fig. 3, B and C), suggesting that SHIP2 regulates ligand-induced EphA2 receptor endocytosis via its SAM domain.

Although SHIP2 can function directly as a phosphoinositide phosphatase, it is interesting to note that it also contains multiple functional domains/motifs that may mediate the recruitment of other signaling molecules. To determine whether SHIP2 regulates EphA2 endocytosis via its phosphatase activity or acting as an adaptor protein, we transfected COS7 cells with a catalytically inactive SHIP2 mutant, D607A (22, 23). As shown in Fig. 3, B and C, the D607A mutant did not inhibit ephrin-A1-induced EphA2 receptor internalization, suggesting that the enzymatic activity of SHIP2 is required for regulation of EphA2 endocytosis.

Enhanced EphA2 Receptor Endocytosis in SHIP2 Knockdown Cells—To understand the role of SHIP2 in EphA2 receptor endocytosis in breast cancer cells, we inhibited the endogenous

FIGURE 1. Activated EphA2 receptor recruits SHIP2 in mammalian cells. A, full-length EphA2 and FLAG-tagged SHIP2 cDNA expression plasmids or vector alone were co-transfected into COS7 cells. Cells were stimulated with ephrin-A1 at the indicated times, and cell lysates were immunoprecipitated (IP) with anti-FLAG, followed by Western blot analysis with anti-EphA2 antibodies. Blots were stripped and reprobed for expression of SHIP2. B, FLAG-tagged SHIP2 or vector were transfected into COS7 cells and stimulated in the presence or absence of ephrin-A1 following a time course. Endogenous EphA2 receptors were immunoprecipitated by anti-EphA2, followed by Western blot analyses with anti-SHIP2. C, MDA-MB-231 cell lysates were added to GST-SHIP2 or control GST resin, and bound proteins were eluted and analyzed by Western blot analysis using anti-EphA2 antibodies. D, MDA-MB-231 cells were stimulated with ephrin-A1 for the indicated time, and cell lysates were immunoprecipitated with anti-SHIP2 or control IgG, followed by Western blot analysis using anti-EphA2 antibodies. E, Myc-tagged SHIP2 and EphA2, A3, or A4 receptors were co-transfected into COS7 cells. SHIP2 was immunoprecipitated with anti-Myc-conjugated resins, followed by Western blot analysis (IB) using anti-EphA2, A3, or A4 antibodies.
SHIP2 expression by retrovirus-mediated siRNA knockdown. Four siRNA duplexes of sequence specific to SHIP2 were tested in MDA-MB-231 breast cancer cells. These siRNAs were stably expressed in MDA-MB-231 cells by retroviral transduction. As shown in Fig. 4A, MDA-MB-231 cells expressing siRNA2 inhibited the expression of endogenous SHIP2 to greater than 80%. siRNAs 1, 3, and 4 also produced a target protein suppression but to a lesser degree (~50%). A control siRNA with two mismatches in sequence 2 was completely inactive.

To investigate whether ephrin-A1-induced EphA2 receptor endocytosis is affected by knockdown of SHIP2 in MDA-MB-231 cells, SHIP2 knockdown or control cells were treated with ephrin-A1, and EphA2 receptor localization was followed by confocal immunofluorescence analysis. As shown in Fig. 4B, in SHIP2 knockdown cells, ephrin-A1 stimulation enhanced the accumulation of internalized EphA2 vesicles, compared with parental MDA-MB-231 (data not shown) or control siRNA-expressing cells. Quantification of these vesicles revealed that there was an ~2-fold increase in vesicle pixel density in SHIP2 knockdown cells, compared with control siRNA-expressing cells, indicating that SHIP2 negatively regulates EphA2 endocytosis.

Internalization of cell surface EphA2 was also tracked using a surface biotinylation assay described by Le et al. (24). MDA-MB-231 cells were surface-biotinylated at 4 °C and then returned to 37 °C following a time course to allow trafficking to resume. Cells were incubated briefly with a dilute trypsin solution to remove cell surface proteins. Internalized EphA2 was sequestered at 37 °C and therefore protected from trypsin digestion. Little if any EphA2 was detected in control cells (Fig. 4C, 0 min), confirming that under these conditions, biotinylated cell surface proteins were efficiently removed by trypsin. In contrast, after 10, 20, and 30 min at 37 °C, a biotinylated pool of EphA2 was detected in cells following trypsin treatment (Fig. 4C, 10, 20, and 30 min), indicating that EphA2 was internalized and protected from tyrosine phosphorylation. Ephrin-A1 induced EphA2 internalization in both control and SHIP2 knockdown cells, but the level of internalized EphA2 was appreciably higher in SHIP2 knockdown cells compared with that in control cells (Fig. 4C).

We also observed a basal level of EphA2 internalization in the absence of ligand stimulation (Fig. 4C, right).}

However, this level is significantly lower than that with ephrin-A1 stimulation. These data provide independent evidence that ephrin-A1 stimulation induces EphA2 internalization, and this process is regulated by SHIP2.

To determine whether increased endocytic vesicles observed in SHIP2 knockdown cells affected ligand-induced receptor degradation, we performed Western blot analysis of EphA2 following ephrin-A1 treatment. The total level of EphA2 receptor decreased with increasing length of ephrin-A1 treatment, and this process was enhanced in SHIP2 knockdown cells (Fig. 4D), indicating that SHIP2 regulates EphA2 degradation.

SHIP2 Regulates EphA2 Endocytosis Through Modulation of Cellular PIP3 Levels—We next explored the mechanisms by which SHIP2 regulates EphA2 receptor endocytosis. As SHIP2 is a phosphoinositide 5-phosphatase, we determined the impact of SHIP2 knockdown on phospholipid PIP3 levels. We measured PIP3 levels by ELISA using an anti-PIP3 monoclonal antibody that is widely used in many studies (25–28). As shown
SHIP2 Regulates EphA2 Endocytosis

A

EphrinA1-Fc  EphA2  EEA1  Overlay

0min

2min

15min

B

EphA2 + Myc-SHIP2  EphA2 + SHIP2ΔSAM  EphA2 + SHIP2 D607A

α-EphA2

α-EphA2 + α-Myc

C

Graph showing vesicle pixel density comparison between Control and Transfected conditions.
in Fig. 5C, compared with those in control cells, PIP$_3$ levels in SHIP2 knockdown cells increased ~2-fold, either at resting phase or stimulated by ephrin-A1. Because phospholipid PIP$_3$ is a product of PI 3-kinase, we tested whether inhibition of PI 3-kinase affects EphA2 endocytosis. The PI 3-kinase inhibitor LY294002 (50 μM) significantly inhibited increased PIP$_3$ levels (Fig. 5C) as well as ephrin-A1-induced EphA2 endocytosis (Fig. 5, A and B). Taken together, these data indicate that SHIP2 regulates EphA2 endocytosis via modulation of cellular PIP$_3$ levels.

SHIP2 Regulates EphA2 Endocytosis through a Rac1 GTPase-dependent Pathway—Previous studies have shown that Vav family guanine nucleotide exchange factors and Rac GTPase activity are required for endocytosis of Eph molecules (7, 9). Since the activation status of guanine nucleotide exchange factors can be regulated by PIP$_3$ levels (29, 30) and PIP$_3$ is a major substrate of SHIP2 (16), we investigated whether ephrin-A1-induced Rac1 GTPase activation is affected in SHIP2 knockdown cells. As shown in Fig. 6A, upon ephrin-A1 stimulation, we detected a transient activation of Rac1 GTPase, with a peak at 2.5–5 min. In SHIP2 knockdown cells, the basal level of GTP-bound Rac1 is increased, and the Rac1 activity is further enhanced upon ephrin-A1 stimulation. In contrast, ephrin-A1 stimulation does not appear to affect Cdc42 activity. The enhanced basal and ephrin-A1-induced Rac1-GTP levels in SHIP2 knockdown cells were blocked by PI 3-kinase inhibitor, LY294002 (Fig. 6A, bottom). These data suggest that, in contrast to PI 3-kinase, SHIP2 negatively regulates Rac1 GTPase activity.

To determine the functional relevance of Rac1 in ligand-induced EphA2 endocytosis, we expressed a wild-type Rac1, a constitutively active mutant of Rac1 (Rac1 V12), or a dominant negative mutant of Rac1 (Rac1 N-17) (31) in COS7 cells. As shown in Fig. 6B, expression of either wild-type Rac1 (green, top) or Rac1 V-12 (red, middle) did not affect EphA2 internalization significantly, but expression of Rac1 N-17 (red, bottom) markedly impaired EphA2 endocytosis, suggesting that Rac1 GTPase activity is required for ligand-induced EphA2 endocytosis.

**DISCUSSION**

In this study, we show that when ephrins bind to Ephs the lipid phosphatase SHIP2 is recruited to the SAM domain of the activated EphA2 receptor through a heterotypic SAM-SAM interaction. We found that overexpression of SHIP2 significantly inhibited ligand-induced EphA2 receptor endocytosis. Silencing of SHIP2 via siRNA-mediated knockdown enhanced the ephrin-A1 ligand-induced increase in PIP$_3$ levels and Rac1 GTPase activity as well as ligand-induced EphA2 receptor endocytosis. Taken together, our data indicate an important role for SHIP2 in regulating phosphoinositol lipids to modulate Eph receptor function in cancer cells.

**Recruitment of SHIP2 to the Activated EphA2 Receptor via a Heterotypic SAM-SAM Domain Interaction—**SHIP1 and SHIP2 have been shown to associate with a number of cell surface receptors (16). Both SHIP1 and SHIP2 contain an N-terminal SH2 domain, a lipid phosphatase domain, proline-rich regions, and NPXY motifs serving as potential protein–protein interaction sites (16). SHIP2 also possesses a C-terminal SAM domain that is not present in SHIP1. In the case of SHIP1, the amino-terminal SH2 domain binds to phosphotyrosine residues to mediate the interactions with a number of signal transduction proteins (16, 32, 33). However, this is not the case in the interaction between SHIP2 and the EGFR. Pesesse et al. (34) reported that the SH2 domain of SHIP2 was unable to precipitate the EGFR, whereas a C-terminal truncated form of SHIP2 that lacks the last 366 amino acids was able to bind to EGFR in EGF-stimulated cells. These results suggest that neither the SH2 nor SAM domain of SHIP2 is capable of binding to the activated EGFR, and a specific EGF binding domain in SHIP2 remains to be identified. Here we show that it is the SAM domain of SHIP2, rather than its SH2 domain (Fig. 2E), that is required for interacting with the activated EphA2 receptor.

SAM domains are protein-protein interaction motifs that can interact homotypically with identical SAM domains or heterotypically with other related SAM domains (35). All known Eph proteins contain SAM domains at the C terminus, which are thought to play a role in receptor clustering. Crystal and solution structures of the EphA4-SAM domain and the EphB2-SAM domain have been resolved (36–38). Despite the potential role of these SAM domains in promoting receptor oligomerization, homotypic SAM–SAM self-association in solution is weak ($K_a > 1$ μM). In light of our data, one major function of the EphA2 receptor SAM domain appears to be mediating heterotypic protein–protein interactions to transduce signals downstream of the Eph receptor. As the EphA2 SAM domain contains three tyrosine residues, one possibility is that the activated receptor phosphorylates its own SAM domain, leading to recruitment of SHIP2. However, our data do not favor this hypothesis. A kinase-dead (D738N) EphA2 mutant and Tyr to Phe mutations in the SAM domain are all still capable of binding to the SHIP2 SAM domain (Fig. 2, A and C). In addition, an SH2 domain deletion mutant of SHIP2 protein retains its ability to interact with the EphA2 receptor (Fig. 2E). The more likely model is that ligand stimulation induces conformational changes in the EphA2 receptor leading to its phosphorylation at the tyrosine residues, promoting the recruitment of SHIP2 to the EphA2 receptor.

**FIGURE 3. Overexpression of SHIP2 inhibits ligand-induced EphA2 receptor endocytosis.** A, kinetics of ligand-induced EphA2 endocytosis. MDA-MB-231 cells were stimulated with ephrin-A1 ligand following a time course. EphA2 receptor (green) is localized diffusely at the cell border at 0 min. Ephrin-A1 stimulation results in EphA2 receptor clustering and internalization (see inset) at 2 min. At 15 min, extensive internalized EphA2 vesicles were observed, co-localizing with EE1, an endosomal marker. B, EphA2 and a Myc-tagged SHIP2, a SHIP2 mutant with SAM domain deletion (SHIP2ΔSAM), or a SHIP2 mutant that lacks phosphatase activity (SHIP2D607A) were co-transfected into COS7 cells, and cells were stimulated with ephrin-A1 for 15 min. EphA2 and SHIP2 are detected by antibodies against EphA2 (green) and Myc (red), respectively. EphA2 endocytosis is significantly inhibited in SHIP2-overexpressing cells ($p < 0.01$) but not in SHIP2ΔSAM or D607A mutant-expressing cells. Arrowhead, transfected cells. C, internalized vesicles in each cell were quantified by counting vesicle pixel density using Metamorph software. Experiments were repeated three times, and ~30 cells/experiment were analyzed. Data are presented as mean ± S.D., and statistical significance was assessed by a two-tailed, paired Student’s $t$ test. W7, wild type.
FIGURE 4. Inhibition of SHIP2 in cancer cells by siRNA-mediated silencing. A, Western blot analysis on SHIP2 siRNA clones. MDA-MB-231 cells were infected with retroviruses expressing siRNA 1, 2, 2A, 3, and 4 and a control mutant 2 siRNA. Pooled clones were subjected to Western blot analysis using an anti-SHIP2 polyclonal antibody. The blot was stripped and reprobed for EphA2 and tubulin for a loading control. B, EphA2 endocytosis is documented by confocal imaging analysis and quantified by Metamorph analysis as described in the legend to Fig. 3. MDA-MB-231 cells were co-stained by anti-EphA2 antibody (green) and endosomal marker EEA1 (red) in SHIP2 knockdown and control cells. Enhanced endocytosis of EphA2 receptor was observed in SHIP2 knockdown cells. C, MDA-MB-231 cells were biotinylated, and surface proteins were either removed immediately as indicated by the 0 time point or removed following a time course in the presence (left panel) or absence (right panel) of ephrin-A1 stimulation. Levels of internalized biotinylated EphA2 were then determined by immunoprecipitation with streptavidin beads followed by Western blot analysis using anti-EphA2 antibody. D, EphA2 protein level is detected by Western blot analysis following ephrin-A1 stimulation in control and SHIP2 knockdown cells. Enhanced EphA2 degradation was observed in SHIP2 knockdown cells.
changes in the EphA2 receptor, allowing SHIP2 to gain access to the EphA2 SAM domain.

SHIP2 and Regulation of Receptor Endocytosis—Ligand-induced Eph receptor endocytosis has been previously reported (7, 39). In these cases, Rac1 was shown to be required for endocytosis of the plasma membrane and reorganization of F-actin. More recently, Vav family proteins were recognized as Rho guanine nucleotide exchange factors to activate Rac GTPase in the growth cone collapse response (9). Since Vav proteins can be regulated through tyrosine phosphorylation and/or binding to PIP3 via the pleckstrin homology domains, one way to regulate receptor endocytosis is to modulate PIP3 levels through PI 3-kinase and/or lipid phosphatases. In support of this idea, we found that the phosphorylated EphA2 receptor interacts with the p85 subunit of PI 3-kinase (data not shown), a result that is consistent with previous findings by Pandey et al. (40). Activated EphA2 also recruits the SHIP2 phosphatase, providing negative feedback to reduce PIP3 levels. Indeed, knockdown of SHIP2 in MDA-MB-231 cells led to an increase in EphA2 receptor endocytosis and degradation. This increased endocytosis of the EphA2 receptor was accompanied by increased ephrin-A1-induced PIP3 levels and activation of Rac1. A PI 3-kinase inhibitor, LY294002, blocked basal and ligand-induced Rac1 activation and significantly inhibited EphA2 endocytosis in SHIP2 knockdown cells. Taken together, these results suggest a switch from a phosphotyrosine-dependent to a SAM-dependent signaling mechanism to regulate EphA2 endocytosis (Fig. 7).

Involvement of a phosphoinositide 5-phosphatase in receptor endocytosis has been described previously (41). Irie et al. (41) reported that activation of EphB2 in neurons induced tyrosine phosphorylation of synaptojanin 1, a phosphatidylinositol 5-phosphatase that is involved in clathrin-mediated endocytosis. Ephrin-induced phosphorylation of synaptojanin 1 inhibits both the interaction with endophilin and the 5-phosphatase activity of synaptojanin 1, resulting in inhibition of internalized vesicle uncoating and blocking entry to endosomes (41). This mechanism apparently is different from regulation of EphA2 endocytosis by SHIP2. Although we also observe increased vesicles in SHIP2 knockdown cells, the elevated numbers of vesicles co-localize with EE1, an endosomal marker. In addition, EphA2 receptor degradation is enhanced in SHIP2 knockdown cells, suggesting that internalized vesicles reach endosomes for protein degradation. Thus, it is likely that SHIP2 acts at an early stage of EphA2 endocytosis, through modulation of Rac1-dependent cytoskeletal dynamics, to regulate EphA2 trafficking.

Aside from modulating Rac1 GTPase activity, SHIP2 may also regulate EphA2 receptor endocytosis via Cbl, a ubiquitinating ligase (E3). Cbl has been recently reported to interact with SHIP2 through the SHIP2 SH2 domain (42, 43) as well as sev-
eral receptor tyrosine kinases, including the EphA2 and the EGF receptors (10, 11, 43). Prasad and Decker (43) proposed that SHIP2 may sequester Cbl, preventing it from binding to the EGF receptor, thereby inhibiting EGF receptor degradation. However, a SHIP2 mutation (D607A) that abolishes phosphatase activity did not affect EphA2 endocytosis (Fig. 3B). Our finding suggests that inhibition of EphA2 endocytosis by SHIP2 is unlikely to be due to sequestering Cbl by SHIP2. Rather, SHIP2 may function as a lipid phosphatase to down-regulate PIP3 levels, which inhibits EphA2 receptor endocytosis.

**Role of SHIP2 in Tumor Cell Malignancy**—SHIP2 belongs to a family of SH2-containing phosphoinositide 5-phosphatases. The closest relative to SHIP2 is SHIP1. SHIP1 expression is restricted to hematopoietic cells and developing spermatogonia, whereas SHIP2 is more widely expressed in many different tissues and cell types (16). The role of SHIPs in the enzymatic conversion of PI 3,4,5-trisphosphate to PI 3,4-bisphosphate raises the question of whether they can act as tumor suppressors like PTEN, which utilizes the same lipid substrate but produces a different lipid product, PI 4,5-bisphosphate. Loss of SHIP1 in mice resulted in a myeloproliferative disease (44). In addition, SHIP1 expression is reduced in both primary cells from leukemic patients and upon induction of BCR-ABL (45). These data suggest that reduced SHIP1 activity may be a prerequisite for the proliferative advantage of some chronic and acute myelogenous leukemic clones. It is currently unclear whether SHIP2 may also play a tumor suppressor-like role. In an earlier study, loss of both SHIP2 and Phox2a in mice led to neonatal lethality and increased sensitivity to insulin (46). Neonatal lethality in this strain renders it difficult to study the role of SHIP2 in cancer, and deletion of Phox2a would confound the results. More recently, deletion of SHIP2 alone in mice resulted in resistance to dietary obesity (47). Since this SHIP2-deficient mouse strain is viable, it provides an opportunity to in-

**FIGURE 6. SHIP2 regulates EphA2 endocytosis via Rac1 GTPase.** A, active GTP-bound forms of Rac1 and Cdc42 were analyzed by Pak-1 binding domain pull-down, followed by immunoblot in lysates from SHIP2 knockdown or control MDA-MB-231 cells stimulated with ephrin-A1, in the presence or absence of PI 3-kinase inhibitor LY294002 (blots). Total Rac1 and Cdc42 levels within the lysate prior to Pak-1 binding domain pull-down were detected by immunoblot. Results from three independent experiments were quantified using Scion Image software and expressed as mean ± S.D. (graphs). B, a wild-type Rac1, a Myc-tagged constitutively active Rac1 mutant (Rac1 V-12), or a dominant negative N17 Rac1 expression construct was transfected into COS7 cells, and ligand-induced EphA2 endocytosis (at 15 min) was analyzed by confocal microscopy. Ephrin-A1-induced EphA2 endocytosis is not affected in wild-type or V12 Rac1-expressing cells but is inhibited in N17 Rac1 expressing cells (red). Arrowhead, transfected cells. Internalized vesicles in each cell were quantified by counting vesicle pixel density using Metamorph software, as detailed under “Experimental Procedures.”
investigate SHIP2 function in tumorigenesis and metastatic progression.

Our data in malignant breast cancer cells suggest that SHIP2 inhibits EphA2 receptor endocytosis. Since EphA2 level is correlated with tumor malignancy, it is possible that enhanced EphA2 receptor endocytosis and degradation in SHIP2 knockdown cells may reduce tumor malignancy. However, it is important to note that SHIP2 also regulates other growth factor receptors, endocytosis of many of which is required for receptor signaling. Thus, it is also possible that ablation of SHIP2 globally may enhance receptor signaling and cell proliferation/migration, resulting in increased cell malignancy. In vivo experiments using relevant animal models are required to resolve this issue.

In summary, our findings reported here implicate a critical role for SHIP2 in regulating ligand-induced EphA2 receptor endocytosis. Since EphA2 level is linked to tumor malignancy, these studies provide a foundation for investigating EphA2 as a potential target for therapeutic intervention.

REFERENCES

1. Pasquale, E. B. (2005) *Nat. Rev. Mol. Cell. Biol.* 6, 462–475
2. Brantley-Sieders, D., Schmidt, S., Parker, M., and Chen, J. (2004) *Carr. Pharm. Des.* 10, 3431–3442
3. Brantley-Sieders, D., and Chen, J. (2004) *Angiogenesis* 7, 17–28
4. Kullander, S., and Klein, R. (2002) *Nat. Rev. Mol. Cell. Biol.* 3, 475
5. Gale, N. W., Holland, S. J., Valenzuela, D. M., Flenniken, A., Pan, L., Ryan, T. E., Henkemeyer, M., Strebhardt, K., Hirai, H., and Wilkinson, D. G. (1996) *Neuron* 17, 9–19
6. Zimmer, M., Palmer, A., Kohler, J., and Klein, R. (2003) *Nat. Cell Biol.* 5, 869–878
7. Marston, D. J., Dickinson, S., and Nobes, C. D. (2003) *Nat. Cell Biol.* 5, 879–888
8. Parker, M., Roberts, R., Enriques, M., Zhao, X., Takahashi, T., Pat Cerretti, D., Daniel, T., and Chen, J. (2004) *Biochem. Biophys. Res. Commun.* 323, 17–23
9. Cowan, C. W., Shao, Y. R., Sahin, M., Shamah, S. M., Lin, M. Z., Greer, P. L., Gao, S., Griffith, E. C., Brugge, J. S., and Greenberg, M. E. (2005) *J. Biol. Chem.* 280, 2693–2700

FIGURE 7. A model for how SHIP2 may regulate ephrin-induced EphA2 endocytosis. Upon binding to ephrins, the EphA2 receptor is tyrosine-phosphorylated. Through the recruitment of the p85 subunit of PI 3-kinase, EphA2 receptor up-regulates phospholipid PIP₂ levels and activates Rac1 GTPase to transduce signals as well as promoting EphA2 receptor endocytosis. EphA2 also recruits SHIP2 through a heterotypic SAM-SAM interaction to mediate negative feedback to reduce PIP₂ levels. Thus, the EphA2 receptor is capable of switching from phosphotyrosine-dependent to SAM-dependent signaling to regulate EphA2 endocytosis.
SHIP2 Regulates EphA2 Endocytosis

(2003) Biochem. Biophys. Res. Commun. 300, 494–500
43. Prasad, N. K., and Decker, S. J. (2005) J. Biol. Chem. 280, 13129–13136
44. Helgason, C. D., Damen, J. E., Rosten, P., Grewal, R., Sorensen, P., Chappel, S. M., Borowski, A., Jirik, F., Krystal, G., and Humphries, R. K. (1998) Genes Dev. 12, 1610–1620
45. Sattler, M., Salgia, R., Shrikhande, G., Verma, S., Choi, J. L., Rohrschneider, L. R., and Griffin, J. D. (1997) Oncogene 15, 2379–2384
46. Clement, S., Krause, U., Desmedt, F., Tanti, J. F., Behrends, J., Pesesse, X., Sasaki, T., Penninger, J., Doherty, M., Malaisse, W., Dumont, J. E., Le Marchand-Brustel, Y., Erneux, C., Hue, L., and Schurmans, S. (2001) Nature 409, 92–97
47. Sleeman, M. W., Wortley, K. E., Lai, K. M., Gowen, L. C., Kintner, J., Kline, W. O., Garcia, K., Stitt, T. N., Yancopoulos, G. D., Wiegand, S. J., and Glass, D. I. (2005) Nat. Med. 11, 199–205