Enzymatic Activity of the Src Homology 2 Domain-containing Inositol Phosphatase Is Regulated by a Plasma Membrane Location*

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The negative regulatory role of the Src homology 2 domain-containing inositol 5-phosphatase (SHIP) has been invoked in a variety of receptor-mediated signaling pathways. In B lymphocytes, co-clustering of antigen receptor surface immunoglobulin with FcγRIIb promotes the negative effects of SHIP, but how SHIP activity is regulated is unknown. To explore this issue, we investigated the effect of SHIP phosphorylation, receptor tyrosine engagement by its Src homology 2 domain, and membrane recruitment of SHIP on its enzymatic activity. We examined two SHIP phosphorylation kinase candidates, Lyn and Syk, and observed that the Src protein-tyrosine kinase, Lyn, is far superior to Syk in its ability to phosphorylate SHIP both in vitro and in vivo. However, we found a minimal effect of phosphorylation or receptor tyrosine engagement of SHIP on its enzymatic activity, whereas membrane localization of SHIP significantly reduced cellular phosphatidylinositol 3,4,5-triphosphate levels. Based on our results, we propose that a membrane localization of SHIP is the crucial event in the induction of its phosphatase effects.

Clustering of the B cell surface immunoglobulin (sIg)1 antigen receptor by binding of foreign antigen initiates a set of biochemical events termed positive signaling which activate B lymphocytes to proliferate and secrete soluble antigen specific Ig (reviewed in Refs. 1–3). The most proximal signaling event is the stimulation of the Src family of protein-tyrosine kinases (PTKs), which phosphorylates tyrosine residues within conserved immunoreceptor tyrosine-based activation motifs (ITAMs), found in receptor-associated proteins (4). Once the tyrosines in the ITAM are phosphorylated, they serve as docking sites for numerous proteins and enzymes containing Src homology 2 (SH2) domains including the PTK Syk and the p85 adapter subunit of phosphatidylinositol 3-kinase (PtdIns 3-kinase; reviewed in Refs. 5 and 6). These activation signals are then propagated through additional tyrosine phosphorylation and protein-protein interactions and result in changes in B cell biology.

PtdIns 3-kinase is comprised of a p85 adapter subunit and a p110 catalytic subunit. By catalyzing the phosphorylation of the D-3 position of the inositol ring (7), PtdIns 3-kinase generates phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3), which acts as an intracellular mediator for several enzymes. PtdIns(3,4,5)P3 binds to pleckstrin homology domains of enzymes (8) such as Akt (9) and Btk (10–12), thereby promoting re-localization to the membrane and providing enzyme access to new lipid substrates or regulatory kinases (13).

In contrast to activating signals generated upon sIg clustering, co-clustering of sIg with the B cell Fc receptor for IgG (FcγRIIb) aborts B cell activation. It has been proposed (14, 15) that co-clustering of sIg and FcγRIIb occurs late in the humoral immune response to block continued Ig production. We have termed sIg-FcγRIIb co-clustering "negative signaling," to contrast with positive signaling initiated by sIg clustering alone and that promotes B cell proliferation. Phillips and Parker (16–18) described an in vitro model using a F(ab′)2 fragment and intact anti-Ig reagents of the IgG class to study biochemical events associated with positive and negative signaling. Earlier studies using this model demonstrated phosphorylation of a tyrosine residue contained within a 13-amino acid motif of the FcγRIIb cytoplasmic tail (19). Additional experiments showed that certain structural features of the FcγRIIb 13-amino acid motif were shared in common with other receptors that likewise conferred an inhibitory function; accordingly, the motif has been termed the immunoreceptor tyrosine-based inhibitory motif (ITIM; reviewed in Ref. 20). Like signaling through the ITAM, the phosphorylated ITIM recruits SH2 domain-containing molecules to carry out negative signaling.

The SH2 domain-containing inositol 5-phosphatase, SHIP, was identified as one of several proteins that bind to the tyrosine-phosphorylated ITIM of FcγRIIb (21, 22). SHIP is a 145-kDa cytosolic protein that contains a single SH2 domain, a central catalytic region, and two tyrosine phosphorylation sites in the C-terminal region (23–25). In addition to the B cell FcγRIIb, SHIP is recruited to and inhibits cellular activation by a variety of other receptors, including numerous cytokine receptors and the mast cell Fce receptor (reviewed in Refs. 26 and 27). Recently, in the B cell model, we demonstrated that SHIP was tyrosine-phosphorylated to high stoichiometry and associated with Ras adapter protein Shc only upon co-clustering sIg with FcγRIIb (28). These events were because of the direct recruitment of the SH2 domain of SHIP to the phosphorylated cytoplasmic tyrosine residue of the FcγRIIb ITIM and hence induces SHIP recruitment to the B cell plasma membrane (29). Similar findings of SHIP membrane translocation...
have been made in T cells stimulated via CD28 (30). The kinase-phosphorylating SHIP is unknown in any cellular system.

Studies of SHIP enzymatic activity revealed an exclusive preference for the hydrolysis of 3-phosphoinositides. As such, SHIP can reverse the action of PtdIns 3-kinase by consuming the PtdIns 3-kinase products (31). Other experiments revealed that co-clustering of slg and FcγRIIb leads to a dramatic reduction of cellular PtdIns-(3,4,5)P$_3$ (32, 33) and reduced activity of distal PtdIns-(3,4,5)P$_3$-responsive enzymes such as Btk (32, 34) and Akt (35, 36). Experiments by our lab (35) and others (37) showed that the reduction of PtdIns-(3,4,5)P$_3$ is not due to inactivation of PtdIns 3-kinase, because we observed no defect in p85 protein association or in membrane translocation of PtdIns 3-kinase. Therefore, stimulation of SHIP enzymatic activity and the resulting hydrolysis of PtdIns-(3,4,5)P$_3$ are most likely the cause of PtdIns-(3,4,5)P$_3$-dependent enzyme inhibition.

Despite the fact that SHIP enzymatic activity is induced by various cytokines and immunoreceptors, it is unclear how the enzymatic activity of SHIP is regulated. Because SHIP was highly phosphorylated upon co-clustering slg with FcγRIIb (21) and recruited to the plasma membrane through the engagement with the phosphorylated receptors (29, 30, 35, 38), we have formulated three distinct hypotheses that can account for the observed increased SHIP enzymatic activity. First, SHIP enzymatic activity may be directly stimulated by tyrosine phosphorylation. Other enzymes such as Vav (39, 40) or phosphatase CY (41) respond in this way to tyrosine phosphorylation. Second, the engagement of a phosphorylated ITIM may promote the elevated activity of SHIP. The SHP-1 tyrosine phosphatase, which binds to the same phosphorylated ITIM of p85 protein association or in membrane translocation of PtdIns 3-kinase, therefore, stimulation of SHIP enzymatic activity and the resulting hydrolysis of PtdIns-(3,4,5)P$_3$ are most likely the cause of PtdIns-(3,4,5)P$_3$-dependent enzyme inhibition.

Regulation of SHIP Activity

Preparation of [32P]PtdIns-(3,4)P$_2$ and [32P]PtdIns-(3,4,5)P$_3$—To produce the substrate and standards for SHIP 5-phosphatase assay, we prepared [32P]PtdIns-(3,4)P$_2$ and [32P]PtdIns-(3,4,5)P$_3$ essentially as described elsewhere (25), using commercial sources of PtdIns-(4,5)P$_2$ and PtdIns-(4)P (Calbiochem), phosphatidylinserine, [γ-32P]ATP, and immobilunoprecipitated PtdIns 3-kinase.

SHIP 5-Phosphatase Assay—All procedures were slightly modified from previous reports (25). Briefly, 1 × 10$^6$ cpm of [32P]PtdIns-(3,4,5)P$_3$ in chloroform/methanol (1:1, vol/vol) were evaporated under vacuum and resuspended by sonication in 300 μl of SHIP assay buffer (50 mM HEPES (pH 7.25), 10 mM MgCl$_2$, 1% Nonidet P-40). For 5-phosphatase assay, 50 μl of substrate in SHIP assay buffer, 0.1 mg/ml bovine serum albumin, and various amounts of recombinant SHIP enzyme were mixed to 30 μl of total volume. The reaction was stopped after 20 min at 37 °C by extraction of phospholipids with 100 μl of chloroform/methanol (1:1) and 100 μl of 2 M KCl. The organic phase containing SHIP substrates and products was washed four times with 100 μl of chloroform; the washes and organic phase were combined and evaporated under vacuum. The dried phospholipids were dissolved in 30 μl of chloroform/methanol (1:1), and the material was separated by thin layer chromatography (TLC) using silica gel 60 plates saturated with 1% 2,6-dichlorophenolindophenol and 1% formic acid in 50% ethanol, as described earlier (25). The identity of PtdIns-(3,4,5)P$_3$ and PtdIns-(3,4)P$_2$ were confirmed by comparison of the mobility of [32P]PtdIns-(3,4,5)P$_3$ and [32P]PtdIns-(3,4)P$_2$ prepared separately using authentic commercial standards on the same TLC plate. PtdIns-(3,4,5)P$_3$ and PtdIns-(3,4)P$_2$ were quantified by a Molecular Dynamics Storm system.

Phosphatase Activity Measurements of SHIP Phosphorylated by In Vitro and In Vivo Kinase Reaction—Lyn from pervanadate (1 mM sodium orthovanadate, 0.6% H$_2$O$_2$-stimulated A20 B cells and Syk from pervanadate-stimulated THP-1 monocytic cells were immunoprecipitated as described earlier (46) and resuspended in kinase buffer (20 mM HEPES (pH 7.4), 10 mM MgCl$_2$, 5 mM MnCl$_2$). To each reaction, 10 μg of cold ATP, 2–4 μCi of [γ-32P]ATP (3000 Ci/mmol), and 2 μg of recombinant SHIP were incubated with the immunoprecipitated kinases for 10 min at 30 °C in a total volume of 25 μl to permit SHIP phosphorylation. The reaction was stopped by adding 5x SDS sample buffer (0.6 M Tris (pH 6.8), 50% glycerol, 12% SDS) and incubating the samples at 95 °C for 5 min. The phosphorylated products, including the autophosphorylated kinases themselves, were analyzed by 7.5% SDS-PAGE, identified by autoradiography, and quantified by a Molecular Dynamics Storm system. To assess the effect of phosphorylation on enzymatic activity, the in vitro kinase reaction was performed as above. Because the Mn$^{2+}$ cation was inhibitory toward SHIP 5-phosphatase activity (see "Results"), we lowered [Mn$^{2+}$] by mixing 23 μM of supernatant containing recombinant, phosphorylated SHIP with 1 and 10 mM EDTA and MgCl$_2$, respectively. After incubation on ice for 10 min, 0.1 μM of recombinant SHIP was subjected to 5-phosphatase assay by addition into COS-7, 10 μg of cDNA encoding Lyn or Syk PTKs were co-transfected with 10 μg of cDNA encoding SHIP. Cells were harvested after 48 h, stimulated with pervanadate, and analyzed by antiphosphotyrosine blotting as described (21). In parallel, Lyn and Syk were immunoprecipitated from the SHIP co-transfected cells and assessed for in vitro kinase activity by autophosphorylation and by antiphospho-
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RESULTS

Generation, Purification, and Assay of Recombinant SHIP—

Purified SHIP enzyme was prepared from yeast transformed with cDNA encoding SHIP and containing a 6× Histidine tag at the C terminus. About 1 mg of SHIP enzyme was purified from yeast lysates derived from a 1-liter culture (Fig. 1A). We tested the purified recombinant enzyme in a phosphatase assay, using [32P]PtdIns-(3,4,5)P3 prepared from commercial PtdIns-(4,5)P2 and immunoprecipitated PtdIns-3-kinase. The results, shown in Fig. 1B, indicated that SHIP generated [32P]PtdIns-(3,4)P2 from [32P]PtdIns-(3,4,5)P3, indicating that the recombinant enzyme had 5-phosphatase activity, as expected. The enzymatic activity was lost in the presence of EDTA, revealing a need for a divalent cation to support SHIP hydrolytic activity toward [32P]PtdIns-(3,4,5)P3. We examined several divalent cations for their ability to support SHIP activity and found (Fig. 2A) that only Mg2+ was active in this regard. SHIP enzymatic activity was greatly reduced in the presence of Mn2+ and the oxidant, H2O2 (Fig. 2B). SHIP displayed a relatively broad pH optimum from pH 6 to pH 8 (Fig. 2C).

The Src Family PTK Lyn Is Superior to Syk in Its Ability to Phosphorylate SHIP—

To investigate whether tyrosine phosphorylation of SHIP affects its enzymatic activity, it was necessary to identify the PTK capable of phosphorylating SHIP. Both Src family PTKs and Syk associate with the B cell antigen receptor and either kinase family member are thus properly cotransfected cells, we stimulated B cells with or without pervanadate for 5 min and immunoprecipitated with anti-SHIP antibody. For measurements of SHIP 5-phosphatase activity, we stimulated B cells with or without pervanadate for 3 min. Lipid extraction and TLC were performed essentially as described earlier (47). [32P]PtdIns-(3,4,5)P3 and [32P]PtdIns-(3,4)P2 were prepared from a PtdIns-3-kinase reaction as described above and used as standards.

Immunofluorescence Studies—

COS-7 cells were transfected with cDNA encoding wild-type SHIP or the CD8-SHIP chimera. 48 h after transfection, cells were labeled by cell dissociation solution (Sigma) and stained with fluorescein isothiocyanate-conjugated anti-CD8 and biotinylated cholera toxin B subunit (to visualize the plasma membrane), followed by Alexa 568 (Molecular Probes, Eugene, OR)-conjugated streptavidin. Additionally, transfected COS-7 cells were permeabilized to identify intracellular proteins with 0.5% Triton X-100 in Tris-buffered saline and immunostained with Alexa 568-conjugated anti-SHIP antibodies. Cells were analyzed by confocal microscopy, and digital images were prepared.

A summary of the findings appears here; the original data were provided for review.

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earlier studies indicated stimulation of SHP-1 phosphotyrosine phosphatase activity upon engagement of a phosphorylated peptide corresponding to the ITIM of FcγRIIb (42). To test the possibility that SHIP activity was similarly regulated, recombinant SHIP was incubated with phosphorylated ITIM peptide or an unrelated phosphorylated peptide for 1 h, and the mixture was subjected to the 5-phosphatase assay (Fig. 5). Control experiments using these peptides in a pull-down assay (Fig. 5A) indicated that the phosphorylated FcγRIIb ITIM peptide but not other peptides were capable of engaging SHIP. However, despite its ability to bind SHIP, the ITIM peptide did not affect SHIP 5-phosphatase activity (Fig. 5, B and C). Thus, unlike SHP-1 phosphatase, SHIP is not activated by engagement of its SH2 domain.

Membrane Localization of SHIP Is the Major Mechanism Promoting Increased Activity upon Co-clustering of αg-FcγRIIb—Enzymes involved in signal transduction are frequently regulated by redistributing their subcellular location to the plasma membrane, which likely promotes enzyme access to substrates or interaction with other molecules. SHIP can be recruited to the membrane by binding of its SH2 domain to the phosphorylated ITIM of FcγRIIb or cytoplasmic tyrosines of other receptors, as shown earlier (29, 30, 38). To investigate whether a membranous location of SHIP leads to enhanced hydrolysis of PtdIns-(3,4,5)P3, the SHIP substrate, we measured cellular PtdIns-(3,4,5)P3 levels in cells harboring SHIP in the cytosol or in the plasma membrane. A membrane-targeted chimera of SHIP was generated by fusing the enzyme with the cytosolic tail of human CD8α. COS-7 fibroblasts were transfected with a plasmid encoding SHIP, the CD8-SHIP chimera, or empty vector. Expression of CD8-SHIP was confirmed by fluorescence-activated cell sorter analysis and indicated that ~17% of transfected cells expressed CD8 on their surface (Fig. 6A). Additionally, cells transfected with the CD8-SHIP chimera displayed a slower migrating form, relative to COS-7 cells transfected with wild-type SHIP, as revealed by SDS-PAGE and immunoblotting with anti-SHIP sera (Fig. 6B). The slower migrating form is because of the presence of the ~30

FIG. 2. Enzymology of recombinant SHIP. A, divalent cation requirement for SHIP for its activity. Upper panel, TLC: lane 1, SHIP 5-phosphatase assay using recombinant purified SHIP; lane 2, SHIP + 10 mM EDTA; lane 3, SHIP + 10 mM Mg2+; lane 4, SHIP + 10 mM Zn2+; lane 5, SHIP + 10 mM Mn2+; lane 6, SHIP + 10 mM Ca2+. Lower panel, quantification of SHIP assay shown above, reported as a ratio of product to substrate plus product. B, inhibition of SHIP 5-phosphatase activity by H2O2 and Mn2+. SHIP enzymatic assay was performed in the presence of 0.5 mM H2O2 and 0.3 mM Mn2+. SHIP assay was performed in the presence of 0.5 mM H2O2 and 0.3 mM Mn2+; the data are shown as the ratio of product to substrate plus enzyme. Lane 1, without added enzyme; lane 2, plus SHIP; lane 3, SHIP plus 0.5 mM H2O2; lane 4, no enzyme; lane 5, plus SHIP; lane 6, SHIP plus 0.3 mM MnCl2. C, pH dependence of SHIP 5-phosphatase activity. SHIP assays were performed at the pH indicated on the x axis, otherwise as described under “Experimental Procedures.” Shown are the TLC analyses of the reaction products (upper panel) and quantified activities as the ratio of product to substrate plus product (lower panel). These results are representative of five separate and similar assays.

phosphorylation on its 5-phosphatase activity, SHIP enzyme was collected from lysates of resting B cells or B cells stimulated with intact anti-mouse IgG, as we have described (38) using the phosphorylated ITIM peptide of FcγRIIb. SHIP obtained in this way was tyrosine-phosphorylated under these stimulation conditions (Fig. 4A, upper panel). The enzyme was eluted by the addition of 100 mM phenylphosphate, and equal amounts of phosphorylated or nonphosphorylated forms, confirmed by Western blot, were applied to the 5-phosphatase assay described above. We found (Fig. 4B) that the rate of PtdIns-(3,4,5)P3 hydrolysis by SHIP was not affected by its tyrosine phosphorylation. To confirm the data, we took advantage of the fact that Lyn phosphorylates SHIP to high stoichiometry in vitro. Thus, recombinant SHIP was phosphorylated by immunoprecipitated Lyn in vitro, as shown in Fig. 3A, and the phosphorylated material, or sham-phosphorylated control, was subjected to the 5-phosphatase assay. We observed no significant difference in enzymatic activity between the nonphosphorylated and phosphorylated forms of SHIP (Fig. 4, C and D). Lastly, SHIP was phosphorylated in vivo by co-transfection into COS-7 cells with Lyn kinase and stimulated by pervanadate treatment. The activity of the phosphorylated and nonphosphorylated forms of SHIP was assessed after immunoprecipitation. The results (Fig. 4, C and D) indicated no significant change in SHIP 5-phosphatase activity upon its phosphorylation, despite efficient tyrosine phosphorylation by co-transfected Lyn. Thus, SHIP catalytic activity is not affected by tyrosine phosphorylation.

The Engagement of the Phosphorylated ITIM of FcγRIIb with SHIP Has No Effect on the Enzymatic Activity of SHIP—Earlier studies indicated stimulation of SHP-1 phosphotyrosine phosphatase activity upon engagement of a phosphorylated peptide corresponding to the ITIM of FcγRIIb (42). To test the possibility that SHIP activity was similarly regulated, recombinant SHIP was incubated with phosphorylated ITIM peptide or an unrelated phosphorylated peptide for 1 h, and the mixture was subjected to the 5-phosphatase assay (Fig. 5). Control experiments using these peptides in a pull-down assay (Fig. 5A) indicated that the phosphorylated FcγRIIb ITIM peptide but not other peptides were capable of engaging SHIP. However, despite its ability to bind SHIP, the ITIM peptide did not affect SHIP 5-phosphatase activity (Fig. 5, B and C). Thus, unlike SHP-1 phosphatase, SHIP is not activated by engagement of its SH2 domain.

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kDa CD8α fused to the ~150-kDa SHIP gene product. To assess the membrane localization of the CD8-SHIP chimera, we performed immunofluorescence studies using confocal microscopy. Cells transfected with wild-type SHIP showed no CD8 on their surface upon immunostaining with anti-CD8, in contrast to those transfected with CD8-SHIP (Fig. 6C). Lastly, cells transfected with CD8-SHIP showed SHIP present in the plasma membrane when immunostained with anti-SHIP antibodies, whereas those transfected with wild-type SHIP showed a diffuse cytosolic location of the enzyme (Fig. 6D).

To measure the effect of membrane-targeted SHIP on 3-phosphoinositide levels, the transfected COS-7 cells were labeled to equilibrium with [32P]orthophosphate for 14 h, trypsinized, and stimulated with pervanadate for 3 min. Equilibrium labeling was done so that changes in 32P counts in 3-phosphoinositides represent changes in their mass levels, rather than rates of label incorporation. These experiments revealed an ~9% decrease in cellular PtdIns-(3,4,5)P3 levels in cells transfected with wild-type SHIP localized to the cytosol, as compared with vector only transfectants (Fig. 6C).

Lower panel, quantification of SHIP phosphorylation. Shown is the extent of SHIP phosphorylation in the presence of two inhibitors as a percentage of that induced in the presence of Me2SO only. Inset, shown is the percent inhibition of Lyn and Syk autophosphorylation was quantitated and expressed as a percentage of the maximum. Inhibition of SHIP phosphorylation in the presence of two inhibitors as a percentage of that induced in the presence of Me2SO only. Inset, shown is the percent inhibition of Lyn and Syk autophosphorylation. We performed immunofluorescence studies using confocal microscopy. Cells transfected with wild-type SHIP showed no CD8 on their surface upon immunostaining with anti-CD8, in contrast to those transfected with CD8-SHIP (Fig. 6C). Lastly, cells transfected with CD8-SHIP showed SHIP present in the plasma membrane when immunostained with anti-SHIP antibodies, whereas those transfected with wild-type SHIP showed a diffuse cytosolic location of the enzyme (Fig. 6D).

It has been proposed that co-clustering of FcγRIIB and sIg on B cells results in an inhibitory signal that provides a negative feedback mechanism for antibody production. Previous findings (32) indicated that sIg-FcγRIIB co-clustering decreased B cell PtdIns-(3,4,5)P3 levels; moreover, this reduction of PtdIns-(3,4,5)P3 was reversed by a catalytically inactive SHIP mutant. These data strongly suggest that the decreased PtdIns-(3,4,5)P3 was because of an increase in the enzymatic activity of SHIP under a sIg-FcγRIIB co-clustering condition, but how the enzymatic activity of SHIP is regulated is unknown. We investigated various known features of the SHIP enzyme that occur under sIg-FcγRIIB co-clustering conditions as potential means to implement activation of 5-phosphatase activity. Our results indicated that cells harboring a membrane-localized form of SHIP showed significantly reduced PtdIns-(3,4,5)P3 levels. We conclude that SHIP-mediated consumption of PtdIns-(3,4,5)P3 and inhibition of PtdIns-(3,4,5)P3-dependent enzymes is initiated by and a consequence of recruitment of the constitutively active enzyme to a phosphorylated receptor within the plasma membrane.

Membrane localization of cytosolic proteins is a common means by which enzymes involved in signaling pathways promote their activation. In this report, we have shown that expression of a CD8-SHIP chimera resulted in depletion of nearly 30% of total cellular PtdIns-(3,4,5)P3, the SHIP substrate, de-
Despite the fact that only 17% of COS-7 cells expressed the chimeric protein. Based on this finding, we propose that SHIP exerts its negative influence on signaling pathways by membrane localization upon SH2 domain engagement of cytoplasmic tyrosine residues within cytokine receptors or immunoreceptors. In earlier reports (29, 38, 50), we proposed a model regarding SHIP-protein interactions in which the phosphorylated ITIM of FcRIIB or cytoplasmic tyrosines of cytokine receptors formed a docking site for the SH2 domain of SHIP. The SH2 domain engagement thus serves two purposes: first, to bring SHIP within the range of its cellular substrate, PtdIns-(3,4,5)P3. Consistent with this interpretation of events, earlier experiments revealed that overexpression of the SH2 domain of SHIP blocked the negative signal delivered by the cytoplasmic tail of FcRIIB (51). It is likely in those cells that the overexpressed SH2 domain bound to the phosphorylated ITIM and prevented conformational change in SHIP. However, and in contrast to this view, experiments of 

Interestingly, we did not see a large decrease in total cellular PtdIns-(3,4,5)P3 of resting cells expressing CD8-SHIP but only in those cells stimulated with pervanadate. The reasons for this observation may be technical; we found only very small levels of PtdIns-(3,4,5)P3 in resting cells, and it may not be possible to detect a decrease in the extremely low background levels. We interpret the need for pervanadate stimulation as indicating the need for induction of PtdIns 3-kinase to elevate PtdIns-

![Regulation of SHIP Activity](image-url)
indicates the spot corresponding to PtdIns-(3,4,5)P$_3$, as determined by and extracted phospholipids were analyzed by TLC. The phospholipids. The cells were stimulated with pervanadate for 3 min, PtdIns-(3,4,5)P$_3$ levels through PtdIns 3-kinase in B cells and vanadate induces SHIP phosphorylation, as well as increased in vivo not be active migration of authentic standards.

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were shown as a percentage of the PtdIns-(3,4,5)P$_3$ amount in the per - shown in (3,4,5)P$_3$ in the transfected fibroblasts to a level in which it is increased PtdIns-(3,4,5)P$_3$ levels.

FIG. 6. Cells harboring membrane-targeted SHIP have decreased PtdIns-(3,4,5)P$_3$ levels. A, COS-7 cells were transfected with vector or CD8-SHIP chimera. 48 h after transfection, the cells were lysed and analyzed by immunoblotting with anti-SHIP. Untransfected COS-7 cells do not express SHIP protein (not shown). The arrowheads indicate the ectopically expressed SHIP or CD8-SHIP chimera. C, COS-7 cells were transfected with the indicated cDNA and stained with anti-CD8 to reveal the CD8-SHIP chimera in the plasma membrane and the B subunit of cholera toxin to outline the plasma membrane. D, COS-7 cells were transfected with the indicated cDNA, permeabilized with detergent, and immunostained with anti-SHIP antisera. E, COS-7 cells were transfected with vector only or vector encoding SHIP or the CD8-SHIP chimera. After 30 h, the transfectants were metabolically labeled to equilibrium with $[^{32}P]$inorganic phosphate to label pools of inositol phospholipids. The cells were stimulated with pervanadate for 3 min, and extracted phospholipids were analyzed by TLC. The arrowhead indicates the spot corresponding to PtdIns-(3,4,5)P$_3$, as determined by migration of authentic standards. F, PtdIns-(3,4,5)P$_3$ levels for the TLC shown in C were quantitated by Molecular Dynamics Storm System and normalized to total phospholipids to control for cell number. The data are shown as a percentage of the PtdIns-(3,4,5)P$_3$ amount in the pervanadate-stimulated, vector only transfected cells. The data are the average of two identical experiments.

(3,4,5)P$_3$ in the transfected fibroblasts to a level in which it is detectable and sensitive to SHIP-mediated hydrolysis. However, this phenomena may also reflect a requirement to induce SHIP phosphorylation; i.e. membrane-associated SHIP may not be active in vitro until it is phosphorylated. Because pervanadate induces SHIP phosphorylation, as well as increased PtdIns-(3,4,5)P$_3$ levels through PtdIns 3-kinase in B cells and fibroblasts, we cannot distinguish between these possibilities.

Activation of SHIP 5-phosphatase activity upon its tyrosine phosphorylation was proposed by several groups. However, reports of SHIP immunoprecipitates from cytokine-activated B6SYA1 (23) or FDC-P1 cells (25) indicated no significant difference in hydrolysis of PtdIns-(3,4,5)P$_3$ or Ins-(1,3,4,5)P$_4$, implying that phosphorylation per se was not causally related to activation of the enzyme. However, it is possible that only a very small amount of phosphorylated SHIP was present in these samples, which might preclude detection of enhanced activity in an in vitro assay. Other studies in yeast indicated that co-expression of the tyrosine kinase Lck and SHIP resulted in the tyrosine phosphorylation of SHIP, accompanied by a 2–3-fold reduction in the level of 5-phosphatase activity (52), suggesting that SHIP enzymatic activity might be reduced upon its phosphorylation.

To more rigorously assess the effect of SHIP tyrosine phosphorylation on its enzymatic activity, it was necessary to identify a kinase capable of phosphorylating SHIP. We examined the Src family member Lyn and Syk in this capacity, because both tyrosine kinases are present upon co-clustering of Ig-FcyRIIb on the B cell surface. Src family PTKs associate with slg in the resting state and to a greater extent upon slg stimulation (53). In addition, FcyRIIb was phosphorylated by Src family PTKs in vitro (54), and FcyRIIb phosphorylation was deficient in ly(-/-) cells (55). Additionally, both Lyn and Syk bind with high affinity to the phosphorylated ITAM-containing proteins associated with slg (56).

We present three distinct arguments to indicate that SHIP is phosphorylated by a Src family kinase. First, the Src family member Lyn but not Syk was capable of phosphorylating recombiant SHIP in vitro. Second, SHIP tyrosine phosphorylation in B cells was sensitive to the Src inhibitor, PP2, but not the Syk inhibitor, piceatannol. Third, co-transfection of SHIP with Lyn promoted strong SHIP phosphorylation in fibroblasts, whereas co-transfection with Syk failed to induce SHIP tyrosine phosphorylation. Although these findings are consistent with a direct role for a Src family PTK in SHIP phosphorylation, we cannot rule out the possibility of an indirect role mediated through another Src-dependent PTK.

We tested the effect of phosphorylation on SHIP enzymatic activity by three methods. First, kinetic measurements indicated no change in 5-phosphatase activity when SHIP was obtained under conditions leading to its high stoichiometry phosphorylation upon stimulation of cells with intact anti-Ig. Second, phosphorylation of SHIP by in vitro kinase reactions did not significantly change its 5-phosphatase activity. Third, SHIP activity was unaffected when derived from in vivo phosphorylation conditions of SHIP and Lyn co-transfected COS-7 fibroblasts. These data argue that phosphorylation of SHIP by co-clustering of slg and FcyRIIb contributes to SHIP protein interactions to affect other cellular functions (38), rather than regulating SHIP enzyme activity.

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Enzymatic Activity of the Src Homology 2 Domain-containing Inositol Phosphatase Is Regulated by a Plasma Membrane Location

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