A Key Role for the Phosphorylation of Ser440 by the Cyclic AMP-dependent Protein Kinase in Regulating the Activity of the Src Homology 2 Domain-containing Inositol 5’-Phosphatase (SHIP1)*

Jun Zhang1, Koki S. Ravichandran5, and James C. Garrison‡,§
From the Departments of 1Pharmacology and §Microbiology, University of Virginia, Charlottesville, Virginia 22908

The Src homology 2 domain-containing inositol 5’-phosphatase 1 (SHIP1) dephosphorylates phosphatidylinositol 3,4,5-trisphosphate to phophatidylinositol 3,4-bisphosphate in hematopoietic cells to regulate multiple cell signaling pathways. SHIP1 can be phosphorylated by the cyclic AMP-dependent protein kinase (PKA), resulting in an increase in SHIP1 activity (Zhang, J., Walk, S. F., Ravichandran, K. S., and Garrison, J. C. (2009) J. Biol. Chem. 284, 20070–20078). Using a combination of approaches, we identified the serine residue regulating SHIP1 activity. After mass spectrometric identification of 17 serine and threonine residues on SHIP1 as being phosphorylated by PKA in vitro, studies with truncation mutants of SHIP1 narrowed the phosphorylation site to the catalytic region between residues 400 and 866. Of the two candidate phosphorylation sites located in this region (Ser440 and Ser774), only mutation of Ser440 to Ala abolished the ability of PKA to phosphorylate the purified, catalytic domain of SHIP1 (residues 401–866). Mutation of Ser440 to Ala in full-length SHIP1 abrogated the ability of PKA to increase the activity of SHIP1 in mammalian cells. Using flow cytometry, we found that the PKA activator, Sp-adenosine 3’,5’-cyclic monophosphorothioate triethylammonium salt hydrate (Sp-cAMPS) blunted the phosphorylation of Akt downstream of B cell antigen receptor engagement in SHIP1-null DT40 B lymphocytes expressing native mouse SHIP1. The inhibitory effect of Sp-cAMPS was absent in cells expressing the S440A mutant of SHIP1. These results suggest that activation of SHIP1 by PKA via phosphorylation on Ser440 is an important regulatory event in hematopoietic cells.

Formation of phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-P3) is central to many intracellular signaling cascades (1). PtdIns-3,4,5-P3 is produced in the inner leaflet of the plasma membrane by a family of phosphatidylinositol 3-kinases that are activated by receptor tyrosine kinases or G protein-coupled receptors (2). The PtdIns-3,4,5-P3 localized at the plasma membrane forms a docking site to attract and regulate downstream signaling molecules containing pleckstrin homology domains, such as the serine kinase, Akt (PKB), that has an essential role in stimulating cell proliferation, growth, survival, and metabolism (2, 3). The level of PtdIns-3,4,5-P3 in the membrane is opposed by two inositol lipid phosphatases, PTEN (phosphatase and tensin homologue) and SHIP (SH2 domain-containing inositol 5’-phosphatase) (4). SHIP opposes the effects of phosphatidylinositol 3-kinases by dephosphorylating the 5’-position on the inositol ring of PtdIns-3,4,5-P3 and producing phosphatidylinositol 3,4-bisphosphate (5).

There are two major members of the SHIP family, SHIP1 and SHIP2, that share a highly homologous catalytic region with less similarity in their C-terminal regions (6). SHIP2 is widely expressed, whereas SHIP1 is only expressed in hematopoietic cells (7). SHIP1 was originally identified based on its ability to bind to cytoplasmic adaptor proteins, such as Shc, Dab-1, and Dok-3 (8). SHIP1 contains an N-terminal SH2 domain that leads to interactions with immune receptors, such as FcγRIIB and FceRI (9), a central inositol 5’-phosphatase domain, and two tyrosines within NPYX motifs in the C-terminal proline-rich region (10). Genetic manipulations of SHIP1 in mice have shown that SHIP1 plays important roles in functions of myeloid cells and B lymphocytes; for example, in mature neutrophils and mast cells, PtdIns-3,4,5-P3 levels and the phosphorylation of Akt activity are significantly elevated in the absence of SHIP1 (11). Similarly, B lymphocytes lacking SHIP1 are insensitive to inhibitory signaling via the FcγRIIB receptor (9). Moreover, SHIP1−/− mice have a decreased viability due to an increased infiltration of myeloid cells into the lungs, perhaps due to activation of migration pathways because of the elevated levels of PtdIns-3,4,5-P3 (12).

Due to its importance in hematopoietic cells, the activity of SHIP1 is tightly regulated. One model for regulation of SHIP1 function envisions translocation of SHIP1 from the cytosol to the membrane (13). Upon stimulation by growth factors, cytokine receptors, or specific signaling receptors on lymphocytes, SHIP1 is recruited via its N-terminal SH2 domain to phosphorylated tyrosine residues in receptor kinases and degrades the elevated levels of PtdIns-3,4,5-P3 near the activated receptor (14). It was also reported that the catalytic activity of SHIP1 can be allosterically enhanced by the binding of its lipid product phosphatidylinositol 3,4-bisphosphate or a small molecule to its

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1 To whom correspondence should be addressed: P.O. Box 800735, University of Virginia, Charlottesville, VA 22908. Tel.: 434-924-5618; Fax: 434-924-5207; E-mail: jcg8b@virginia.edu.
2 The abbreviations used are: PtdIns-3,4,5-P3, phosphatidylinositol 3,4,5-trisphosphate; PKA, cyclic AMP-dependent protein kinase; Sp-cAMPS, Sp-adenosine 3’,5’-cyclic monophosphorothioate triethylammonium salt hydrate; SH2, Src homology 2; BCR, B cell antigen receptor; MFI, mean fluorescence intensity.
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C2 domain (15, 16). SHIP1 has also been found to undergo phosphorylation on two tyrosine residues by tyrosine kinases (14, 17). However, it is not clear that this phosphorylation event can regulate SHIP1 activity (14). We have demonstrated that SHIP1 can be phosphorylated and activated by the cyclic AMP-dependent protein kinase (PKA) both in vitro and in cells, highlighting a new mode of SHIP1 regulation (18).

Although the activation of SHIP1 by PKA is a potentially important regulatory event, the site(s) of phosphorylation within the SHIP1 molecule have not been identified. In the present study, through mass spectrometry and site-specific mutations, we identified and characterized the potential PKA-mediated phosphorylation sites within SHIP1. We also created SHIP1−/− DT40 cell lines stably expressing native and mutated SHIP1 molecules to evaluate the importance of selected phosphorylation sites in the regulation of SHIP1 by PKA. These results demonstrate that Ser440 within the catalytic region of the molecule is the site phosphorylated by PKA to activate SHIP1 and that the phosphorylation event is functionally relevant in B lymphocytes.

EXPERIMENTAL PROCEDURES

Materials—Anti-SHIP antibodies (catalogue nos. SC-8425 (P1C1) to the C terminus and SC-14503 (D20) to the phosphatase domain) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-FLAG M2 monoclonal antibody and anti-FLAG M2 affinity gel were purchased from Sigma. The FLAG peptide was synthesized by Bio Basic Inc. (Markham, Canada). The unlabeled phospho-Akt (Thr308), the Alexa Fluor 488-conjugated phospho-Akt (Thr308), the total Akt, and the Alexa Fluor 647-conjugated anti-FLAG antibodies were purchased from Cell Signaling Technology (Danvers, MA). The goat F(ab′)2 anti-mouse IgM and mouse anti-chicken IgM (M4) antibodies were obtained from Southern Biotech (Birmingham, AL). Phosflow Fix Buffer I and Perm Buffer III were purchased from BD Biosciences. The sources of all other reagents have been published (18).

Truncated SHIP1 Construction and Site-directed Mutagenesis—The cDNA encoding mouse SHIP1 was subcloned as described previously into the pCMVTag2C vector (Stratagene), which adds a FLAG epitope to the N terminus of the expressed protein (18). To allow expression of and one-step purification of truncated regions of the SHIP1 protein, cDNA constructs encoding truncated regions of SHIP1 were generated by PCR and subcloned into the pCMVTag2C vector. The resulting truncated proteins contained amino acids 401–866, 401–1190, or 1–866 of SHIP1. Full-length SHIP1 cDNAs encoding single or double point mutations of putative phosphorylation sites (see “Results”) were generated with the QuikChange II XL site-directed mutagenesis kit (Stratagene) in the pCMVTag2C vector. All cDNA constructs used to express the full-length, truncated, or mutant SHIP1 proteins were sequenced to ensure fidelity.

Cell Culture and Transfection—HEK-293 cells were cultured in DMEM supplemented with 10% fetal bovine serum and transfected with the plasmids indicated under “Results” using Lipofectamine 2000 (Invitrogen) at a 1:1 ratio (DNA/Lipofectamine, w/v) as described (18). DT40 cell suspensions (9) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, 2 mM l-glutamine, 1% (v/v) chicken serum (Sigma), and 50 μM 2-mercaptoethanol as described (18). Stable DT40 cell lines expressing full-length, wild type SHIP1 and SHIP1 carrying two single point mutations of putative phosphorylation sites at Ser440 (S440A) or Ser774 (S774A) were generated for these experiments as follows. Thirty μg of linearized cDNA encoding wild type, S440A, or S774A SHIP1 was co-transfected by electroporation at 250 V and 960 microfarads into SHIP-deficient DT40 cells (9) in combination with 3 μg of linearized pApurol plasmid DNA to provide a selectable puromycin marker. Following electroporation, cells were allowed to stabilize for 24 h in a T-flask, the medium was removed by centrifugation, and the cells were plated in 96-well plates in RPMI 1640 medium containing all of the above supplements plus 0.5 μg/ml puromycin to select for cells expressing the SHIP1 proteins. The clonal DT40 cells stably expressing wild type SHIP1 or the two point mutants were expanded and lysed, the supernatant was run on an SDS gel, and the expressed SHIP1 proteins were identified by Western blot with anti-SHIP and anti-FLAG antibodies. The DT40 lines stably expressing native SHIP1 and the two mutants were expanded in suspension culture as above. The stable DT40 cell line expressing the truncated SH2-401-900 SHIP1 protein was cultured as described (19).

Purification of Recombinant SHIP1 Proteins—The FLAG-tagged wild type, truncated, or phosphorylation site mutant SHIP1 proteins were overexpressed in confluent HEK-293 cells for 48 h, and the cells were solubilized in 0.5 ml of lysis buffer containing 25 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM MgCl2, 1% (v/v) Triton X-100, and a mix of protease and phosphatase inhibitors. This mixture contained 200 mM microcystin, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 100 μg/ml benzamidine, and 100 μg/ml pefabloc SC. The cell debris was removed by centrifugation at 20,000 × g for 15 min at 4 °C, and the recombinant proteins were purified by immunoadfinity chromatography using 40 μl of anti-FLAG beads as described (18). To purify SHIP1 from the clonal DT40 cells stably expressing wild type or mutant SHIP1 proteins, 1 × 108 cells were pelleted by centrifugation and solubilized in 0.5 ml of the above lysis buffer, and the proteins were purified by immunoadfinity chromatography. To determine the yield and purity of each recombinant protein, fractions collected from the FLAG column were loaded on an 8% SDS gel, separated by electrophoresis, and stained with Coomassie Blue (Simply Blue, Invitrogen). The gels were scanned with a Bio-Rad GS-800 densitometer, and the concentration of SHIP1 was determined from the optical density of the protein bands as compared with a standard curve of β-galactosidase ranging from 0 to 500 ng/lane.

In Vitro Phosphorylation of SHIP1—To phosphorylate the purified SHIP1 protein in vitro, wild type, truncated, or phosphorylation site mutants of SHIP1 were incubated with 30 units of PKA catalytic subunit in 10 μl of buffer containing 20 mM Hepes, pH 7.4, 200 mM microcystin, 0.1 mg/ml BSA, 12.5 mM magnesium acetate, and 1.25 mM EGTA (phosphorylation buffer). The reaction was started by the addition of 1 μl of 1.25 mM ATP containing 2.2 × 106 dpm of [γ-32P]ATP/tube and...
incubated for 0–30 min at 30 °C with frequent gentle mixing. Reactions were terminated by adding SDS sample buffer and heating to 95 °C. Aliquots were run on an 8% SDS gel, stained with Coomassie Blue or silver, and dried, and an autoradiograph was prepared with Kodak X-Omat LS film.

Mapping PKA-phosphorylated Serine/Threonine Residues in SHIP1 by Mass Spectrometry—The SHIP1 protein has 32 potential phosphorylation sites, as predicted by the KinasePhos computer program (20). In an effort to identify the majority of these sites via mass spectrometry (MS), 3 μg of pure, wild type SHIP1 was phosphorylated with a large amount of PKA (10,000 units) for 60 min at 30 °C in the above phosphorylation buffer without radiolabeling. A control reaction was run with 3 μg of SHIP1, the PKA storage buffer (20 mM Hepes, pH 7.4, 100 mM KCl, 1 mM DTT, 10% glycerol), and no added kinase. Reactions were terminated by adding SDS sample buffer and heating to 95 °C. The reaction mix was run on an 8% SDS-polyacrylamide gel, and the phosphorylated residues in SHIP1 were identified by the Biomolecular Research Facility at the University of Virginia using a Finnigan LTQ-FT MS system (21). Briefly, the gel pieces were washed and destained in 200 μl of 50% methanol for 16 h and then dehydrated, rehydrated, and digested with trypsin overnight at 37 °C. The tryptic peptides were extracted from the gel with two 30-μl aliquots of 50% acetonitrile, 5% formic acid. These extracts were combined and evaporated to 15 μl for MS analysis. The digest was analyzed using the MS/MS capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine the amino acid sequence. The data were analyzed using the Sequest search algorithm against the mouse SHIP1 sequence. Spectra that were thought to contain phosphorylated peptides were manually examined and verified. To reduce the interference from keratin in the mass spectrometry analysis, special care was taken while purifying the SHIP1 protein and during the phosphorylation reaction by wearing gloves and by filtering all buffers through a 0.22-μm filter.

Assay of SHIP1 or Phosphorylated SHIP1 Activity—The protocols for the assay of SHIP1 activity have been published (18). Briefly, the assay was carried out in a mixture of 20 mM Hepes, pH 7.4, 10 mM MgCl2 and 100 μM di-o-octanoylglycerol-PtdIns-3,4,5-P3. The amount of inorganic phosphate released by dephosphorylating diolein for 16 h and then dehydrated, rehydrated, and digested with trypsin overnight at 37 °C. The tryptic peptides were extracted from the gel with two 30-μl aliquots of 50% acetonitrile, 5% formic acid. These extracts were combined and evaporated to 15 μl for MS analysis. The digest was analyzed using the MS/MS capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine the amino acid sequence. The data were analyzed using the Sequest search algorithm against the mouse SHIP1 sequence. Spectra that were thought to contain phosphorylated peptides were manually examined and verified. To reduce the interference from keratin in the mass spectrometry analysis, special care was taken while purifying the SHIP1 protein and during the phosphorylation reaction by wearing gloves and by filtering all buffers through a 0.22-μm filter.

Measurement of Akt Phosphorylation by Western Blotting—Suspension cultures of DT40 cells were washed and resuspended at 2 × 107 cells/ml in serum-free RPMI 1640. The resuspended cells were aliquoted into separate tubes containing vehicle (H2O) or Sp-cAMPS and incubated for 15 min at 37 °C (to phosphorylate SHIP1) prior to stimulation of the B cell antigen receptor (BCR). The BCR was activated by adding a secondary antibody (2 μg/ml F(ab)2 fragment of goat anti-mouse IgM) for 5 min before adding a mouse anti-chicken IgM (M4) antibody at 0.5 μg/ml. After 0, 1, 3, and 5 min of stimulation, aliquots containing 1 × 107 cells were withdrawn, pelleted in a microcentrifuge for 5 s, and solubilized in 100 μl of lysis buffer as described (18). The mixture was centrifuged at 12,000 × g for 10 min at 4 °C. Ten μl of the supernatant was resolved on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed for phospho-Akt (Thr308) and total AKT.

Analysis of Phospho-AKT in DT40 Cells by Flow Cytometry—Flow cytometry was used to select the DT40 cells expressing FLAG-tagged SHIP1 and accurately measure the phosphorylation state of Akt without potential artifacts due to cell lysis and Western blotting. The procedures used for Sp-cAMPS treatment and BCR stimulation of DT40 cells were the same as described above. Two min after BCR stimulation, 3 × 106 cells were immediately pelleted and resuspended in 0.5 ml of prewarmed BD Phosflow Fix Buffer I. Tubes were incubated for 10 min in a 37 °C water bath to fix the cells and then chilled for 1 min on ice. Cells were washed once with 1 ml of incubation buffer (0.5% BSA in PBS) and then permeabilized in 0.5 ml of BD Phosflow Perm Buffer III at 4 °C for 1 h. After two washes with the incubation buffer, cells were incubated with fluorochrome-conjugated anti-FLAG and anti–phospho-Akt (Thr308) at 1:100 dilution in incubation buffer for 1 h at room temperature. Finally, cells were washed with 1 ml of incubation buffer and resuspended in 0.5 ml of PBS.

Samples were then analyzed on a BD FACS Canto II flow cytometer equipped with 488- and 633-nm lasers and emission filters for FITC (to detect the Alexa 488-conjugated phospho-Akt antibody) and allophycocyanin (to detect the Alexa 647-conjugated anti-FLAG antibody). At least 10,000 events were collected for every sample. Gating on FLAG-positive cells was used to assess SHIP1 expression within three different DT40 stable lines that expressed FLAG-tagged WT or mutant SHIP1. To measure the phosphorylation state of Akt, the level phospho-Akt (Thr308) in FLAG-positive cells was analyzed with FlowJo software (Tree Star, Inc.).

Data Presentation and Statistical Analysis—Averaged data are presented as means ± S.E. Control SHIP1 activity presented in Figs. 4 and 6 is normalized to 100%. Statistical difference between two individual treatments was examined via unpaired t tests using Statview software (SAS Institute Inc.).

RESULTS

Identification of the Serine/Threonine Residues Phosphorylated in the SHIP1 Protein by Mass Spectrometry—We previously demonstrated that SHIP1 is phosphorylated by PKA and that phosphorylation increased its catalytic activity (18). The SHIP1 protein has 32 serines or threonines within consensus phosphorylation sites that might be phosphorylated by PKA. To determine if these residues in SHIP1 were indeed phosphorylated by PKA, pure, recombinant SHIP1 obtained from HEK-293 cells was incubated with an excess of PKA in vitro and digested with trypsin, and the resulting peptides were analyzed by mass spectrometry. Table 1 presents a summary of the peptides containing serine or threonine residues that were found to be phosphorylated in the SHIP1 protein using this technique. In total, 17 peptides containing serine or threonine residues were identified; eight were phosphorylated in the “native” protein as
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Phosphorylation of Truncated SHIP1 — As shown in Fig. 1, the serine or threonine phosphorylation sites identified in SHIP1 by mass spectrometry tend to be distributed to the N- or C-terminal regions of the molecule. Previous work has demonstrated that fragments of the SHIP1 protein that contain the conserved 5'-inositol phosphatase domain (residues 401–866) have catalytic activity (19). Thus, to pursue which of the 17 phosphorylated residues might be important in the activation of SHIP1, we first generated N-terminal FLAG-tagged versions of full-length SHIP1 and three truncation mutants of the molecule. These truncation mutants were designed to code for an active molecule by virtue of containing the catalytic region (residues 401–866) but also to contain either the N-terminal region (residues 1–866) or the C-terminal region (residues 401–1190) (see schematic in Fig. 1).

To determine if the truncation mutants could be phosphorylated in vitro, the four constructs were transiently expressed in HEK-293 cells, and the proteins were purified using anti-FLAG beads and incubated with 30 units of the catalytic subunit of PKA and [γ\textsuperscript{32}P]ATP at 30 °C for 10 min. Fig. 2A shows that all four SHIP1 proteins can be phosphorylated when equal amounts of SHIP1 protein were incubated with PKA. Note that, as expected, the full-length SHIP1 (left) was found to contain higher amounts of [32P] than the N- and C-terminal truncation mutants or the 401–866 SHIP1 catalytic domain. Fig. 2B shows that all four SHIP1 proteins maintained the ability to dephosphorylate PtdIns-3,4,5-P\textsubscript{3}. Consistent with our previous report (18), phosphorylation of full-length SHIP1 (residues 1–1190) with PKA increased its activity about 2-fold (left bars). More importantly, phosphorylation of the catalytic domain of SHIP1 (residues 401–866) increased its activity over 3-fold, whereas phosphorylation of either the N-terminal (residues 1–866) or C-terminal regions (residues 401–1190) of SHIP1 did not increase their activities (Fig. 2B). Also of importance is the observation that all three truncation mutants of SHIP1 had higher specific activities than the wild type protein. The effect was particularly dramatic with the mutant missing its C-terminal region (residues 1–866), whose specific activity was 8–10 times higher than that of full-length SHIP1 (hatched bars in Fig. 2B). The latter observation suggests an important role for the C-terminal region of SHIP1 in the regulation of its activity. Although not observed previously with SHIP1, this result is in keeping with recent studies on the related SHIP2 isozyme showing that the C terminus and SH2 domains of the molecule confer an inhibitory effect to lower the basal activity of SHIP2 under unstimulated conditions (27).

Identification of the PKA Phosphorylation Site by Mutation of Serine/Threonine Residues — To search for the amino acid residue phosphorylated by PKA that activates SHIP1, we generated six single and two double point mutations of serines or threonines that were phosphorylated in the context of full-length SHIP1 protein (as shown

**TABLE 1**

**Phosphopeptides detected by mass spectrometry in pure, mouse SHIP1 treated with PKA**

| Peptide Site | MH\textsuperscript{a} |
|--------------|-----------------------|
| DGSFLVRASESI\textsuperscript{PR} Ser\textsuperscript{20} and Ser\textsuperscript{26} | 1693.735 |
| AP\textsuperscript{E}VTRISLSETF\textsuperscript{LQ} Ser\textsuperscript{163} | 1926.968 |
| LG\textsuperscript{Q}MDTSGLPEEHLK Ser\textsuperscript{171} | 1780.782 |
| LFDQQLSPGLR Ser\textsuperscript{240} | 1533.656 |
| PQV\textsuperscript{Q}G\textsuperscript{E}P\textsuperscript{M}\textsuperscript{E}P\textsuperscript{I}M\textsuperscript{V}K Ser\textsuperscript{250} | 1600.770 |
| LQ\textsuperscript{S}Q\textsuperscript{L}T\textsuperscript{S}L\textsuperscript{L}S\textsuperscript{I}ED\textsuperscript{K} Ser\textsuperscript{260} | 1613.803 |
| TRDD\textsuperscript{S}AD\textsuperscript{Y}PI\textsuperscript{D}HYV\textsuperscript{G}TG\textsuperscript{Q}D\textsuperscript{P}LE\textsuperscript{G}KEK Ser\textsuperscript{400} | 3027.362 |
| SQ\textsuperscript{E}G\textsuperscript{E}N\textsuperscript{E}C\textsuperscript{E}G\textsuperscript{E}G\textsuperscript{E}L\textsuperscript{V}R Ser\textsuperscript{724} | 1927.792 |
| STL\textsuperscript{S}P\textsuperscript{D}D\textsuperscript{Q}L\textsuperscript{F}AW\textsuperscript{S}YD\textsuperscript{Q}DLPK Ser\textsuperscript{934} | 2258.038 |
| GEG\textsuperscript{P}P\textsuperscript{T}P\textsuperscript{P}Q\textsuperscript{Q}P\textsuperscript{S}\textsuperscript{L}PK Thr\textsuperscript{986} and Ser\textsuperscript{971} | 1745.755 |
| KS\textsuperscript{F}S\textsuperscript{S}\textsuperscript{T}T\textsuperscript{N}R Ser\textsuperscript{1002} | 1107.483 |
| VE\textsuperscript{X}L\textsuperscript{I}QLE\textsuperscript{D}E\textsuperscript{L}LT\textsuperscript{K}\textsuperscript{P}M\textsuperscript{E}F\textsuperscript{P}EN\textsuperscript{L}G\textsuperscript{Y}SSF\textsuperscript{P}PK Ser\textsuperscript{1092} | 3490.710 |
| STFC\textsuperscript{S}SSA\textsuperscript{E}GR | 1268.461 |
| AS\textsuperscript{A}SSA\textsuperscript{Q}AP\textsuperscript{Q}V\textsuperscript{P}VK Ser\textsuperscript{1113} | 1221.587 |
| SPF\textsuperscript{A}VL\textsuperscript{Q}L\textsuperscript{Q}HSK Ser\textsuperscript{1160} | 1287.646 |

**FIGURE 1.** Phosphorylation sites detected in SHIP1. The schematic diagram shows positions of phosphorylated serine or threonine residues as determined by mass spectrometry. The SH2 domain, inositol 5'-phosphatase domain (5'-Pase), proline-rich sequences (dark bars), and the tyrosines in the C terminus are depicted. The phosphorylated serine or threonine residues found by mass spectrometry are indicated above the line. The known phosphorylated tyrosine residues are indicated below the line. The regions of the molecule encoded by the catalytic core (residues 401–866), the catalytic core and the C terminus (residues 401–1190), and the N terminus and the catalytic core (residues 1–866) are shown below the full-length molecule.
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FIGURE 2. Comparison of the effect of phosphorylation on the activity of three SHIP1 truncation mutants, 401–866, 401–1190, and 1–866. A, the upper panel presents an autoradiograph showing the ability of PKA to phosphorylate the three purified SHIP1 truncation mutants in vitro. Each reaction contained 30 ng of SHIP1 protein and was incubated with 30 units of PKA for 10 min. The lower panel presents the silver-stained 8% SDS gel used to make the autoradiograph and shows equal loading of the phosphorylated fragments. B, the purified, full-length SHIP1 protein and the truncation mutants were treated with 30 units of PKA for 10 min, and the activity of SHIP1 was measured using the Malachite Green assay. Phosphorylation of full-length SHIP1 and the 401–866 mutant containing the catalytic core increased their activity by 2- and 3-fold, respectively. NS, not significant; *, p < 0.05; ***, p < 0.001, n = 6. Error bars, S.E.

FIGURE 3. Mutation of Ser$^{440}$ to Ala abolished the ability of PKA to phosphorylate the 401–866 catalytic core of SHIP1. A, upper panel, an autoradiograph showing the ability of PKA to phosphorylate a series of single or double mutant serine or threonine mutants of the full-length, 1–1190 SHIP1 molecule. Each reaction contained 30 ng of SHIP1 protein and was incubated with 30 units of PKA for 10 min. Lower panel, Coomassie Blue-stained 8% SDS gel used to make the autoradiograph. B, 100 ng of the S440A and S774A mutants in the full-length SHIP1 molecule (upper part of B) and wild type, S440A, S774A, and S440A/S774A double mutant in SHIP1 401–866 (lower part of B) were phosphorylated by 30 units of PKA in the presence of [γ-$^{32}$P]ATP for the indicated time. PKA cannot phosphorylate the S440A single mutant and S440A/S774A double mutant in SHIP1 401–866.

in Table 1) and incubated them with PKA. The single residues modified to Ala were Ser$^{440}$, Ser$^{774}$, Ser$^{1022}$, Thr$^{1091}$, Ser$^{1113}$, and Ser$^{1160}$. The double alanine mutations were made for adjacent serine residues and include Ser$^{30}$/Ser$^{36}$ and Ser$^{971}$/Ser$^{976}$. As shown in Fig. 1, these residues are located throughout the SHIP1 molecule, including the SH2 domain, the 5'-phosphatase domain, and the C-terminal region of SHIP1.

The eight mutant SHIP1 proteins were expressed in HEK-293 cells and immunopurified with a FLAG antibody column, and the mutant proteins were incubated with 30 units of PKA for 10 min with [γ-$^{32}$P]ATP. All of the mutant SHIP1 proteins were highly expressed and efficiently purified. Fig. 3 (A and the upper part of B) shows that 30 units of PKA was able to phosphorylate all eight of the mutants although the double mutant, S30A/S36A, and the single mutant, T1091A, were phosphorylated less well than native SHIP1 or other SHIP1 mutants. Given that the 401–866 truncated mutant of SHIP1 was markedly activated by PKA (Fig. 2B) and contained only two phosphorylated residues (Ser$^{440}$ and Ser$^{774}$), we made additional point mutations in the truncated 401–866 protein. Single mutations to Ala were made at Ser$^{440}$ or Ser$^{774}$ along with the double Ser$^{440}$ and Ser$^{774}$ mutant. These proteins were purified and tested in the in vitro kinase assay with PKA. The lower part of Fig. 3B demonstrates that PKA can phosphorylate the unmodified 401–866 SHIP1 fragment and the fragment harboring the S774A mutation. However, PKA did not phosphorylate S440A or the S440A/S774A double mutant of the truncated SHIP1 molecule, even after 30 min of incubation. It is noteworthy that S440A and the double mutant S440A/S774A run slower than the S774A and the unmodified 401–866 SHIP1 fragment (Fig. 3B, bottom). These experiments indicate that Ser$^{440}$ is the most likely residue within SHIP1 that, when phosphorylated by PKA, increases the catalytic activity of the inositol phosphatase.

Effect of Mutations at Ser$^{440}$ and Ser$^{774}$ on the Activation of SHIP1 by PKA—The observation that only the activity of the 401–866 fragment of SHIP1 was increased by phosphorylation suggests that the phosphatase domain in SHIP1 may be an important target for regulation by PKA. The functional importance of modifications of the residues in the phosphatase domain is supported by the finding that naturally occurring mutations of Arg or Lys residues in the catalytic domain of INPP5E (type IV 5'-phosphatase) in patients with Joubert syndrome result in impaired 5'-phosphatase activity. Examination of fibroblasts isolated from these patients found altered ratios of phosphatidylinositol lipids, suggesting elevated phosphatidylinositol 1,4,5-trisphosphate levels. When cDNAs encoding the mutant INPP5E proteins were transfected into HEK-293T cells, the cells displayed a larger than normal phosphorylation of Akt following application of PDGF (28). Both of these results point to the hypothesis that modification of the amino acid residues in the catalytic domain may alter the activity of the
FIGURE 4. Phosphorylation of Ser\textsuperscript{440} in SHIP1 is required for activation of SHIP1 by PKA \textit{in vitro} and in intact HEK-293 cells. \textit{A}, the activity of the purified wild-type protein, the full-length S440A mutant, and the S774A mutant SHIP1 protein were compared using the Malachite Green assay following treatment with 30 units of PKA for 10 min. Activity was stimulated only with the wild-type and S774A mutant SHIP1 proteins. \( NS \), not significant. ***, \( p < 0.001 \), \( n = 8 \). \textit{B}, plasmids expressing the wild-type or mutant SHIP1 proteins were co-transfected into HEK-293 cells with or without a plasmid expressing the PKA catalytic subunit and incubated for 40 h. The recombinant SHIP1 proteins were immunopurified, and the activity of 10 ng of SHIP1 protein was measured using the Malachite Green assay. Transfection of the catalytic subunit only increased the activity of the wild type and S774A mutant SHIP1 proteins. \( NS \), not significant; **, \( p < 0.01 \); ***, \( p < 0.001 \), \( n = 5 \). The rate of PtdIns-3,4,5-P\(_3\) hydrolysis for control samples ranged between 1.4 and 1.9 pmol of P\(/\text{min}/\text{ng of SHIP1} and was normalized to 1.0. \( \text{Error bars, S.E.} \).

5'-phosphatase with direct consequences on signaling. Therefore, we mutated either Ser\textsuperscript{440} or Ser\textsuperscript{774} to Ala in the full-length protein and investigated the functional impact of these mutations on the phosphorylation and activation of SHIP1 both \textit{in vitro} and in cells.

The wild-type and full-length SHIP1 harboring the S440A or S774A mutations were expressed in HEK-293 cells and purified, and the 5'-phosphatase activity of the recombinant proteins was measured following incubation with 30 units of PKA for 10 min. Fig. 4A shows that, as expected, the activity of the wild-type protein is increased following phosphorylation with PKA (left bars); however, the protein harboring the S440A mutation was not activated following incubation with PKA (middle bars). The protein with the S774A mutation did show a small but significant increase in activity following incubation with PKA (right bars). To determine how mutations of S440A or S774A affect the activation of the protein by PKA in intact cells, experiments analogous to those shown in Fig. 4A were performed by co-transfecting HEK-293 cells with cDNA encoding wild-type or mutant SHIP1 proteins and a plasmid expressing the active PKA catalytic subunit. The proteins were purified, and their 5'-phosphatase activity was measured. Expression of an exogenous PKA catalytic subunit stimulated the activity of the wild type and S774A SHIP1 activity about 1.4-fold. In contrast, cotransfection of S440A SHIP1 with PKA did not stimulate the activity of the protein purified from the cells (Fig. 4B). As above, these results indicate that Ser\textsuperscript{440} may be the most likely residue to regulate the activity of SHIP1 via phosphorylation.

Ser\textsuperscript{440} in SHIP1 is Functionally Relevant in B Lymphocytes—The above data suggested that Ser\textsuperscript{440} is an important regulatory site in SHIP1. We chose DT40 B lymphocytes to determine whether the phosphorylation of Ser\textsuperscript{440} (or Ser\textsuperscript{774}) regulates the function of SHIP1 and inositol lipid metabolism in a cellular model. PtdIns-3,4,5-P\(_3\) levels are markedly stimulated in DT40 cells by engagement of the BCR, and the activity of SHIP1 plays a key role in opposing the increase in PtdIns-3,4,5-P\(_3\) caused by BCR stimulation (2, 18). Importantly, the DT40 cell line has a high rate of homologous recombination activity, and variant DT40 lines with specific proteins genetically knocked out are easily created (9, 19). The availability of SHIP1\textsuperscript{−/−} DT40 cells (9) and the ease of developing new stable lines expressing modified SHIP1 proteins (19) makes them an especially attractive cell system to study how the SHIP1 protein is regulated by phosphorylation. The first SHIP1\textsuperscript{−/−} DT40 line we examined expressed a truncated version of SHIP1 containing only the N-terminal SH2 domain and the 401–900 minimal catalytic region of SHIP1 (19). This 80-kDa protein contains two of the important functional domains of SHIP1, the SH2 sequence needed for translocation to the membrane and an active catalytic region that includes the Ser\textsuperscript{440} phosphorylation site. It was reasoned that the activity of this truncated protein would respond to phosphorylation by PKA, but it had no other domains to complicate the response (19).

Immunoblotting results presented in Fig. 5A demonstrate that the truncated, SH2-401-900 SHIP1 protein was properly expressed in the SHIP1\textsuperscript{−/−} DT40 cells as a 80-kDa protein. Raising PtdIns-3,4,5-P\(_3\) levels in these cells by ligation of the BCR caused a 2-fold increase in the phosphorylation of Akt on Thr\textsuperscript{308} over a 5-min time period (Fig. 5B). Pretreatment of the cells with 100 \( \mu \text{M} \) Sp-cAMPS for 15 min markedly reduced the BCR-induced phosphorylation of Akt at the 1 and 3 min time points, suggesting that the SHIP1 protein was activated. This result is consistent with the \textit{in vitro} result shown in Fig. 2B with pure proteins and implies that the 401–900 catalytic region of SHIP1 is modified via phosphorylation with PKA.

We then generated new stable lines expressing wild-type, S440A, or S774A mouse SHIP1 proteins. Between two and eight clones were obtained for each stable line, and the expression level of SHIP1 was monitored using an anti-SHIP1 antibody. Fig. 6A presents a Western blot showing high expression of SHIP1 in the three DT40 lines used in the flow cytometry experiments described below. Fig. 6B provides quantitative data on the expression of wild-type SHIP1 and the two mutants as measured by flow cytometry using a fluorescent antibody against the FLAG epitope on the protein. Fig. 6B shows that 92.4%–96% of the cells in each of the three new lines express SHIP1. Importantly, the shape of the peaks suggests that most cells in each population express similar amounts of SHIP1. We then used each of the three lines to measure the phosphorylation of Akt following BCR engagement as an index of PtdIns-
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We next examined the phospho-Akt levels in SHIP1\(^{-/-}\) DT40 cells expressing the full-length mouse SHIP1 molecule harboring specific point mutations (S440A or S774A) using similar protocols. Fig. 6D presents an experiment performed with cells expressing the full-length S440A point mutation of SHIP1. The increase in phospho-Akt (peak shift) caused by BCR engagement was similar to that observed in cells expressing the wild type protein, but pretreatment of the cells with Sp-cAMPS did not inhibit the phospho-Akt response (peak shift) to BCR engagement. The quantitative analysis of the changes in fluorescent intensity is shown in the bottom panel of Fig. 6D. The level of phospho-Akt was increased about 3.9-fold following BCR engagement (solid bar), but the increase was still about 3.6-fold after treatment with Sp-cAMPS (hatched bars). There is no significant difference in the increase in MFI (increase in phospho-Akt levels) between the control and Sp-cAMPS-treated cells (p = 0.08). Fig. 6E presents an analogous experiment performed with DT40 cells expressing the S774A point mutation. As observed with cells expressing the wild type protein, BCR engagement caused a marked increase in phospho-Akt (a 3.2-fold increase in MFI), and pretreatment of the cells with Sp-cAMPS markedly blunted the response (only about a 1.7-fold increase in MFI). This result is consistent with the hypothesis that Ser\(^{774}\) is not essential for the regulation of SHIP1 via phosphorylation by PKA. Taken together, these results suggest that activation of SHIP1 via phosphorylation of Ser\(^{440}\) is an important regulatory event in hematopoietic cells.

Effect of Sp-cAMPS on the Activity of SHIP1 Purified from DT40 Cells—To address how phosphorylation of SHIP1 with the endogenous DT40 cell PKA affects its enzymatic activity, SHIP1 was immunopurified from the DT40 cell lines overexpressing the wild type and S440A point mutants of SHIP1. The cells were treated with Sp-cAMPS for 15 min, and the proteins were purified and assayed for their ability to dephosphorylate di-o-octanoylglycerol-PtdIns-3,4,5-P\(_3\) in vitro. As shown in Fig. 7, the activity of native SHIP1 purified from DT40 cells was increased about 1.5-fold following treatment with Sp-cAMPS. The activity of the S440A mutant of SHIP1 was not increased by treatment of the cells with Sp-cAMPS. The right two bars present experiments performed with a second, S440A-expressing clone (Clone 2), which provided identical results. Collectively, the results shown in Figs. 6 and 7 strongly support a role for Ser\(^{440}\) as a relevant PKA phosphorylation site on SHIP1 in cells.

**DISCUSSION**

The two major isoforms of the SH2 domain containing phosphatidylinositol phosphatase, SHIP1 and SHIP2, are important enzymes that lower the level of the PtdIns-3,4,5-P\(_3\) signal (5, 6). SHIP1 is highly expressed in hematopoietic cells and is an important regulator of the phosphatidylinositol 3-kinase/AKT pathway in these cells (5, 6). We recently demonstrated that the activity of SHIP1 could be stimulated by phosphorylation with PKA (18); this finding provides a new mode of SHIP1 regulation by G protein-coupled receptors that raise cyclic AMP. Because of the biological and therapeutic significance of SHIP1 (5), identification of the phosphorylation sites in SHIP1 is an
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FIGURE 6. Mutation of Ser^{440} to Ala blunted the inhibitory effect of Sp-cAMPS on the BCR-induced phosphorylation of Akt in DT40 cells. DT40 cells were transfected with wild type and mutant mouse SHIP1 proteins, stable cell lines were established, and the phosphorylation state of Akt was measured as a reflection of PtdIns-3,4,5-P3 levels in the cells. A, immunoblots showing that the wild type (WT), S440A, and S774A SHIP1 molecules are expressed in the stable DT40 lines. Immunoblotting was performed with an anti-SHIP1 antibody, P1C1. B, the percentage of FLAG-positive cells in WT, S440A, or S774A SHIP1-expressing DT40 lines was quantified as described under “Experimental Procedures.” The FLAG-negative SHIP1−/− DT40 cells were used as the control. The x axis presents the fluorescence intensity of the anti-FLAG Alexa 647 antibody using a log scale. The y axis (percentage of maximum) shows the number of cells with a given fluorescence intensity divided by the number of cells in the bin containing the largest number of cells. C, the effect of Sp-cAMPS on the BCR-induced phosphorylation of Akt in SHIP1−/− DT40 cells expressing the wild type SHIP1 protein. Upper panel, the distribution of fluorescence intensity from the Alexa 488-conjugated anti-phospho-Akt antibody in control cells and those treated with the anti-chicken M4 antibody to activate the BCR for 2 min. Light gray peaks represent the phospho-Akt fluorescence intensity in cells before BCR engagement, and the dark gray peaks show the cells after 2 min of BCR engagement. The upper pair of peaks were from cells pretreated with 100 μM Sp-cAMPS for 15 min. Lower panel, the fold change of geometric MFI of the phospho-Akt (Thr^{308}) response caused by BCR engagement calculated as described under “Experimental Procedures.” Left two bars, the effect of 2 min of BCR engagement caused a 3.4-fold increase in intensity in control cells; right two hatched bars, minimal increase following pretreatment with Sp-cAMPS. D, an experiment analogous to that presented in C performed with SHIP1−−− DT40 cells expressing the S440A mutant of full-length SHIP1. Treatment of these cells with Sp-cAMPS did not affect the ability of BCR engagement to stimulate the level of phospho-Akt (right hatched bars). E, experiments analogous to those presented in C and D performed with SHIP1−/− DT40 cells expressing the S774A mutant of full-length SHIP1. Treatment of these cells with Sp-cAMPS inhibited the ability of BCR engagement to stimulate the level of phospho-Akt equally well as in cells expressing wild type SHIP1. The data are representative of three separate experiments with each mutant. NS, not significant; **, p < 0.01; ***, p < 0.001, n = 3. Error bars, S.E.

FIGURE 7. Measurement of the activity of SHIP1 extracted from DT40 cells. DT40 SHIP1−/− cells expressing the wild type and S440A full-length SHIP1 proteins were treated with 100 μM Sp-cAMPS to activate the endogenous PKA for 15 min. Two independent S440A clones were examined, Clone 1 and Clone 2. The expressed SHIP1 was immunopurified, the protein concentration was determined, and the activity of 10 ng of SHIP1 was measured with the Malachite Green assay. The activity of the wild type SHIP1 was increased 1.5-fold. ***, p < 0.001, n = 6. The activity of SHIP1 was not increased by Sp-cAMPS treatment in either of the DT40 cell clones expressing the S440A full-length SHIP1. NS, not significant. The rate of PtdIns-3,4,5-P3, hydrolysis for control samples ranged between 3.5 and 3.7 pmol of P/min/ng of SHIP1 and was normalized to 1. Error bars, S.E.
This conclusion is based on six lines of evidence. First, the phosphopeptide containing Ser$^{440}$ that was identified by mass spectrometry (TRDDSADYIPHTDYQTEDPLGEK) harbors the RXS(S/T) consensus motif for phosphorylation by PKA (24–26). It is noteworthy that the KTRDDSDAD sequence is conserved among all five known SHIP1 homologues in Homo sapiens, Rattus norvegicus, Mus musculus, Xenopus laevis, and Salmo salar (Fig. 8). Second, mutation of Ser$^{440}$ to Ala in the truncated, catalytic domain of the molecule (residues 401–866) abolished the phosphorylation of SHIP1 by PKA in vitro (Fig. 3B). Third, mutation of Ser$^{440}$ to Ala in the full-length molecule (residues 1–1190) greatly reduced the ability of PKA to activate SHIP1 in vitro (Fig. 4A). Fourth, overexpression of the PKA catalytic subunit with the full-length wild type SHIP1 protein in HEK-293 cells results in an activated SHIP1 (Fig. 4B); the full-length protein containing an S440A point mutation is not activated in this protocol. Fifth, flow cytometry analysis demonstrates that transfection of a full-length S440A mutant protein into SHIP$^{+/−}$ DT40 cells yields a cell in which the phosphoryoAkt response is not inhibited by activation of PKA (Fig. 5D). This finding suggests that the phosphorylation of SHIP1 is a physiologically relevant process in hematopoietic cells. Finally, when SHIP$^{−/−}$ DT40 cells expressing the wild type SHIP1 protein are treated with Sp-cAMPS to activate PKA, the enzyme is activated. If a full-length S440A point mutation is expressed in the cells, treatment with Sp-cAMPS does not activate SHIP1 (Fig. 7). Taken together, these data identify Ser$^{440}$ as the site in the molecule that is phosphorylated by PKA to activate SHIP1.

The phosphorylation site identified, Ser$^{440}$, is not in one of the domains in SHIP1 previously thought to be involved in its regulation (6). The major regulatory domains of SHIP1 include an SH2 domain in its N-terminal region, a C2 domain located at the C-terminal end of its phosphatase domain, and a C terminus containing a proline-rich region and two NPXY amino acid sequences. These domains have been shown to act as important docking sites for signaling molecules, adaptor proteins, allosteric ligands, and immunoreceptors (8–10, 15, 16). The SH2 domain mediates interaction with tyrosine-phosphorylated proteins, the immunoreceptor tyrosine-based inhibitory motifs, and immunoreceptor tyrosine-based activation motifs (8, 9). The C2 domain is the binding site for potential allosteric activators, such as phophatidylinositol 3,4-bisphosphate, and novel small molecules, such as AQX-MN100 (15, 16). The proline-rich region of the C terminus of SHIP1 interacts with the SH3 domain of Grb2 or PLCγ following activation of the immunoreceptor tyrosine-based activation motif-containing FceRI in RBL-2H3 cells (29). The two NPXY sequences, when phosphorylated following stimulation with antibodies, bind proteins such as Shc and Dok2, which have phosphotyrosine binding domains (17, 30). No phosphorylation or direct regulation of the central catalytic core (the 5′-phosphatase region itself) has been previously reported. The current model for SHIP1 regulation in various physiological situations is primarily based on the translocation of SHIP1 from the cytosol to the membrane following activation of receptor tyrosine kinases (growth factors, cytokine receptors, or immunoreceptors). In this translocation model, no significant change has been detected in the 5′-phosphatase activity of SHIP1 (13). Our results indicate that SHIP1 activity can be regulated by PKA phosphorylation on Ser$^{440}$ in the catalytic core and highlight a new mode of SHIP1 regulation.

Although Ser$^{440}$ may be a primary phosphorylation site involved in SHIP1 regulation, the possibility remains that other serine/threonine residues (Ser$^{30}$, Ser$^{36}$, Thr$^{979}$, Ser$^{1113}$, and Ser$^{1163}$) identified by mass spectrometry may also be involved in the overall regulation. As shown in Fig. 2B, two of the truncated constructs (mutants 401–1190 and 1–866) were not activated by phosphorylation with PKA despite the presence of Ser$^{440}$. Perhaps more importantly, the SHIP1 molecule lacking its C terminus (truncation mutant 1–866) is activated about 8–10-fold (see Fig. 2B). In this regard, it is most interesting that truncation of the homologous SHIP2 molecule by removal of the C-terminal region (truncation mutant 1–822) has also been shown to activate the enzyme (27). The finding that the C-terminal region of SHIP2 seems to inhibit its catalytic activity is very similar to our finding with an analogous mutant of SHIP1 (Fig. 2B). Thus, besides directly affecting the catalytic activity of the enzyme, Ser$^{440}$ phosphorylation could direct a conformational change in the protein, which relieves an inhibitory effect of the molecule’s C-terminal tail. The N terminus may also play a role in regulating the activity of SHIP1 (see Fig. 2B), but more information is needed to clarify this possibility. Taken together, the data suggest that phosphorylation of Ser$^{440}$ initiates events that activate the catalytic domain and may also relieve an inhibitory constraint by the C terminus of the molecule.

SHIP2 is a 142-kDa protein that contains a catalytic core structure similar to SHIP1 (7). Unlike the case with SHIP1, the phosphorylation of certain tyrosines in SHIP2 can enhance its 5′-phosphatase activity as measured in immunoprecipitates (27, 31). For example, if HeLa cells are transfected with SHIP2 and treated with EGF or H$_2$O$_2$, there is a marked increase in the tyrosine phosphorylation state of SHIP2 and a 2-fold increase in the activity of the enzyme (27, 31). Mutational analysis of tyrosines in SHIP2 showed that Tyr$^{986}$/Tyr$^{987}$ and Tyr$^{1135}$ were the most likely sites phosphorylated in response to EGF (27). Importantly, there are three potential regulatory serine/threonine residues (Thr$^{958}$, Ser$^{1003}$, and Ser$^{982}$) in the C-terminal region of SHIP2 (32–34). These three residues are located near Tyr$^{986}$, a well known tyrosine phosphorylation site in the NPXY motif of the molecule. Therefore, it has been suggested that the phosphorylation of Thr$^{958}$, Ser$^{1003}$, or Ser$^{982}$ could affect the affinity of Tyr$^{986}$ for its tyrosine kinase (32). Although there are a number of serine or threonine residues in the corresponding NPXY region of SHIP1 (see Fig. 1), based on our mutational analysis, they do not appear to be involved in the regulation of SHIP1 activity.
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There is recent genetic and structural evidence that modification of amino acid residues in the catalytic region of the inositol phosphatase can markedly alter their activity. Genetic studies with the type IV 5\' -phosphatase (INPP5E), a lipid phosphatase that hydrolyzes phosphate at the 5\' -position from PtdIns-3,4,5-P\textsubscript{3}, phosphatidylinositol 4,5-bisphosphate, and PtdIns-3,4,5-P\textsubscript{3} hint at how regulation of 5\' -phosphatase activity could be modified by alteration of the residues in the 5\' -phosphatase catalytic domain (28). INPP5E is a highly active 5\' -phosphatase of PtdIns-3,4,5-P\textsubscript{3} that is widely expressed and found in brain, testis, breast, hemopoietic, and other cell types (35). Patients with human ciliary diseases were found to have single-nucleotide polymorphisms in the INPP5E gene in the region of the molecule corresponding to the catalytic core of SHIP1 (28). These single-nucleotide polymorphisms mutated arginine or lysine residues centered in the phosphatase domain and greatly impaired the 5\' -phosphatase activity of the enzyme. Study of primary fibroblasts cultured from individual patients showed altered cellular phosphatidylinositol lipid ratios and transfection of the mutated proteins into HEK-293 cells resulted in phenotypes with high basal levels of phospho-Akt and an exaggerated response to PDGF (28). The authors modeled these mutations on the crystal structure of synaptojanin, a yeast 5\' -inositol phosphatase (36), and found the residues to be clustered in the catalytic core of the molecule near the binding pocket for PtdIns-3,4,5-P\textsubscript{3} (28). These data suggest that modification of the amino acids in the 5\' -phosphatase catalytic region of SHIP1 is capable of inducing meaningful changes in the enzyme's activity and provide additional support for our hypothesis that phosphorylation of Ser\textsuperscript{440} in the catalytic core of SHIP1 is important for regulation of PtdIns-3,4,5-P\textsubscript{3} signaling.

In conclusion, although tyrosine phosphorylation and activation of SHIP1 and SHIP2 is a highly studied area, there are few comprehensive reports on the regulation of SHIP1 or SHIP2 by serine/threonine kinases. PKA is the best characterized member of the serine/threonine protein kinase family, and phosphorylation of its substrates is important for regulating many cellular processes. Our previous observation that phosphorylation of SHIP1 with PKA markedly activates its function provided insight into an important new mode of SHIP1 regulation. In the present study, we have identified the Ser\textsuperscript{440} residue in SHIP1 as a major PKA phosphorylation site with functional implications. These results outline a new role for G protein-coupled receptors and PKA in regulating PtdIns-3,4,5-P\textsubscript{3} levels and inflammatory signals via the activation of SHIP1.

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