SH2-containing 5'-inositol Phosphatase, SHIP2, Regulates Cytoskeleton Organization and Ligand-dependent Down-regulation of the Epidermal Growth Factor Receptor

Nagendra K. Prasad and Stuart J. Decker

From the Department of Basic Medical Sciences and Purdue Cancer Center, Purdue University, West Lafayette, Indiana 47907 and the Life Sciences Institute, University of Michigan, Ann Arbor, Michigan 48105

Phosphoinositide lipid second messengers are integral components of signaling pathways mediated by insulin, growth factors, and integrins. SHIP2 dephosphorylates phosphatidylinositol 3,4,5-trisphosphate generated by the activated phosphatidylinositol 3'-kinase. SHIP2 down-regulates insulin signaling and is present at higher levels in diabetes and obesity. SHIP2 associates with p130Cas and filamin, regulators of cell adhesion/migration and cytoskeleton, influencing cell adhesion/spreading. Type I collagen specifically induces Src-mediated tyrosine phosphorylation of SHIP2. To better understand SHIP2 function, we employed RNA interference (RNAi) approach to silence the expression of the endogenous SHIP2 in HeLa cells. Suppression of SHIP2 levels caused severe F-actin deformities characterized by weak cortical actin and peripheral actin spikes. SHIP2 RNAi cells displayed cell-spread defects involving a notable absence of focal contact structures and the formation of multiple slender membrane protrusions capped by actin spikes. Furthermore, decreased SHIP2 levels altered distribution of early endocytic antigen 1 (EEA1)-positive endocytic vesicles and of vesicles containing internalized epidermal growth factor (EGF) and transferrin. EGF treatment of SHIP2 RNAi cells led to the following: enhanced EGF receptor tyrosine kinase activity; increased EGFR ubiquitination; and decreased EGFR phosphorylation of SHIP2. To better understand SHIP2 function, we employed RNA interference (RNAi) approach to silence the expression of the endogenous SHIP2 in HeLa cells. Suppression of SHIP2 levels caused severe F-actin deformities characterized by weak cortical actin and peripheral actin spikes. SHIP2 RNAi cells displayed cell-spread defects involving a notable absence of focal contact structures and the formation of multiple slender membrane protrusions capped by actin spikes. Furthermore, decreased SHIP2 levels altered distribution of early endocytic antigen 1 (EEA1)-positive endocytic vesicles and of vesicles containing internalized epidermal growth factor (EGF) and transferrin. EGF treatment of SHIP2 RNAi cells led to the following: enhanced EGF receptor tyrosine kinase activity; increased EGFR ubiquitination; and increased EGFR association with c-Cbl ubiquitin ligase. Taken together, these experiments demonstrate that SHIP2 functions in the maintenance and dynamic remodeling of actin structures as well as in endocytosis, having a major impact on ligand-induced EGFR internalization and degradation. Accordingly, we suggest that, in HeLa cells, SHIP2 plays a distinct role in signaling pathways mediated by integrins and growth factor receptors.

SH2-containing inositol phosphatase-2 (SHIP2), also called INPPL1 (inositol polyphosphate 5'-phosphatase-like protein-1), is a lipid phosphatase that dephosphorylates phosphorylated phosphatidylinositol (PI) and inositol molecules on the 5'-position of the inositol ring (1). In particular, phosphatidylinositol 3,4,5-trisphosphate (PIP₃), produced by PI 3'-kinase (PI3K), is an important substrate of SHIP2 (2, 3). PI3K products are critical second messengers in cell signal transduction pathways involving growth factors (e.g. EGF, vascular endothelial growth factor), hormones (e.g. insulin), and cell-cell (cadherins) and cell-matrix interactions (integrins) (4). PI lipids interact with the pleckstrin homology (PH) domain containing proteins, leading to their membrane recruitment and/or allosteric activation (5). Examples of PH domain-containing enzymes include protein kinase B/Akt, phosphoinositide-dependent kinase 1, Tec family tyrosine kinases, and guanine nucleotide exchange factors for Ras and Rho family GTPases (e.g. Sos1 and Vav1). PIP₃ is converted to phosphatidylinositol 3,4-bisphosphate (PI(3,4-P2)) by SHIP2 action. The role of PI(3,4-P2) in signaling is unclear as some suggest a lack of function for this intermediate perhaps due to its rapid rate of turnover (6), whereas some evidence supports that PI(3,4-P2) may have a regulatory role in the signaling pathways because it binds to several PH domain proteins including Akt, which it activates in vitro (7, 8). Moreover, Akt cannot be fully activated by PIP₃ when PI(3,4-P2) is absent in vivo (9). In addition to PIP₃, SHIP2 substrates include phosphatidylinositol 4,5-bisphosphate (PIP₂) and a soluble inositol phosphate, inositol 1,3,4,5-tetrakisphosphate, suggesting that SHIP2 may play a role in processes other than PI3K signaling (6, 10).

SHIP2 mRNA is expressed ubiquitously (2). SHIP2 is thought to down-regulate insulin signaling (11). The genetic knockout of SHIP2 in mice led to neonatal lethality due to hypoglycemia caused by insulin hypersensitivity (12). However, Clement et al. (12) have acknowledged recently that this SHIP2 knockout mouse strain had inadvertent deletion of the adjacent Phox2a gene. Thus, they were unsure whether the observed insulin hypersensitivity phenotype can be attributed solely to the absence of SHIP2 function (13). However, studies in adipocytes and skeletal myocytes indicate that SHIP2 down-regulates insulin signaling (14, 15). Other evidence suggests a possible link between increased SHIP2 levels and high fat diet, obesity, and diabetes (16–19). The effect of SHIP2 overexpression on Akt activation and cell cycle progression remains contentious, because two reports describe conflicting results (6, 20). Another report (21) describes a dual role for SHIP2 in vascular smooth muscle cells: 1) an anti-apoptotic function...
SHIP2 Regulates the Cytoskeleton and EGFR Down-regulation

dependent on the SH2 domain; and 2) a growth inhibitory function dependent on the catalytic activity. Thus, the functions of endogenous SHIP2 in different cell types and its role in signaling pathways that are mediated by growth factors, integrins, and cadherins remain poorly understood.

SHIP2 contains an amino-terminal SH2 domain, a carboxy-terminal proline-rich region, and a central inositol phosphatase domain. In addition, SHIP2 contains a sterile α-motif domain at the carboxyl terminus and a NPXY motif. Previously, we showed that SHIP2 is tyrosine-phosphorylated in response to treatment of cells with growth factors and insulin and is associated with Shc adaptor protein when treated with EGF and platelet-derived growth factor but not when treated with insulin (22). We have also demonstrated an essential role for SHIP2 in adhesion and spreading of HeLa cells and have established a role for Src family tyrosine kinases in the regulation of SHIP2 during cell adhesion and spreading (23, 24). Others (25) have reported a role for SHIP2 in submembrane actin regulation through interaction with an actin-binding protein, filamin.

In this report, we employed RNA interference (RNAi) to suppress the endogenous SHIP2 levels in HeLa cells to further elucidate SHIP2 function. SHIP2 knockdown in these cells caused marked actin abnormalities and cell-spread defects. Also, alterations in the endocytic vesicular distribution were evident upon SHIP2 silencing, having major impact on the ligand-dependent EGF receptor (EGFR) internalization and degradation pathway. Accordingly, we suggest that, in HeLa cells, SHIP2 functions to regulate actin remodeling and receptor endocytosis, specifically that of EGFR.

EXPERIMENTAL PROCEDURES

Materials—For RNAi experiments, single-stranded or duplex RNA oligonucleotides were purchased from Dharmaco Research. We obtained RNA Extraction kit from Qiagen, DNA-Free kit and 18 S rRNA primers from Ambion, and RT-PCR first-strand cDNA synthesis kit and high fidelity PCR mixture from Invitrogen. Generation of rabbit polyclonal antibodies against SHIP2 and EGFR were described earlier (22, 26). Antibodies to c-Cbl (Chl), EEA-1, and ubiquitin were from BD Transduction Laboratories. Anti-FLAG (M2) antibody and TRITC or fluorescein isothiocyanate-conjugated antibodies were from Sigma. We obtained antibodies against Akt and horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG from Cell Signaling Technologies, rhodamine-tagged EGF and Oregon Green-conjugated antimouse IgG from Molecular Probes, and rhodamine-conjugated transferrin from Rockland, Inc.

Cell Culture and RNA Interference Experiments—HeLa and human embryonic kidney 293/T cells were routinely cultured in Dulbecco’s modified Eagle’s medium (with high glucose, pyridoxine hydrochloride, and L-glutamine and without sodium pyruvate) containing 7.5% fetal bovine serum. Single-stranded RNA oligomers were annealed for 1 h at 37 °C in 100 mM potassium acetate, 30 mM Hepes, pH 7.4, 2 mM magnesium acetate. Synthetic duplex or laboratory-annealed duplex RNA molecules (50 nm) were transfected using Lipofectamine (Invitrogen) reagent according to the manufacturer’s instructions. At the indicated intervals following transfection, cell lysates were routinely assayed for silencing effect by Western blots. Unique SHIP2-targeted oligomers (confirmed through BLAST analyses) contained the following sequences: S2 (nucleotides 2058–2076), S2-a (nucleotides 191–200), S2-a (nucleotides 191–200), S2-a (nucleotides 191–200), S2-a (nucleotides 191–200), and S2-a (nucleotides 191–200), which were mismatches in the sequence S2-a. A sequence (denoted by Ds), 5′-AGCCACAAGCTGGATACTGG-3′, with no significant homology to any published gene sequences was used as control.

For RT-PCR, total RNA was prepared at the indicated intervals and was rendered free of DNA. First-strand reverse transcription reactions were carried out using random hexamer primers. PCR reactions were carried out using SHIP2-specific primers and high fidelity PCR mixture along with a 1:9 ratio of 18 S RNA primers.

Generation of expression constructs of FLAG-tagged wild type SHIP2 and mutant SHIP2 (R47G and A593YF) were described previously (23, 24). Transient transfection of 293/T cells was performed using modified calcium phosphate method (Stratagene) as described previously (23).

**Fig. 1.** A, SHIP2 mRNA levels as determined by RT-PCR after RNAi. RT-PCR was carried out using RNA from HeLa cells transfected with siRNA oligomers after 48 and 72 h of transfection. B. SHIP2 knockdown in these cells caused marked actin abnormalities and cell-spread defects. Also, alterations in the endocytic vesicular distribution were evident upon SHIP2 silencing, having major impact on the ligand-dependent EGF receptor (EGFR) internalization and degradation pathway.
produced a target protein suppression of nearly 70–80%, an efficiency of silencing commonly achieved by this approach (Fig. 1, top and middle panels). Gene knockdown lasted between 70 and 100 h post-transfection in a reproducible and consistent manner as shown with the samples from five independent experiments in Fig. 1B. A duplex siRNA oligomer with two mismatches in the sequence S2-a (depicted as MS2-a in Fig. 1B, bottom panel) was completely inactive. SHIP2 protein levels returned to pre-transfection levels in 6–8 days post-transfection (data not shown). The silencing was evident at concentrations as low as 1 nM specific siRNA duplexes, whereas an siRNA for firefly luciferase (GL3) did not alter SHIP2 protein levels at concentrations of 50 nM (see supplementary data). Data from experiments with S2 siRNA are described below, whereas similar results were also obtained with S2-a siRNA as well (see supplementary data).

**Actin Cytoskeleton Abnormalities upon SHIP2 Knockdown**

One of the striking results of SHIP2 knockdown was alteration in cell morphology. Given our earlier findings of an important role for SHIP2 in cell adhesion and spreading events (23, 24), we reasoned that we should examine these changes at the level of actin cytoskeleton organization by staining for F-actin. Staining with phalloidin showed profound abnormalities in actin cytoskeleton following SHIP2 knockdown (Fig. 2). In the presence of serum, ~10–15% SHIP2 RNAi cells displayed long transversely oriented actin filaments or stress fibers along with depletion of submembranous cortical actin (Fig. 2B). A majority of cells (nearly 75%) also presented long filamentous fascicles of actin that protruded at the periphery and at the extremities. These actin filamentous “spikes” became quite prominent (thick arrows), whereas stress fibers disappeared after 24 h of serum starving (Fig. 2D). The weakening of submembranous cortical actin upon suppression of SHIP2 was clearly evident in the absence of stress fibers (thin arrows point to cortical actin in all of the panels; Fig. 2D). Actin abnormalities were induced by SHIP2-specific siRNAs at low concentrations (10 nM), whereas the siRNA for luciferase (GL3) did not cause overt defects in the actin cytoskeleton (see supplementary data).

Rho family small G proteins are central regulators of actin cytoskeleton dynamics. Important among them are RhoA, Rac1, and Cdc42. Together, these enzymes play a coordinated role in cell migration process (28). F-actin spikes and spreading defects are commonly observed in experimental dysfunction of one or more of these proteins (29). Stress fibers are induced by activated Rho A, whereas actin spikes are formed upon activation of Rac1 or Cdc42. Formation of lamellipodia is determined by the actions of PI3K and Rac1. Therefore, morphological changes seen after SHIP2 silencing suggest that SHIP2 may regulate coordinated functioning of Rho family members. We tested this notion by expressing dominant negative (d.n.) forms of Rac1 or Cdc42 in SHIP2 RNAi cells (a gift from Dr. A. Hall, University College, London, United Kingdom). Expression of d.n. Rac1 reduced the severity of F-actin deformities to a large extent in nearly 90% of the cells, whereas d.n. Cdc42 was somewhat less effective in this assay, which rescued the actin abnormalities in ~40% cells (Fig. 2E). In control cells (transfected with Ds siRNA), expression of d.n. Rac1 caused no overt phenotypic change with the exception of a modest decrease in the F-actin network (data not shown).

In HeLa cells, a phosphatase-inactive mutant of SHIP2 prevents cell spreading and movement of the tyrosines in the NPXY motif of SHIP2 disrupts lamellipodia extension on collagen I (23, 24). Therefore, we tested whether suppression of SHIP2 similarly affected the spreading ability of HeLa cells. Fig. 3 depicts an experiment where control or SHIP2 siRNA-transfected cells were allowed to spread on type I collagen for 60 min. Although there were no significant differences between control and SHIP2 RNAi in the number of cells attached to the collagen-coated surface, striking actin deformities and spreading defects were seen following SHIP2 silencing. After SHIP2 RNAi, newly spreading cells showed a lack of formation of “focal contacts” (also called “contact points,” initial transitory structures formed upon cell contact with matrix proteins; see thin arrows in control cells in panel A) and failed to extend lamellipodia (see thick arrows in control cells in panel A). Instead, SHIP2 RNAi cells displayed numerous long mem-

---

**Fig. 2. Cytoskeleton abnormalities in SHIP2 knock-down cells.**

A–D, 72 h after transfection with double-stranded control siRNA (panels A and C) or SHIP2-specific siRNA (panels B and D). HeLa cells were fixed and stained with phalloidin–TRITC. Cells shown in panels A and B were cultured in the presence of 7.5% fetal bovine serum, whereas those in panels C and D were serum-starved (Dulbecco’s modified Eagle’s medium with 0.5% bovine serum albumin) for 24 h prior to staining. Thin arrows denote submembranous cortical actin, and thick arrows denote protruding actin spikes. Panel E, altered functioning of Rho family GTPases upon SHIP2 suppression. Cells were co-transfected with S2 siRNA along with expression constructs for Myc-tagged N17 Rac1 or HA-tagged T17 Cdc42. Three days post-transfection, cells were stained with epitope-specific antibodies and counterstained with TRITC-phalloidin. Cells positive for Rac1 or Cdc42 expression were scored (n = 60) for F-actin abnormality in comparison with those that were negative for GTPases expression and with the control (Ds) siRNA-transfected cells. White arrows point to the N17 Rac1- and T17 Cdc42-positive cells. Photomicrographs in the top row show that the expression of N17 Rac1 decreased the peripheral actin spikes as seen in the adjacent untransfected cell (scored Rescue), whereas the bottom row pictures show no major effect of T17 Cdc42 expression on the actin stress fibers or the peripheral actin spikes (scored No rescue/round). Table shows the extent of phenotypic rescue by d.n. Rac and Cdc42 in SHIP2 RNAi cells.
brane protrusions capped at their extremities by a fascicle of actin filaments. As many as 65–70% SHIP2 RNAi cells displayed these actin deformities. The cells maintained this phenotype for as long as 3 h after plating and eventually were able to spread but with a somewhat distorted morphology. Together, these results demonstrate an essential function for SHIP2 in the maintenance and dynamic remodeling of actin structures in HeLa cells.

Cytoplasmic Vesicles following SHIP2 Silencing—Another striking feature of cells undergoing silencing of SHIP2 via RNAi was the accumulation of phase-bright cytoplasmic vesicular structures, indicative of disruption of vesicular trafficking (Fig. 4). These vesicles were easily detected in the perinuclear area in <10% cells (Fig. 4, middle panel, denoted by the arrow). Serum starvation did not cause significant alteration in these vesicles; but treatment with EGF (50 ng/ml) for 24 h markedly enhanced the extent of vesicular accumulation in some cells and increased the total number of cells displaying the phenotype as well (Fig. 4, bottom panel, denoted by the arrow). Some but not all of these enlarged vesicles were positive for the early endosome marker, EEA1, whereas markers for late endosomes and the lysosomes (LAMP1, LysoTracker dye, respectively) failed to co-localize to these enlarged vesicles (data not shown). Interestingly, after SHIP2 knockdown, endocytic vesicles identified by the presence of EEA1 markedly differed in their size and the distribution when compared with the control cells (Fig. 5). EEA1-positive vesicles appeared significantly larger than those in control cells and were distributed randomly in the cytoplasm of SHIP2 siRNA-transfected cells, in contrast to the perinuclear accumulations seen in a majority of control cells (Fig. 5, A and B). Increased numbers of small (pin-point) EEA1-positive vesicles were seen in the peripheral cytoplasm of SHIP2 RNAi cells immediately after treatment with EGF (50 ng/ml) for 5 min (Fig. 5D). Control cells displayed similar changes in response to EGF but to a reduced extent (Fig. 5C). EEA1-positive vesicles remained distributed in the cytoplasm in nearly 50–60% SHIP2 RNAi cells, whereas these vesicles re-accumulated at the perinuclear region in control cells when treated for 30 min with EGF (Fig. 5, E and F).

Defective Receptor Endocytosis upon SHIP2 RNAi—The accumulation of cytosolic vesicles and the observed differences in the distribution of EEA1-positive vesicles after EGF treatment prompted us to question whether EGF-induced endocytosis of EGFR might be affected by SHIP2 knockdown. To this end, we performed pulse-chase experiments with a 5-min pulse treatment of EGF (25 ng/ml) followed by chase in the absence of EGF for intervals up to 3 h. Immediately following the 5-min EGF treatment (at 0 h chase), internalized EGF in endocytic vesicles appeared as mere specks distributed throughout cytoplasm (Fig. 6). Microscopic observation suggested an apparent increase in the number of EGF-containing vesicles upon SHIP2 RNAi when compared with the controls (Fig. 6, A and B). The distribution of EGF-containing vesicles was dramatically different after 1–3 h of chase in the absence of EGF. At 1 h of chase, EGF-positive vesicles in control siRNA-transfected cells moved to the perinuclear location and accumulated unilaterally. The signal intensity (or vesicular density) decreased by 3 h of chase (Fig. 6, C and E). On the other hand, EGF carrying...
vesicles remained randomly distributed throughout cytoplasm with no clear accumulation in SHIP2 RNAi cells, even after 3 h of chase (Fig. 6, D and F). This result clearly indicated a disruption in the routing of internalized EGF following the suppression of SHIP2 levels. This disruption was evident when the concentrations of SHIP2-specific siRNAs were lowered from 50 to 10 nM and was absent at high concentrations of luciferase (GL3) siRNA (see supplementary data).

By a similar assay, we examined the internalization of rhodamine-tagged transferrin to test whether SHIP2 function regulates endocytosis of receptors other than the EGFR. Upon SHIP2 RNAi, endocytic vesicles containing rhodamine-tagged transferrin were enlarged and remained scattered in the cytoplasm with an apparent increase in their intensity. Also, unlike the control cells, perinuclear accumulation of sorting and recycling endosomes was largely absent in SHIP2 knock-down cells after 30 min of chase (Fig. 6G). Thus, SHIP2 knockdown had a somewhat different effect on transferrin endocytosis but the intracellular routing of these vesicles was disrupted as it was in case of EGF.

To test whether the changes observed in routing of EGF-containing vesicles following SHIP2 knockdown altered ligand-induced EGFR internalization and degradation, we carried out anti-EGFR Western blots of whole cell extracts after SHIP2 RNAi in HeLa cells. These studies showed that EGFR degradation was enhanced following SHIP2 RNAi when compared with double-stranded scrambled sequence control (Fig. 7A). Enhanced degradation of EGFR was evident within 1 h of treatment with high concentrations of EGF (50 ng/ml), but the effect was clearly noticeable after 3 h of treatment with low EGF concentrations (5 ng/ml).

**Altered Cbl-mediated Ubiquitination of the EGFR in SHIP2 Knock-down Cells**—Ubiquitination of the EGFR is known to be critical for ligand-dependent internalization and subsequent lysosomal sorting of the receptor (30, 31). Therefore, we examined the possible involvement of this regulatory mechanism in the enhanced degradation of the EGFR observed following transfection with SHIP2-specific siRNA. Immunoprecipitation of EGFR coupled with anti-ubiquitin Western blot was used to examine the ubiquitination status of the receptor. These studies demonstrated as much as 90% increase in the ubiquitination of EGFR in response to EGF upon SHIP2 knockdown (Fig. 7B). This effect was readily detectable within 5 min of EGF treatment at both low (5 ng/ml) and high (50 ng/ml) concentrations of EGF. We also tested whether association of EGFR with Cbl, E3 ubiquitin ligase that is responsible for ubiquitination of EGFR, is affected by the loss of SHIP2. As shown in Fig. 7C, anti-Cbl Western blots of EGFR immunoprecipitations revealed that SHIP2 knock-down cells displayed an increased amount of Cbl that associated with (and therefore co-precipitated with) the EGFR at both low and high concentrations of EGF.

**Association of SHIP2 with Cbl**—To further examine the mechanism through which SHIP2 knockdown effects EGFR down-regulation, we tested whether SHIP2 directly interacted with Cbl. In co-immunoprecipitation experiments, SHIP2 associated with Cbl in non-stimulated conditions. This interaction appeared to be modestly increased upon EGF treatment (Fig. 8A). In transient transfection assays using 293T cells, FLAG-tagged wild type SHIP2 co-precipitated endogenous Cbl, whereas a SH2 domain mutant (R47G) failed to do so (Fig. 8B). However, disruption of phosphotyrosine-binding domain-interacting NPXY motif in SHIP2 did not interfere with SHIP2-Cbl association. This demonstrates the requirement for the SH2 domain and tyrosine phosphorylation of SHIP2 and/or Cbl for SHIP2-Cbl interaction. Together, our studies in HeLa cells demonstrate an important role for SHIP2 in the pathways involved in ligand-dependent EGFR down-regulation via regulation of EGFR interaction with Cbl and subsequent EGFR ubiquitination and degradation.

**DISCUSSION**

**SHIP2 Function in HeLa Cells**—In this report, we describe a novel role for SHIP2 in the maintenance and remodeling of actin cytoskeleton. Our data also demonstrate a novel role for SHIP2 in the regulation of receptor endocytosis and in ligand-dependent EGFR down-regulation. In light of the essential role of phosphoinositide lipids in actin cytoskeleton regulation and in vesicular trafficking, these significant alterations observed in these pathways after SHIP2 knockdown are not totally unforeseen (32). However, it was surprising that the knockdown of SHIP2 did not lead to overt changes in cell growth or cell survival. It is possible that such effects were not readily evident because of the transient nature of our assays. Nevertheless, our studies clearly demonstrate that SHIP2 function is critical for those aspects of cell biology involving cell adhesion/migration and EGFR receptor functioning. Previously, we have demonstrated the importance of SHIP2 in regulating actin remodeling, specifically in response to type I collagen (23, 24). Therefore, we suggest that SHIP2 may modulate the ability of cells to migrate and attach in collagen-rich environments. A loss of focal contacts and a decreased ability to spread in a coordinated fashion suggest a defective cell locomotion, which in turn may profoundly affect cancer cell migration/invasion during metastasis.
tasis (33). Detailed studies in models that allow evaluation of cell migration under both in vitro and in vivo conditions could reveal a potential role for SHIP2 in cancer cell migration.

**Molecular Mechanisms Underlying a SHIP2 Role in Actin Remodeling**—Members of Rho family small G proteins, RhoA, Rac1, and Cdc42, are known to be the primary regulators of cytoskeleton dynamics (28). Our data suggest that morphologic changes seen after SHIP2 silencing are due to altered functioning of Rac1 and Cdc42 (Fig. 2E). We propose (below) a model for regulation of Rho proteins by SHIP2 based on data reported for a functional relationship among SHIP2, p130Cas,Src, Shc, and filamin (23–25). Localization of p130Cas to the sites of integrin clustering followed by its tyrosine phosphorylation by focal adhesion kinase and Src may provide the necessary binding sites on p130Cas for recruitment of SHIP2. Upon interaction with p130Cas, SHIP2 is accessible for phosphorylation by Src on NPXY motif, leading to the interaction with Shc adaptor protein. Strategic localization of SHIP2 to the proximity of active PI3K at the adhesion complexes could cause two possible downstream effects. Firstly, SHIP2 phosphatase activity could lead to the generation of local pools of PI-3,4-P2, thereby regulating the activity of one or more guanine nucleotide exchange factors specific for Rho family proteins (29). This function of SHIP2 in conjunction with PI3K activity may aid in generating

**FIG. 6.** SHIP2 knockdown modulates endocytic uptake and routing of EGF. A—F, 48 h after transfection with double-stranded (Ds) control siRNA (panels A, C, and E) or SHIP2-specific siRNA (S2) (panels B, D, and F), cells were serum-starved for an additional 24 h. All of the samples then were treated with rhodamine-tagged EGF (25 ng/ml) for 5 min. Following the washing of cells to remove tagged EGF, cells were chased for the indicated durations (panels A and B, 0 h/no chase; panels C and D, 1 h; and panels E and F, 3 h) in serum-free medium. Cells then were fixed and examined under fluorescence microscope for the distribution of internalized EGF. Panel G, localization of internalized TRITC-conjugated transferrin. Three days post-transfection with indicated 50 nm duplex siRNA oligomers, serum-starved HeLa cells were treated with TRITC-transferrin (5 μg/ml) for 20 min on ice and examined after 30 min of chase in the absence of conjugated transferrin. siRNA oligomers used are as follows: siRNA specific to firefly luciferase (GL3) and two siRNAs specific to SHIP2 (S2 and S2-a) (two fields are shown).

**FIG. 7.** A, silencing of SHIP2 enhances ligand-induced EGFR degradation. HeLa cells were transfected with double-stranded control siRNA (Ds) or SHIP2-specific siRNA (S2). 48 h post-transfection, cells were serum-starved for 24 h prior to the treatment with EGF (as indicated in the figure). Anti-EGFR immunoblots of whole cell lysates are shown. B, enhanced Cbl-mediated ubiquitination of EGFR upon SHIP2 RNAi. Anti-EGFR or control pre-immune (Pre) immunoprecipitations (IP) from HeLa cells 72 h post-RNAi were blotted with anti-ubiquitin or anti-EGFR antibodies (loading control). Cells were treated as described above for A, but EGF treatment lasted for 5 min. The numbers reflect the quantitation of relative densities of signals in the respective blots. C, increased binding of Cbl to EGFR after SHIP2 silencing. The experiment was performed similar to B with the exception that the anti-EGFR IPs were blotted with anti-Cbl antibody. Treatment with low (5 ng/ml) and high (50 ng/ml) concentrations of EGF was carried out for 2 or 5 min as indicated in the figure.
SHIP2 expression constructs. Anti-FLAG IPs from untreated or EGF-a gradient of phosphoinositides that modulates cell spreading. (SH2-defective, transfected with FLAG-tagged wild type (SHIP2 interaction with Cbl. human embryonic kidney 293/T cells were immunoprecipitations (SDS-PAGE and blotted with anti-c-Cbl or anti-SHIP2. Such an outcome of general endocytic deficiency could have lineate the changes in these vesicular transport mechanisms, by phospholipids and through cross-talk with Rho family proteins. However, SHIP2 may have an indirect effect on the actin cytoskeleton and endocytosis regulation. This view is supported by the observation of altered EEA1- and subsequent internalization and degradation of the receptor. The effects of SHIP2 in this pathway could be direct or indirect. Direct effects would be mediated by constitutive association of SHIP2 with Cbl. Such an interaction may regulate this degradation process of EGFR. SHIP2-Cbl interaction may regulate this degradation process of EGFR. Clearly, there are remarkable similarities between these observations and our study on SHIP2. As noted before, SHIP2 is known to dephosphorylate PIP2. Because of the relative abundance of PIP2 in unstimulated cells, it is possible that it could be a major physiological substrate of SHIP2. If this is true, a loss of SHIP2 could lead to vesicular accumulation and actin disorganization as seen with synaptojanin deletion. No such abnormalities were reported in SHIP2-deficient mice (12). Although these data must be re-examined as a consequence of the accidental deletion of another gene in these mice (13). Possible existence of a redundant PIP2 5-phosphatase in normal tissues could explain the absence of cytoskeleton/vesicular trafficking defects in these animals. In cancer cells, such as HeLa, we suggest that altered genetic background may be responsible for the distinct and essential function of SHIP2 in actin cytoskeleton and endocytosis regulation.

Acknowledgments—We thank Robert Topping and Robert Nieto for technical assistance, Dr. Ronald Hullinger for critical reading of the manuscript and for inspiring discussions, and Dr. Ourania Andrisani and members of the laboratory for helpful discussions and comments.

REFERENCES
1. Woscholski, R., and Parker, P. J. (1997) Trends Biochem. Sci. 22, 427–431
2. Pesesse, X., Deleu, S., De Smedt, F., Drayer, L., and Erneux, C. (1997) Biochem. Biophys. Res. Commun. 239, 697–700
3. Wisniewski, D., Strife, A., Swendeman, S., Erdjument-Bromage, H., Geromides, S., Kavanaugh, W. M., Tempst, P., and Clarkson, B. (1999) Blood 93, 2707–2720
4. Cantrell, D. A. (2001) J. Cell Sci. 114, 1439–1445
5. Vanhaesebroeck, B., Levers, S. J. A., Ahmad, K., Timms, J., Kats, R., Dirsoll, P. C., Woscholski, H., Parker, P. J., and Waterfield, M. D. (2001) Annu. Rev. Biochem. 70, 535–602
6. Taylor, V., Wong, M., Brando, C., Brilly, L., Dean, N. M., Cowert, L. M., Moodie, S., and Stokoe, D. (2000) Mol. Cell. Biol. 20, 6860–6871
7. Klippel, A., Kavanaugh, W. M., Pot, D., and Williams, L. T. (1997) Mol. Cell. Biol. 17, 338–344
8. Franke, T. F., Kaplan, D. R., Cattley, L. C., and Toker, A. (1997) Science 275, 665–668
9. Scheid, M. P., Huber, M., Damen, J. E., Hughes, M., Kang, V., Neilsen, P., Prestwich, G. D., Krystal, G., and Duronio, V. (2002) J. Biol. Chem. 277, 9027–9035
10. Chi, Y., Zhou, B., Wang, W. Q., Chung, S. K., Kwon, Y. U., Ahn, Y. H., Chang, Y. T., Tsujishita, Y., Hurley, J. H., and Zhang, Z. Y. (2004) J. Biol. Chem. 279, 44967–44975
11. Baumgartener, J. W. (2003) Curr. Drug Targets Immune Endocr. Metabol. Disord. 3, 291–298
12. Clement, S., Krause, U., Desmedt, F., Tanti, J. F., Behrends, J., Pesesse, X., Sasaki, T., Penninger, J., Doherty, M., Malaissa, W., Dumont, J. E., Le Marchand-Brustel, Y., Erneux, C., Hue, L., and Schurmans, S. (2001) Nature 409, 92–97
13. Clement, S., Krause, U., Desmedt, F., Tanti, J.-F., Behrends, J., Pesesse, X., Sasaki, T., Penninger, J., Doherty, M., Malaissa, W., Dumont, J. E., Le Marchand-Brustel, Y., Erneux, C., Hue, L., and Schurmans, S. (2004) Nature 431, 874
SHIP2 Regulates the Cytoskeleton and EGFR Down-regulation

14. Wada, T., Sasaoka, T., Funaki, M., Hori, H., Murakami, S., Ishiki, M., Haruta, T., Asano, T., Ogawa, W., Ishihara, H., and Kobayashi, M. (2001) Mol. Cell. Biol. 21, 1633–1646

15. Sasaoka, T., Hori, H., Wada, T., Ishiki, M., Haruta, T., Ishihara, H., and Kobayashi, M. (2001) Diabetes 51, 2387–2394

16. Hori, H., Sasaoka, T., Ishihara, H., Wada, T., Murakami, S., Ishiki, M., and Kobayashi, M. (2002) Diabetes 51, 1258–1267

17. Marion, E., Kaisaki, P. J., Pouillon, V., Gueydan, C., Levy, J. C., Bodson, A., Krzentowski, G., Dubessere, J. C., Mocek, J., Behrends, J., Servais, G., Sapirer, C., Kruys, V., Gauguier, D., and Schurmans, S. (2002) Diabetes 51, 2012–2017

18. Murakami, S., Sasaoka, T., Wada, T., Fukui, K., Nagira, K., Ishihara, H., Usui, I., and Kobayashi, M. (2004) Endocrinology 145, 3215–3223

19. Kaisaki, P. J., Delepine, M., Woon, P. Y., Sebag-Montefiore, L., Wilder, S. P., Menzel, S., Vionnet, N., Marion, E., Riveline, J. P., Charpentier, G., Schurman, S., Levy, J. C., Lathrop, M., Farrall, M., and Gauguier, D. (2004) Diabetes 53, 1900–1904

20. Choi, Y., Zhang, J., Murga, C., Yu, H., Koller, E., Monia, B. P., Gutkind, J. S., and Li, W. (2002) Oncogene 21, 5289–5300

21. Sasaoka, T., Kikuchi, K., Wada, T., Sato, A., Hori, H., Murakami, S., Fukui, K., Ishihara, H., Aota, R., Kimura, I., and Kobayashi, M. (2003) Endocrinology 144, 4204–4214

22. Habib, T., Hiepa, J. A., Moses, E. E., and Decker, S. J. (1998) J. Biol. Chem. 273, 18505–18509

23. Prasad, N., Topping, R. S., and Decker, S. J. (2001) Mol. Cell. Biol. 21, 1416–1428

24. Prasad, N., Topping, R. S., and Decker, S. J. (2002) J. Cell Sci. 115, 3807–3815

25. Dyson, J. M., O'Malley, C. J., Becanovic, J., Munday, A. D., Berndt, M. C., Coghill, I. D., Sandurkar, H. H., Ooms, L. M., and Mitchell, C. A. (2001) J. Cell Biol. 153, 1053–1079

26. Decker, S. J. (1989) J. Biol. Chem. 264, 17641–17644

27. Prasad, N., Topping, R. S., Zhou, D., and Decker, S. J. (2000) Biochemistry 39, 6929–6935

28. Schmitz, A. A., Vekov, E. E., Bottner, B., and Van Aelst, L. (2000) Exp. Cell Res. 261, 1–12

29. Raftopoulos, M., and Hall, A. (2004) Dev. Biol. 261, 23–32

30. Wiley, H. S., and Burke, P. M. (2001) Traffic 2, 12–18

31. Longva, K. E., Bystad, F. D., Stang, E., Larsen, A. M., Johannessen, L. E., and Madhus, I. H. (2002) J. Cell Biol. 156, 843–854

32. Yin, H. L., and Janney, P. A. (2003) Ann. Rev. Physiol. 65, 781–789

33. Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T., and Horwitz, A. R. (2003) Science 302, 1704–1709

34. Haugh, J. M., Codazzi, F., Teruel, M., and Meyer, T. (2000) J. Cell Biol. 151, 1289–1290

35. van der Flier, A., and Sonnenberg, A. (2001) Biochim. Biophys. Acta 1538, 99–117

36. Klemke, R. L., Leng, J., Molander, R., Brooks, P. C., Vuori, K., and Cheresh, D. A. (1998) J. Cell Biol. 140, 961–972

37. Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., and Matsuda, M. (1998) Genes Dev. 12, 3331–3336

38. Collins, L. R., Ricketts, W. A., Yeh, L., and Cheresh, D. (1999) J. Cell Biol. 147, 1561–1568

39. Vandenbroere, I., Paternotte, N., Dumont, J. E., Erneux, C., and Pirson, I. (2003) Biochem. Biophys. Res. Commun. 300, 494–500

40. Mitchell, C. A., Gurung, R., Kong, A. M., Dyson, J. M., Tan, A., and Ooms, L. M. (2002) IUBMB Life 55, 25–36

41. Cremona, O., Di Paolo, G., Wenk, M. R., Luthi, A., Kim, W. T., Takei, K., Daniell, L., Nemoto, Y., Shears, S. B., Flavell, R. A., McCormick, D. A., and De Camilli, P. (1999) Cell 99, 179–188

42. Malecz, N., McCabe, P. C., Spaargaren, C., Qiu, R., Chuang, Y., and Symons, M. (2000) Curr. Biol. 10, 1383–1386