Regulation of the Src Homology 2-containing Inositol 5-Phosphatase SHIP1 in HIP1/PDGFβR-transformed Cells*

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It has been shown previously that the Huntingtin interacting protein 1 gene (HIP1) was fused to the platelet-derived growth factor β receptor gene (PDGFβR) in leukemic cells of a patient with chronic myelomonocytic leukemia. This resulted in the expression of the chimeric HIP1/PDGFβR protein, which oligomerizes, is constitutively tyrosine-phosphorylated, and transforms the Ba/F3 murine hematopoietic cell line to interleukin-3-independent growth. Tyrosine phosphorylation of a 130-kDa protein (p130) correlates with transformation by HIP1/PDGFβR and related transforming mutants. We report here that the p130 band is immunologically related to the 125-kDa isoform of the Src homology 2-containing inositol 5-phosphatase, SHIP1. We have found that SHIP1 associates and colocalizes with the HIP1/PDGFβR fusion protein and related transforming mutants. These mutants include a mutant that has eight Src homology 2-binding phosphotyrosines mutated to phenylalanine. In contrast, SHIP1 does not associate with H/P(KI), the kinase-dead form of HIP1/PDGFβR. We also report that phosphorylation of SHIP1 by HIP1/PDGFβR does not change its 5-phosphatase-specific activity. This suggests that phosphorylation and possible PDGFβR-mediated sequestration of SHIP1 from its substrates (PtdIns(3,4,5)P$_3$ and Ins(1,3,4,5)P$_4$) might alter the levels of these inositol-containing signal transduction molecules, resulting in activation of downstream effectors of cellular proliferation and/or survival.

Chronic myelomonocytic leukemia is a type of myelodysplastic syndrome characterized by dysplastic monocytosis, variable bone marrow fibrosis, and progression to acute leukemia. Chromosomal translocations involving the platelet-derived growth factor β receptor gene (PDGFβR) are recurring cytogenetic abnormalities associated with chronic myelomonocytic leukemia. These translocations result in cellular expression of fusion proteins such as TEL/PDGFβR (1, 3), which contain dimerizing amino-terminal motifs fused to the transmembrane and tyrosine kinase domains of PDGFβR.

The TEL/PDGFβR and HIP1/PDGFβR fusions both exhibit constitutive kinase activity due to dimerization mediated through the TEL and HIP1 domains. Expression of these fusion proteins transforms the IL-3-dependent murine hematopoietic cell line, Ba/F3, to IL-3-independent growth and induces hematopoietic malignancies in murine models of leukemia (3–5). Both the kinase activity of the PDGFβR portion and the oligomerization motifs of the TEL or HIP1 portions of the fusions are necessary for transformation.

We have recently examined in more detail via mutational analysis the roles of both HIP1 and the PDGFβR in the fusion protein-mediated transformation (6). For the HIP1 portion, stepwise deletions were made to find the minimal oligomerization domain. For the PDGFβR portion, we tested the effect of several tyrosine to phenylalanine substitutions that abrogate binding of signal transduction molecules, including PtdIns 3-kisane and PLCγ. These studies demonstrated that dimerization and autophosphorylation alone were insufficient for transformation. Furthermore, key mitogenic pathways mediated by PLCγ and PtdIns 3-kisane, that are normally activated by native PDGFβR, were not necessary for transformation by HIP1/PDGFβR. This raised the question of which signaling pathways, activated by HIP1/PDGFβR, are necessary for transformation.

During this mutational analysis, we found that regardless of the transforming HIP1/PDGFβR mutant used, cells contained a hyperphosphorylated 130-kDa protein (p130) and constitutively active STAT5. Since STAT5 is known to promote DNA synthesis and cell division (7–9), its constitutive activation might result in a transformed phenotype. In addition, there is strong evidence that constitutive STAT5 activation is not only correlated with transformation but is also necessary for leukemogenesis (10, 11). To further clarify our understanding of the molecular events leading to cellular transformation by HIP1/PDGFβR, we have focused in this study on the identification and characterization of p130.

During purification of p130 from transformed cells, p130 was found to comigrate on SDS-PAGE gels with one of the in vivo processed forms of the SH2-containing inositol 5-phosphatase SHIP1. SHIP1 is an enzyme that catalyzes the hydrolysis of PtdIns(3,4,5)P$_3$ (1, 3) that contain dimerizing amino-terminal motifs fused to the transmembrane and tyrosine kinase domains of PDGFβR.

The abbreviations used are: IL-3, interleukin-3; PAGE, polyacrylamide gel electrophoresis; PtdIns, phosphatidylinositol; PtdIns(3,4,5)P$_3$, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P$_4$, phosphatidylinositol 3,4,5,6-tetrasphosphate; Ins(1,3,4)P$_3$, inositol 1,3,4-trisphosphate; PLCγ, phospholipase Cγ; SH2, Src homology 2; HPLC, high pressure liquid chromatography.

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both PtdIns(3,4)P_2 and Ins(1,4,5)P_3, resulting in the formation of PtdIns(3,4)P_2 and Ins(1,3,4)P_2 respectively. It is phosphorylated on tyrosyl residues in response to growth factor stimulation and activation of immune receptors (12–14) and is expressed solely in hematopoietic and developing hematopoietic tissues (15, 16). During murine development, SHIP1 is first expressed at embryonic day 7.5, coincident with the onset of hematopoiesis. This is consistent with the phenotype of mice homozygous for a SHIP1 deletion, which, although viable and fertile, overproduce granulocytes and macrophages and suffer from progressive splenomegaly, massive myeloid infiltration of the lungs, and a shortened lifespan (17). The granulocyte/macrophage progenitors from these mice are more sensitive to many cytokines compared with similar cells from their wild type littermates.

Based on the phenotype of SHIP1 gene disruption and the hydrolysis of PtdIns(3,4)P_2 and Ins(1,4,5)P_3, SHIP1 is likely to be a negative regulator of cellular signaling involving PtdIns 3-kinase. Consistent with this hypothesis, it has recently been shown that SHIP1 protein levels were reduced in primary neoplastic cells from patients with chronic myelogenous leukemia, as might be expected with the myeloproliferative phenotype observed in SHIP−/− mice (18). Furthermore, ectopic expression of BCR/ABL in Ba/F3 cells led to a rapid reduction in the level of SHIP1 protein. The involvement of SHIP1 in the regulation of hematopoietic proliferation, along with the electrophoretic comigration of p130 with SHIP1 in our transforming HIP1/PDGFBR extracts led us to pursue the idea that p130 might be identical or related to SHIP1.

We demonstrate here that the p130 phosphorylated protein observed in HIP1/PDGFBR-transformed Ba/F3 cells is indeed related to the 125-kDa isoform of SHIP1. Although SHIP1 protein levels were not consistently altered in these cells, tyrosine phosphorylation of SHIP1 and its physical association with the HIP1/PDGFBR fusion proteins might contribute to transformation, sequestering SHIP1 from its normal inositol-containing substrates and altering their cellular levels. Since SHIP1 is only expressed in hematopoietic tissue and developing hematopoietia, we predict it would be an excellent therapeutical target for hematologic malignancies such as chronic myelomonocytic leukemia.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture—**Ba/F3, 32D, and FL5.12 cells were grown in RPMI 1640 and 10% fetal calf serum with or without IL-3 (1 ng/ml). 293T cells were grown in Dulbecco’s modified Eagle’s medium and 10% fetal calf serum.

**Immunoprecipitations—**Protein extracts (500 μg of total protein) were incubated at 4°C for 1 h with 4 μl of 4G10 anti-TyrP antibody (Upstate Biotechnology, Inc.), 16 μl of anti-SHIP antibody (Pharminogen), 5 μl of anti-tail PDGFβR antibody (Pharminogen), or 5 μl of anti-HIP1 immune serum (see below). Protein G-Sepharose (Amersham Pharmacia Biotech) was used to precipitate the immune complexes. The protein G beads were washed three times with 500 μl of lysis buffer (50 mM triis, pH 7.4, 150 mM NaCl, 1% Triton X-100, protease inhibitors, 30 mM sodium pyrophosphate, 50 mM NaF, and 100 μM sodium orthovanadate).

**Western Blotting—**Extracts (100 μg of total protein) or immunoprecipitates were separated on 7% SDS-PAGE, transferred to nitrocellulose (APB, Hybond-ECL), and blocked with TBST, 5% bovine serum albumin. Primary antibodies (1:1000–1:5000 dilutions depending on antibody) were incubated with the blocked membrane in 5% bovine serum albumin/TBST. The membranes were washed with TBST, and then anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:5000 in TBST) were used to develop the blots with ECL (Amersham Pharmacia Biotech).

**DNA Constructs—**A Myc-tagged SHIP1a DNA insert in pVL1393 (kindly provided by Philip Majerus, Washington University, St. Louis) was digested with EcoRI. The released Myc-SHIP fragment was ligated into the EcoRI site of pcDNA3. HIP1/PDGFBR, TEL/PDGFBR, and the various mutants have been previously described (6). The glutathione

S-transferase-HIP1 fusion construct that was used to generate anti-HIP1 rabbit serum contained glutathione S-transferase fused in frame to HIP1 amino acid sequences starting at the internal EcoRI site (nucleotide 1250) and ending at the native stop codon (nucleotide 3010). pcDNA3/HIP1 was digested with EcoRI, and the 3-kilobase pair HIP1 fragment was cloned into the EcoRI site of pGEX4T-1 (Amerham Pharmacia Biotech) to obtain a construct designated pGEX-3’ HIP1.

**Stable Expression of Fusion Proteins in Ba/F3 Cells—**All PDGFBR fusion constructs were cloned into the EcoRI site of pGEX4T-1 (Amerham Pharmacia Biotech) to obtain a construct designated pGEX-3’ HIP1.

**Transient Expression in 293T Cells—**Murine 293T cells were passaged 1:2 onto 15-cm plates in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, 10 units/ml penicillin/streptomycin. When confluent, cells were passaged at a dilution of 1:4 onto 10-cm plates. Approximately 48 h after plating, cells were transfected with 15 μg of DNA in 450 μl of Dulbecco’s modified Eagle’s medium and 90 μl of Superfect transfection reagent as described by the supplier (Qiagen). A 48-h post-transfection supernatant (1 ml) was then added to 106 Ba/F3, 32D, or FL5.12 cells (1 ml) (all kindly provided by W. Fear, University of Pennsylvania, Philadelphia) in the presence of 10% FCS and cycloheximide as described previously (19). Cells with stable expression were selected in the presence of G418 and IL-3 as described (4).

**Antibody Production—**The pGEX-3’-HIP1 construct was used to express and purify recombinant protein as described by the supplier of the pGEX vector (Amerham Pharmacia Biotech). The purified protein was dialyzed to remove glutathione and treated with thrombin to release glutathione S-transferase, and the free glutathione S-transferase was removed by adding a second aliquot of glutathione-Sepharose and collecting the unbound fraction as antigen. For the initial immunization, 100 μg of purified antigen were dissolved in complete Freund’s adjuvant and injected subcutaneously into a rabbit at multiple sites. Purified antigen (50 μg) was mixed with incomplete Freund’s adjuvant and used for the secondary immunizations.

**Enzyme Assays—**The phosphatase activity of immunoprecipitates derived from the Ba/F3 cell line and 293T cells was assessed using Ins(1,3,4,5)P_4 as a substrate. Protein extract (1 mg of total protein) used for the immunoprecipitations was prepared as described above. Prior to use in phosphatase assays, immunoprecipitates were washed three times with 1 ml of phosphatase assay buffer (50 mM Tris, pH 7.5, 10 mM MgCl2) to remove phosphatase inhibitors. Phosphatase assays were carried out at 37°C as described for SHIP2 (20) using a malachite green-based assay system (21).

**HPLC—**For analysis of products formed after incubation of Ins(1,3,4,5)P_4 with immunoprecipitates, the reactions were carried out using 3H-labeled substrate and separated by anion HPLC using a Partisil SAX column. Separations were achieved using an ammonium phosphate gradient consisting of buffer A (10 mM ammonium phosphate, pH 3.8) and buffer B (1.0 M ammonium phosphate, pH 3.8) as follows: 0–10 min, 0% B; 10–40 min, 0% B to 12.5% B; 40–80 min, 12.5% B to 100% B; 80–89 min, 100% B; 89–90 min, 100% B to 0% B; 90–115 min, 0% B at a flow rate of 1 ml/min. An online radioisotope detector was used with Beckman Ready Flow III scintillant.

**Subcellular Fractionation—**Crude homogenates of 293T cells were made by sonication at 4°C in buffer without the Triton detergent. The first centrifugation step precipitated cellular debris and nuclei for 5 min at 1,500 g. The 1,500 × g supernatant was subsequently centrifuged for 30 min at 142,000 × g. This was designated the total membrane fraction. The remaining supernatant was defined as the cytosolic fraction.

Aliquots of the total membrane fraction were suspended twice at 2 mg/ml in 0.5 M NaCl, 10 mM Tris (pH 7.4), and protease inhibitors. The pellet achieved by washing once at 4°C in buffer without the Triton detergent. The first centrifugation step precipitated cellular debris and nuclei for 5 min at 1,500 × g. The 1,500 × g supernatant was subsequently centrifuged for 30 min at 142,000 × g. This was designated the total membrane fraction. The remaining supernatant was defined as the cytosolic fraction.

All samples were diluted to 1× with 5× sample buffer and loaded onto SDS-PAGE gels after boiling. Western blotting was performed as described above.
results

p130 Is Immunologically Related to the 125-kDa Form of SHIP1—Recently, Sattler et al. (18) demonstrated that SHIP1 RNA and protein levels were down-regulated in BCR/ABL-transformed Ba/F3 cells. Based on these results, we wished to determine whether SHIP1 protein levels were similarly altered in HIP1/PDGFβR-transformed Ba/F3, 32D, and FL5.12 cell lines. Levels of SHIP1 protein were measured in these cells by immunoblotting with various anti-SHIP1 antibodies. In contrast to the situation with BCR/ABL, we found that all four SHIP1 isoforms (designated as 110-, 125-, 135-, and 145-kDa isoforms) showed similar levels in control and HIP1/PDGFβR-transformed cells (Fig. 1A). However, one of the four HIP1/PDGFβR protein bands migrated in a similar position to our previously unidentified p130 (6), as shown in three independent pairs of HIP1/PDGFβR-transformed Ba/F3 cell lines in Fig. 1. The same phosphorylation pattern was seen in HIP1/PDGFβR-expressing 32D and FL5.12 hematopoietic cell lines (data not shown).

Following this observation, p130 immunoprecipitation by anti-SHIP1 antibodies was tested. As shown in Fig. 2, anti-SHIP1 antibodies specifically immunoprecipitated p130 from Ba/F3 cells transformed with the HIP1/PDGFβR fusion protein. When the anti-phosphotyrosine blot of the anti-SHIP1 immunoprecipitates was stripped and reprobed with anti-SHIP1 antibodies, p130 co-migrated with the 125-kDa SHIP1 isoform. Fig. 2 also shows that p130 was cleared from the supernatant with anti-SHIP1 antibodies after three serial immunoprecipitations of the supernatant. On average, ~50% of p130 was cleared from the supernatant when most of the SHIP1 125-kDa isoform was cleared, suggesting that this band may contain another phosphoprotein. It also remains a possibility that SHIP1 associates with a currently unidentified co-migrating phosphoprotein. We have previously eliminated CBL, RAS-GAP, CAS, JAK1–3, TYK2, interleukin-β receptor, PLCγ, Ptdlns 3-kinase, and focal adhesion kinase as candidates for p130 (6) and more recently tested to see if the SIRPα/β p130 protein was part of the phospho-p130 in our transformed extracts. A monoclonal antibody specific to SIRPα/β was used to immunoprecipitate p130, and while all of SIRPα/β was precipitated from the supernatant, all of p130 remained unprefecipitated (data not shown).

Fig. 2 also demonstrates that anti-SHIP1 antibodies were able to immunoprecipitate p130 in the H/P(F8)-transformed cells but not in the H/P(KI)-expressing nontransformed cells grown in IL-3. H/P(F8) is a mutant of the HIP1/PDGFβR fusion protein that has eight of the SH2-binding site tyrosines mutated to phenylalanines. H/P(KI) is a construct that has a point mutation at arginine 634 of the PDGFβR, where the substituted lysine leads to a kinase-dead mutant (6). As shown previously (6) as well as in Fig. 1, p130 was not detectable in cells transfected with vector alone, suggesting that the tyrosine phosphorylation of SHIP1 can be attributed directly to expression of the fusion protein. Additionally, p130 co-migrated with SHIP1 and was recognized by SHIP1 antibodies in all of the previously described HIP1/PDGFβR- and TEL/PDGFβR-transformed lines (data not shown). This included the H/P(F8) mutant (Fig. 2).

SHIP1 Is Tyrosine-phosphorylated and Associates with HIP1/PDGFβR and TEL/PDGFβR Fusion Proteins When Co-expressed in 293T Cells—To determine whether the tyrosine phosphorylation of SHIP1 is mediated by the HIP1/PDGFβR or TEL/PDGFβR fusion proteins, we initially analyzed protein extracts derived from cells heterologously co-expressing Myc-tagged SHIP1 with either HIP1/PDGFβR or TEL/PDGFβR. As shown in Fig. 3A, Myc-SHIP1 was tyrosine-phosphorylated when co-expressed with HIP1/PDGFβR or TEL/PDGFβR (lanes 5 and 6). SHIP1 phosphorylation was not detected when it was expressed alone (lane 2) or in cells transfected with empty vector (lane 1). It should be noted that endogenous SHIP1 phosphorylation was not detected in 293T cells transfected with fusion constructs alone (lanes 3 and 4) because SHIP1 was expressed in these cells. Expression of the recombinant SHIP1 was confirmed by immunoblotting with anti-Myc antibody as shown in Fig. 3B (lanes 2, 5, and 6). These results strongly suggest that tyrosine phosphorylation of SHIP1 was catalyzed directly by the PDGFβR kinase domain of the fusion proteins or a kinase activated by the fusion proteins. As illustrated in Fig. 3A (lanes 3 and 4), both fusion proteins undergo autophosphorylation on tyrosine residues as expected.

We next tested whether SHIP1 could physically associate with the HIP1/PDGFβR or TEL/PDGFβR fusion proteins when co-expressed in 293T cells. We surmised that association of SHIP1 with autophosphorylated HIP1/PDGFβR or TEL/PDGFβR, via its N-terminal SH2 domain, might play a role in
regulating its phosphorylation by these proteins. To address this question, immunoprecipitations were performed from lysates of 293T cells expressing the various combinations of SHIP1 and the fusion proteins, as described above, using an antibody directed toward the C terminus of the PDGFRβ. As seen in Fig. 4A, tyrosine-phosphorylated SHIP1 co-immunoprecipitates with either HIP1/PDGFRβ or TEL/PDGFRβ (lanes 5 and 6), and immunoprecipitation with anti-Myc antibodies showed that Myc-SHIP1 was only phosphorylated in HIP1/PDGFRβ and TEL/PDGFRβ co-expressing extracts (Fig. 4B, lanes 5 and 6). SHIP1 expression in extracts of all cells transfected with pcDNA3-MycSHIP1 was confirmed by immunoblotting using anti-Myc antibody as shown in Fig. 3B (lanes 2, 5, and 6).

To address the specificity of the interaction between SHIP1 and the PDGFR fusion proteins, we next tested the transforming H/P(F8) and nontransforming kinase-inactive R634K also known as H/P(KI) mutants of HIP1/PDGFRβ for their ability to associate with and promote phosphorylation of SHIP1. The H/P(F8) mutant, which has eight known SH2 phosphoryosine binding sites mutated to phenylalanine, still associated with and promoted tyrosine phosphorylation of SHIP1 (Fig. 2, right panel, lane 2; Fig. 5A, lane 4; and Fig. 6A, lane 4), consistent with its previously reported transforming properties (6). The binding site for SHIP1 on the fusion protein may be an auto-phosphorylation site, since the H/P(KI) mutant of the HIP1/PDGFRβ fusion neither promoted phosphorylation (Fig. 2, lanes 3, and Fig. 5A, lane 6) nor associated with SHIP1 (Fig. 6A, lane 4). Expression of SHIP1 and H/P(KI) was confirmed (Fig. 5B, lane 6 for SHIP1; Fig. 5C, lane 6 for H/P(KI)), and all of the HIP1/PDGFRβ mutants were immunoprecipitated equally well by the anti-HIP1 antibody (Fig. 6C, lanes 3–6). The data also indicate that the anti-HIP1 antibody is specific for the fusion protein or fusion protein complexes, since no SHIP1 was immunoprecipitated by the HIP1 antibody (Fig. 6B, lanes 2 and 6). Although the levels of expression of the fusion protein and SHIP1 in 293T cells were nonphysiologic, the lack of association between the kinase-dead fusion protein, H/P(KI), and SHIP1 provides evidence that the interaction of SHIP1 with HIP1/PDGFRβ was phosphorylation-dependent and specific and suggests that there was direct phosphorylation of SHIP1 by the transforming fusion protein. The exact phosphorylation sites of SHIP1 and interaction site(s) of both proteins remain to be determined, since the known SH2-binding domains of the PDGFRβ do not appear to be involved.

SHIP1 Inositol 5-Phosphatase Activity Is Not Altered by Tyrosine Phosphorylation in HIP1/PDGFRβ-expressing Cells—Since SHIP1 is known to function as a negative regulator of cell
growth and antiapoptotic signal transduction pathways, we tested the possibility that its phosphatase activity might be inhibited by tyrosine phosphorylation in HIP1/PDGFβR-transformed Ba/F3 cells. To address this issue, in vitro phosphatase assays were performed with SHIP1 immunoprecipitated from Ba/F3 transformed cells, using Ins(1,3,4,5)P₄ as a substrate. Unexpectedly, we found that SHIP1 activity was not significantly affected by tyrosine phosphorylation. The in vitro phosphatase activity of SHIP1 immunoprecipitated with anti-SHIP1 antibodies exhibited no significant changes in transformed cells when compared with cells transfected with vector alone, suggesting that tyrosine phosphorylation was not a principal mechanism of SHIP1 regulation (data not shown). Even when SHIP1 along with HIP1/PDGFβR or TEL/PDGFβR was overexpressed in 293T cells, tyrosine phosphorylation did not result in a dramatic change in SHIP1 phosphatase activity (Fig. 7A). In Fig. 7A, ~20% of the Ins(1,3,4,5)P₄ phosphatase activity was eliminated with the anti-SHIP1 antibody in all extracts (HIP1/PDGFβR, TEL/PDGFβR, or control extracts) and ~5% with the anti-phosphotyrosine antibody in the HIP1/PDGFβR- and TEL/PDGFβR-expressing extracts as compared with 0.5% in the control extracts. These results suggest that the increase in 5-phosphatase activity found in the anti-phosphotyrosine immunoprecipitates of the HIP1/PDGFβR- or TEL/PDGFβR-expressing cells was a result of increased immunoprecipitation of SHIP1 mass due to increased tyrosine phosphorylation rather than stimulation of enzyme-specific activity.

The data from these activity studies further support the identification of p130 as SHIP1, since the anti-phosphotyrosine antibody immunoprecipitates from HIP1/PDGFβR-expressing cells contained more intrinsic inositol 5-phosphatase activity than extracts from control cells. Consistent with the substrate specificity of SHIP1, the HPLC elution positions of the reaction products generated by these anti-phosphotyrosine immunoprecipitates using radiolabeled Ins(1,3,4,5)P₄ as a substrate demonstrate that the phosphatase activity is specific for the 5' position in both transformed and nontransformed cells (Fig. 7B). The product was exclusively Ins(1,3,4)P₃ with no additional or alternative hydrolysis of other positions on the inositol ring.

SHIP1, HIP1/PDGFβR, and TEL/PDGFβR Are Colocalized in Vivo—Recently, Phee et al. suggested that the enzymatic activity of SHIP1 is regulated by its localization to the plasma membrane (22). These authors also reported that neither phosphorylation of SHIP1 by Lyn (a Src-related protein tyrosine kinase) and Syk, nor receptor binding of SHIP1 changed its intrinsic enzymatic activity. Rather, it was SHIP1 membrane localization that significantly reduced PtdIns(3,4,5)P₃ levels. Phee et al. (22) concluded that membrane localization of SHIP1 is required for its negative regulation of cellular signaling pathways. Our work extends these findings and explains how phosphorylation of SHIP1 might contribute to transformation. We suggest that in the HIP1/PDGFβR-transformed cells, physical association of the fusion protein with SHIP1 sequesters SHIP1 from binding to tyrosine kinase receptors that are known to provide docking sites for the SH2 domain of SHIP1. This could deny SHIP1 access to its substrates, PtdIns(3,4,5)P₃ and Ins(1,3,4,5)P₄, thus prolonging their steady-state levels.

In addition to the data presented above, which suggests that phosphorylated SHIP1 binds directly to the transforming fusion proteins, we determined the cellular localization of SHIP1 and the PDGFβR fusions. Consistent with this hypothesis, we found that the majority of SHIP1 in 293T cells was localized to the cytoplasm rather than the membrane (Fig. 8). Although HIP1/PDGFβR was co-localized with SHIP1 in the cytoplasm, HIP1/PDGFβR was distributed equally in the peripheral membrane and cytosol, whereas SHIP1 was mostly cytosolic. Fig. 8 also shows that co-expression of SHIP1 and the fusion proteins did not dramatically alter either of their subcellular locales. Similar co-localization results were obtained when TEL/PDGFβR and SHIP1 were co-expressed in 293T cells (data not shown).
SHIP1 Regulation in PDGFβR Fusion-transformed Cells

In this paper, we provide evidence that a previously unidentified 130-kDa phosphorylated protein, from Ba/F3 cells transformed by PDGFβR fusion proteins, appears highly related, if not identical, to the 125-kDa isoform of SHIP1. We show that HIP1/PDGFβR and TEL/PDGFβR physically associate with SHIP1, suggesting that SHIP1 phosphorylation may be catalyzed by the PDGFβR tyrosine kinase moiety of these fusion proteins. This conclusion is further supported by the observation that the kinase-dead mutant form of the fusion, H/P(KI), which is not phosphorylated in the H/P(KI)-expressing cells, does not associate with or promote phosphorylation of SHIP1. This is consistent with our previous experiments showing that the H/P(KI) mutant is nontransforming and that H/P(KI)-expressing Ba/F3 cells do not contain phospho-p130 (6).

We also find that the enzymatic activity of SHIP1 is not altered by tyrosine phosphorylation in transformed cells, leading us to propose that SHIP1 may be physically sequestered away from activated receptor tyrosine kinases such as the IL-3 receptor and therefore unable to negatively regulate signaling via PtdIns(3,4,5)P3 or Ins(1,3,4,5)P4. This would lead to constitutive activation of cell growth signaling pathways. In support of this hypothesis, we find that the PDGFβR fusion proteins coimmunoprecipitate with SHIP1 and that SHIP1 and the fusion proteins are co-localized in the cell. It is also noteworthy that cells transformed with the H/P(F8) mutant, in which eight recognized SH2-binding phosphotyrosines are mutated to phenylalanine, still exhibit tyrosine phosphorylation of SHIP1. Since the H/P(F8) mutant is recognized by the anti-Tyr(P) antibody, it is likely that the association of SHIP1 with the fusion protein is mediated through an as yet unidentified autophosphorylation site in the PDGFβR kinase domain. Tyrosine phosphorylation of SHIP1 in the absence of PtdIns 3-kinase and PLCγ activation offers a possible explanation for the ability of the H/P(F8) mutant to transform Ba/F3 cells (6, 9). For example, although we have previously found that PtdIns 3-kinase is not constitutively activated in the H/P(F8) transformed cells, SHIP1 could be inhibited from negatively regulating PtdIns(3,4,5)P2-dependent signals. Although normally generated by activated PtdIns 3-kinase phosphorylation of PtdIns (4,5)P2 sequestration of SHIP1 away from its PtdIns(3,4,5)P3 substrate provides an alternative mechanism by which to increase the PtdIns(3,4,5)P3 growth signaling in the absence of elevated PtdIns 3-kinase activity. A similar explanation applies to PLCγ activation, which ultimately generates the Ins(1,3,4,5)P4 signal by generating Ins(1,4,5)P3 from PtdIns(4,5)P2. Ins(1,4,5)P3 is then converted to Ins(1,3,4,5)P4 by an Ins(1,4,5)P3-specific 3-kinase. Inhibition of Ins(1,3,4,5)P4 degradation by SHIP1 phosphorylation/sequestration away from its Ins(1,3,4,5)P4 substrate provides an alternative pathway to increase levels of Ins(1,3,4,5)P4, thus making constitutive activation of PLCγ unnecessary for transformation.

We did not find substantial changes in SHIP1 protein levels, Akt phosphorylation, or association of either Grb2 or Shc with SHIP1 (p130) in the HIP1/PDGFβR- or TEL/PDGFβR-transformed Ba/F3 cells (data not shown). The SHIP1 polypeptide has been shown to exist in at least four different molecular weight forms. The largest are the 145- and 135-kDa forms, which have C-terminal tyrosine phosphorylation sites that bind the SH2-domain of the adaptor protein Shc (23). In addition, proline-rich sequences found within the carboxyl-terminal of the larger SHIP1 isoforms bind the SH3 domain of Grb2 (24). Shc and Grb2 are adaptor proteins that were first identified as important in growth factor receptor signaling through the RAS/mitogen-activated protein kinase pathway. The mitogen-activated protein kinase pathway was not constitutively activated in our HIP1/PDGFβR-transformed cells, despite multiple attempts to show this (6). The inability of Grb2 and Shc to bind p130 (possibly 125-kDa SHIP1) is not unexpected, since the C-terminal region of the 145- and 135-kDa SHIP1, to which these proteins bind, is not present in the 125-kDa isoform of SHIP1. Hence, the lack of a high affinity association of p130 with Grb2 and Shc is consistent with the possible identification of p130 as the 125-kDa form of SHIP1.

However, the lack of constitutive or enhanced Akt activation in HIP1/PDGFβR transformed Ba/F3 cells observed in our experiments was unexpected. Enhanced Akt phosphorylation was expected, since Akt activation was observed in response to IL-3 in SHIP1 null mice. In that study, it was predicted that SHIP1 would function as a negative regulator of Akt kinase activity by lowering cellular levels of PtdIns(3,4,5)P3, the primary activator of Akt, and that SHIP1 deletion would lead to Akt activation (25). However, in HIP1/PDGFβR-transformed Ba/F3 cells, we have not detected excess Akt activation either constitutively or as a hyperresponsive activation in response to IL-3. We see similar Akt activation in response to IL-3 stimulation in all cell lines whether transformed with HIP1/PDGFβR or not. Hence, the mechanism by which SHIP1 phosphorylation in HIP1/PDGFβR-transformed cells confers IL-3-independent growth remains unclear. It is also possible that SHIP1 phosphorylation in response to IL-3 may not be sufficient to alter Akt activation but may be sufficient to mediate positive effects on proliferation by, for example, altering levels of its water-soluble substrate (Ins(1,3,4,5)P4) or reaction product (Ins(1,3,4,5)P3).

It is relevant that Phee et al. (22) have found that the SHIP1-mediated decrease in endogenous PtdIns(3,4,5)P3 and resultant inhibition of PtdIns(3,4,5)P3-dependent kinases depends on recruitment of the constitutively active SHIP1 enzyme to a tyrosine-phosphorylated plasma membrane growth...
factor receptor. Our results suggest that the PDGFβR fusion proteins may sequester SHIP1 from binding to activated receptor tyrosine kinases, as suggested by Phee et al. (22). This provides a mechanism whereby the fusion proteins could abrogate negative signaling via SHIP1.

In addition to the absence of Akt activation, we did not detect an increase in PtdIns(3,4,5)P3 in resting or IL-3-stimulated PDGFβR fusion-transformed cells compared with control cells (data not shown). However, this observation could be due to low sensitivity of our assay, since we find very low and variable levels of PtdIns(3,4,5)P3 in IL-3-stimulated Ba/F3 cells. Therefore, detecting altered PtdIns(3,4,5)P3 levels in these cells after IL-3 stimulation or constitutively may require more refined and as yet undeveloped techniques. Alternatively, IL-3-mediated changes in cellular levels of other phosphoinositides may play a role in Akt activation. This is supported by data from a previous study that identified Akt activation as a downstream signaling event in IL-3-stimulated Ba/F3 cells. This activation required PtdIns 3-kinase activity (26). However, this effect was not demonstrated to be mediated directly through PtdIns(3,4,5)P3, suggesting the possibility that SHIP1 may function as a regulator of distinct phosphoinositide effectors other than PtdIns(3,4,5)P3.

It has been proposed by several groups that SHIP1 5-phosphatase activity is stimulated when SHIP1 is tyrosine-phosphorylated by activated receptor tyrosine kinases. However, tyrosine phosphorylation of SHIP1 has in fact not been consistently shown to increase or decrease enzymatic activity (24). For example, one study in yeast has shown that co-expression of Lck with SHIP1 results in phosphorylation of SHIP1 and a 3-fold decrease in 5-phosphatase activity (14). Additionally, activity measurements of SHIP1 immunoprecipitates from cytokine-activated B6YTA1 cells (24) and FDC-P1 cells (27) show no difference in hydrolysis of PtdIns(3,4,5)P3 or Ins(1,3,4,5)P3. This is consistent with our finding that tyrosine-phosphorylation does not alter enzymatic activity. It remains possible that in each of these studies in vitro assays may not be sensitive enough to detect differences in enzymatic activity when only a very small amount of tyrosine-phosphorylated SHIP1 is present in the samples.

Finally, the molecular basis and significance of the differential tyrosine phosphorylation of the 125-kDa SHIP1 isoform, but not other SHIP1 isoforms, in our transformed cells remains unclear. It is possible that the 125-kDa form has a binding site for the fusion proteins that is masked on the 145- or 135-kDa forms or that may be truncated in the 110-kDa form. It has been previously suggested that the truncated forms may have different signaling properties. For example, the 110-kDa form was found to be exclusively localized to the cytoskeleton (24).

Phosphorylation of SHIP1 in cells transformed with the PDGFβR fusions is a novel finding. We have reported previously that PtdIns 3-kinase and PLCγ were phosphorylated in HIP1/PDGFβR-transformed cells. However, Tyr to Phe mutants that prevent PtdIns 3-kinase and PLCγ phosphorylation by the PDGFβR fusions did not affect cell transformation (6). The difference described herein is that SHIP1 phosphorylation is seen in all transformed cells examined to date. Additional studies using SHIP1 dominant negative constructs and SHIP1 null mice will be necessary to clarify whether SHIP1 phosphorylation is necessary for transformation. Future work in our laboratory will focus on identifying the exact mechanism and role(s) of both SHIP1 phosphorylation and its physical interaction with the fusion tyrosine kinase oncogenes in cellular transformation.

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Regulation of the Src Homology 2-containing Inositol 5-Phosphatase SHIP1 in HIP1/PDGF βR-transformed Cells  
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