Phosphatidylinositol (3,4,5)P$_3$ Is Essential but Not Sufficient for Protein Kinase B (PKB) Activation; Phosphatidylinositol (3,4)P$_2$ Is Required for PKB Phosphorylation at Ser-473

STUDIES USING CELLS FROM SH2-CONTAINING INOSITOL-5-PHOSPHATASE KNOCKOUT MICE

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Using bone marrow derived mast cells from SH2-containing inositol-5-phosphatase (SHIP) +/- and --/-- mice, we found that the loss of SHIP leads to a dramatic increase in Steel Factor (SF)-stimulated phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P$_3$), a substantial reduction in PI(3,4)P$_2$, and no change in PI(4,5)P$_2$ levels. We also found that SF-induced activation of protein kinase B (PKB) is increased and prolonged in SHIP--/-- cells, due in part to more PKB associating with the plasma membrane in these cells. Pretreatment of SHIP--/-- cells with 25 μM LY294002 resulted in complete inhibition of SF-induced PI(3,4)P$_2$ while still yielding PI(3,4,5)P$_3$ levels similar to those achieved in SHIP+/- cells. This offered a unique opportunity to study the regulation of PKB by PI(3,4,5)P$_3$, in the absence of PI(3,4)P$_2$. Under these conditions, PKB activity was markedly reduced compared with that in SF-stimulated SHIP+/- cells, even though more PKB localized to the plasma membrane. Although phosphoinositide-dependent kinase 1 mediated phosphorylation of PKB at Thr-308 was unaffected by LY294002, phosphorylation at Ser-473 was dramatically reduced. Moreover, intracellular delivery of PI(3,4)P$_2$ to LY294002-pretreated, SF-stimulated SHIP--/-- cells increased phosphorylation of PKB at Ser-473 and increased PKB activity. These results are consistent with a model in which SHIP serves as a regulator of both activity and subcellular localization of PKB.

The src homology 2 (SH2)-containing inositol phosphatase (SHIP) is a 145-kDa hematopoietic-specific signaling protein (1–3) that becomes both tyrosine-phosphorylated and associated with the adapter protein Shc in response to many cytokines and to B and T cell receptor engagement (4). SHIP has been shown to inhibit immune receptor activation in both mast cells and B cells by binding to the tyrosine-phosphorylated immunoreceptor tyrosine-based inhibition motif of the inhibitory co-receptor FcεRIIB and inhibiting FcεRI- and B cell receptor-induced calcium influx, respectively (5, 6). In addition, SHIP has been shown, even in the absence of FcεRIIB co-clustering, to play a “gatekeeper” role in IgE-mediated mast cell degranulation by setting the threshold for and limiting the degranulation process (7, 8).

In 1996 when we and others first reported the cloning of SHIP (1–3), we demonstrated its ability, in vitro, to hydrolyze the 5'-phosphate from phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P$_3$) but not from PI(4,5)P$_2$. More recently, however, by modifying the in vitro assay conditions, SHIP was found capable of readily hydrolyzing PI(4,5)P$_2$ to PI(4)P (9, 10). To resolve its phospholipid substrate specificity and to gain some insight into the normal role that SHIP plays in vivo, we generated a SHIP knockout mouse by homologous recombination in embryonic stem cells (11). Although these mice are viable and fertile, they suffer from progressive splenomegaly, massive myeloid infiltration of the lungs, wasting, and a shortened lifespan (11, 12). Interestingly, granulocyte/macrophage progenitors from these mice are substantially more responsive to multiple cytokines than their wild type littermates (11, 12). These mice have allowed us to ask whether one of SHIP's normal functions is to hydrolyze PI(3,4,5)P$_3$ and/or PI(4,5)P$_2$ in vivo. Specifically, in the present study we have utilized bone marrow-derived mast cells (BMMCs) from SHIP--/-- and +/- littermates to determine if SHIP affects PI(4,5)P$_2$ levels and if it plays a significant role in regulating the activation and function of these cells.

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The abbreviations used are: SH2, src homology 2; BMMCs, bone marrow-derived mast cells; PKD1, phosphoinositide-dependent kinase 1; PDK1, phosphatidylinositol 3-kinase; PI(3,4)P$_2$, phosphatidylinositol 3,4-bisphosphate; PI(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P$_3$, phosphatidylinositol 3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog on chromosome 10; di-C$_8$, dicapryloyl; di-C$_{16}$, dipalmityl. 

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role in hydrolyzing the Steel Factor (SF)-stimulated increase in PI(3,4,5)P3 in vivo. We have also used these two cell types to examine the role of SHIP in the activation of the proto-oncogene, protein kinase B (PKB) (also referred to as Akt or RAC) (13), and more specifically, the role of PI(3,4,5)P3 and PI(3,4)P2 in mediating the activation of PKB. Our data reveal that SHIP is the primary enzyme responsible for hydrolyzing PI(3,4,5)P3 in SF-stimulated normal BMMCs and that PI(3,4,5)P3 is the major source for PI(3,4)P2 in these cells. However, the presence or absence of SHIP does not have any significant effect on PI(4,5)P2 levels. Interestingly, although the loss of SHIP increases the levels of PI(3,4)P2 and enhances PKB activity, we show that the generation of PI(3,4)P2 is also essential to fully activate PKB, because this lipid is important for mediating phosphorylation of PKB at Ser-473.

MATERIALS AND METHODS
Antibodies—Antibodies to PKB, and the p85 subunit of PI3K were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phospho-PKB antibodies were from Cell Signaling Technologies (Beverly, MA). Anti-SHIP2 antibody was kindly provided by Dr. Bayard Clarkson, Memorial Sloan-Kettering Cancer Center, New York.

Generation of Mast Cells from Mouse Bone Marrow—Bone marrow from 4- to 8-week-old SHIP+/+ and −/− littersmates was plated in methycellulose (StemCell Technologies, Methocult M3234) supplemented with 30 ng/ml murine interleukin-3, 50 ng/ml murine SF, and 10 ng/ml human interleukin-6 for 10-14 days and then harvested and grown in suspension in IMDM containing 15% FCS (StemCell Technologies, Methocult M3234) supplemented with 20 mM HEPES, pH 7.4, and 1 mCi/ml [32P]orthophosphate (carrier free, ICN) for 90 min at 37 °C. Reactions were initiated by pretreating 5 × 106 cells with or without 25 μM LY294002 for 7 min. The lipid-shuttle complex, IP6-shuttle, or a control inositol head group (IP6) (Echelon Research Laboratories) were used to complex with histone (20 μg) as a shuttle to facilitate entry into cells (18) by sonication in a water-bath sonicator for 2 min. Other experiments used di-C 16-PI(3,4)P2, with di-C 16-PI(3,5)P2 as a control. Reactions were initiated by pretreating 5 × 106 cells with or without 25 μM LY294002 for 7 min. The lipid-shuttle complex, IP6-shuttle, or empty shuttle was added to cells and incubated for 5 min, followed by incubation with 100 ng/ml SF for 2 min. Reactions were terminated by quickly centrifuging the cells, aspirating off the supernatant, and quick freezing the pellets in methanol/dry ice.

RESULTS
SHIP Hydrolyzes SF-induced PI(3,4,5)P3 to PI(3,4)P2 in Normal BMMCs—To determine whether SHIP functions in vivo to convert PI(3,4,5)P3 to PI(3,4)P2, and/or PI(4,5)P2 to PI(4)P2, we compared SF-induced changes in PI(4,5)P2, PI(3,4,5)P3, and PI(3,4)P2 levels within BMMCs from SHIP+/+ and −/− littersmates. Previous studies had established that SF stimulates the binding of PI3K to the activated SF receptor, c-kit, in murine BMMCs and that PI3K inhibitors abrogate SF-induced membrane ruffling, adhesion, and enhancement of degranulation of these cells (19). As well, we had shown previously that SHIP becomes tyrosine-phosphorylated and associated with Shc in response to SF in various hemopoietic cell lines (16) and in normal BMMCs. 2HPLC analysis of SF-labeled deacylated phospholipids revealed no significant difference in the level of PI(4,5)P2 (based on 10 separate experiments) either before or after SF stimulation, between SHIP+/+ and −/− BMMCs. However, as can be seen from the HPLC elution profiles shown in Fig. 1A, a 2-min exposure to a saturating concentration of SF (100 ng/ml) induced a far more dramatic elevation of PI(3,4,5)P3 in the SHIP−/− (total cpm under the peak = 21,055 cpm in the example shown) than in SHIP+/+ (1265 cpm) BMMCs. In contrast, the SF-induced increase in the level of PI(3,4)P2 was lower in the SHIP−/− (2895 cpm in the example shown) than in the SHIP+/+ (4710 cpm) BMMCs. Even in unstimulated cells (i.e. following 14 h of starvation in 10% FCS and 90 min in serum-free, phosphate-free medium), although not obvious because of the different scales employed in Fig. 1A, the level of PI(3,4)P2 in the SHIP−/− cells was consistently higher than in the SHIP+/+ cells (525 cpm compared with 195 cpm in the example shown), while PI(3,4)P2 levels were similar. These differences between SHIP+/+ and −/− BMMCs most likely reflect differences in mass rather than specific activity of the labeled phosphoinositides, because, as mentioned above, M. Huber and G. Krystal, unpublished.
FIG. 1. PI(3,4,5)P₃ levels are dramatically elevated and PI(3,4)P₂ levels substantially reduced in SF-stimulated SHIP⁻/⁻ BMMCs. A, Partisol 10 SAX HPLC elution profiles of deacylated PI(3,4,5)P₃ and PI(3,4)P₂ from SHIP⁺/+ and −/− BMMCs following 2 min with (closed circles) and without (open circles) 100 ng/ml SF. The asterisk indicates the elution position of PI(3,4)P₂. The profiles are representative of four separate experiments. B, PI(3,4,5)P₃ (left panel) and PI(3,4)P₂ (right panel) levels were measured in SHIP⁺/+ and −/− BMMCs following 2 min of stimulation with the indicated concentrations of SF. **C, PI(3,4,5)P₃ (left panel) and PI(3,4)P₂ (right panel) levels were measured in SHIP⁺/+ and −/− BMMCs following stimulation with 30 ng/ml SF for the indicated times. Results shown in B and C are the mean ± S.D. of duplicate determinations and are representative of three separate experiments.

FIG. 2. SHIP2 is tyrosine phosphorylated in response to SF stimulation of SHIP⁻/⁻ BMMCs. SHIP (left panels) or SHIP2 (right panels) were immunoprecipitated from equal numbers of BMMCs derived from SHIP⁻/⁻ or SHIP⁺/+ mice that were either untreated (−) or stimulated with 200 ng/ml SF (+) for 2 min. Immunoblots were performed using 4G10 anti-phosphotyrosine antibody. The lower panels are from the same immunoblot as the upper panels, but these are different exposures. A 20-min exposure was necessary to reveal SHIP and SHIP2 bands, whereas a 2-min exposure was sufficient to show the co-immunoprecipitated tyrosine-phosphorylated SHC (pSHC). Results are representative of two separate experiments.

the radioactivity in PI(4,5)P₂, as well as in other lipid species, was similar. Furthermore, total recovered chloroform/methanol-soluble radioactivity between SHIP⁺/+ and −/− BMMCs never differed by more than 30%. SF concentration studies revealed that a 2-min exposure to SF, at concentrations ranging from 5 to 100 ng/ml SF, induced a detectable increase in both PI(3,4,5)P₃ and PI(3,4)P₂ in both SHIP⁺/+ and −/− BMMCs but that, at all SF concentrations tested, PI(3,4,5)P₃ levels were significantly higher and PI(3,4)P₂ levels significantly lower in SHIP−/− BMMCs (Fig. 1B). Interestingly, even though the SF-induced increase in PI(3,4)P₂ was less in SHIP−/− BMMCs, the fact that there was still a detectable increase in this phosphoinositide in these cells indicates that another 5′-phosphatase might be present in these cells. Consistent with this notion we have detected low levels of SHIP-2 (20) by Western blot analysis in both SHIP⁺/+ and −/− BMMCs, with slightly elevated levels in the SHIP−/− BMMCs. Furthermore, we tested the ability of SF to stimulate tyrosine phosphorylation of SHIP2, as an indication of activation of this enzyme. As shown in Fig. 2, the phosphorylation of SHIP2 was undetectable in SHIP⁺/+ BMMCs, but a low level of tyrosine-phosphorylated SHIP2 was observed in SHIP−/− BMMCs. As expected, SF-stimulated SHIP tyrosine phosphorylation was observed only in cells from SHIP⁺/+ mice. The co-immunoprecipitation of tyrosine-phosphorylated Shc from both SHIP and SHIP2 was also observed (Fig. 2), which provides additional supporting evidence for activation of SHIP2. There is a small amount of phosphorylated Shc in the anti-SHIP immunoprecipitates from SHIP−/− cells and in anti-SHIP2 immunoprecipitates from SHIP⁺/+ cells, but there was increased Shc co-immunoprecipitated with SHIP2 from the SHIP−/− cells treated with SF. Therefore, in the absence of SHIP, it is likely that SHIP2 is able to provide some 5′-phosphatase activity that generates PI(3,4)P₂.

Alternatively, or in addition to the involvement of SHIP2, there might be a PI3K or PI4K activity that generates a low level of PI(3,4)P₂ by directly phosphorylating PI(4)P or PI(3)P,

³ J. Kalesnikoff and G. Krystal, unpublished results.
PI(3,4,5)P3 levels did not remain elevated significantly longer than in SHIP+/− BMMCs. Surprisingly, although PI(3,4,5)P3 levels were substantially higher and PI(3,4)P2 levels dramatically lower at 0.5, 2, and 15 min following 30 ng/ml SF, PI(3,4,5)P3 levels did not remain elevated significantly longer in the SHIP−/− cells, returning to unstimulated levels by 30 min (Fig. 1C, left panel). Given that PI(3,4)P2 levels in SHIP−/− cells remained lower or the same as that in +/+ cells at all time points examined (Fig. 1C, right panel), this suggested that a substantial proportion of the SF-generated PI(3,4,5)P3 was being metabolized to something other than PI(3,4)P2. One candidate is PI(4,5)P2 via the 3′-phospholipid phosphatase, PTEN (23). Relevant to this, no PI(3,5)P2 was generated, as assessed by HPLC, in either SHIP+/+ or −/− BMMCs in response to SF, ruling out a 4′-phospholipid phosphatase playing a role in this process. Also relevant to this, the level of PI(4,5)P2 is approximately two orders of magnitude higher than that of PI(3,4,5)P3 following 2 min of SF stimulation. Therefore, it is not surprising that, if PTEN is indeed responsible for the drop in PI(3,4,5)P3 with time in SHIP−/− BMMCs, no obvious increase in PI(4,5)P2 was observed. This is consistent with reports in other systems (59).

To rule out that the rapid increase in PI(3,4,5)P3 in SF-stimulated SHIP−/− BMMCs was due to increased PI3K activity, PI3K assays were carried out with anti-c-kit immunoprecipitates from control and 1-min SF-stimulated SHIP+/+ and −/− BMMCs. There was a similar, low level of PI3K activity in both cell types prior to stimulation and, following the addition of SF, the level of c-kit-associated PI3K activity was consistently equal or slightly lower in SHIP−/− cells (data not shown). Consistent with this result, anti-c-kit immunoprecipitation, followed by Western blotting using anti-p85 antibodies, revealed that the level of PI3K associated with c-kit was minimal before stimulation and increased following 1 min of stimulation with SF to a similar extent in the two cell types (data not shown).

Loss of SHIP Increases SF-induced Activation of PKB in BMMCs—Having demonstrated the critical role that SHIP plays in regulating PI(3,4,5)P3 and PI(3,4)P2 levels, we then used the SHIP+/+ and −/− BMMCs to assess the relative importance of PI(3,4,5)P3 or PI(3,4)P2 in the activation of PKB in vivo. To do this we first compared PKB activation in SHIP+/+ and −/− BMMCs following exposure of these cells for various times to a saturating concentration of SF (200 ng/ml). As can be seen in Fig. 3A, even in the absence of SF stimulation, PKB was reproducibly more active in SHIP+/− cells after 14 h of cytokine withdrawal, consistent with the slightly elevated PI(3,4,5)P3 levels in these cells under these conditions. Following treatment of cells with 200 ng/ml SF, PKB was activated to the same extent following 1 min of treatment in the two cell types. However, with time, PKB activity dropped off substantially faster in the SHIP+/+ BMMC. This was consistent with PI(3,4,5)P3 (or the combined total amount of PI(3,4,5)P3 + PI(3,4)P2) rather than PI(3,4)P2 being the key regulator of PKB activation. It is also apparent from these results that maximal activation of PKB can be achieved with the levels of PI(3,4,5)P3 and PI(3,4)P2 formed in SHIP+/+ cells, and no greater activation is observed in SHIP−/− cells, even though there was a massive increase in the level of PI(3,4,5)P3.

We next carried out SF dose-response studies with the two cell types. Similar to the results obtained with 200 ng/ml SF, we found that a 2-min treatment with 100 ng/ml SF gave the same level of PKB activation in the two cell types (Fig. 3B). However, following 2 min of exposure to lower concentrations of SF, PKB was significantly more activated in the SHIP−/− than in the +/+ BMMCs. Interestingly, addition of only 5 ng/ml SF to SHIP−/− BMMCs yielded PKB activity close to that obtained when 100 ng/ml SF was added to SHIP+/+ cells (Fig. 3B). Thus, at doses of SF closer to physiological levels, i.e. 3 ng/ml in serum (24), loss of SHIP has a more pronounced effect on the activation of PKB.

SHIP−/− BMMCs Show Enhanced PKB Phosphorylation at Low Concentrations of SF—To gain further insight into the activation mechanism of PKB in SHIP+/+ and −/− BMMCs we stimulated these cells with various concentrations of SF for 2 min and then subjected cell lysates to immunoprecipitation with an anti-PKB antibody, followed by immunoblot analysis with an anti-phospho-PKB antibody (which recognizes the phosphorylation at Ser-473). As can be seen in Fig. 4A, following 2 min of stimulation with 1 or 5 ng/ml SF, the phosphorylation of PKB at Ser-473 was greater in the SHIP−/− BMMCs, indicating either increased activity of a Ser-473 kinase, increased recruitment of PKB to the plasma membrane, or both. Reprobing of this blot with anti-PKB confirmed similar loading for these lanes (Fig. 4A, lower panel). It was also of interest that at 1 ng/ml SF, two bands were observed in the SHIP−/− lane while only the more rapidly migrating band was seen in the SHIP+/+ lane. This was consistent, perhaps, with some
PKB being phosphorylated at both Ser-473 and Thr-308 in the SHIP−/− cells (and thus fully activated) whereas only Ser-473 was phosphorylated in the SHIP+/+ cells at this low concentration of SF. This also suggested that there was increased PDK1 activity toward PKB in SHIP−/− cells. Also consistent with Fig. 3B, the equal levels of slowly migrating, phosphorylated PKB at 100 ng/ml SF in the two cell types coincided with equal, maximal activity. As well, consistent with PKB activity and PI(3,4,5)P3 measurements, there was some partially phosphorylated PKB consistently observed in the SHIP−/− BMMCs in the absence of stimulation (i.e. following 14 h of growth factor deprivation in 10% FCS) and is most likely the result of low levels of SF and perhaps other mast cell mitogens in FCS.

**PKB Is Attracted to PI(3,4,5)P3 at the Plasma Membrane**—We next examined the membrane localization of PKB in SHIP+/+ and −/− BMMCs. If PI(3,4,5)P3 (or PI(3,4,5)P3 + PI(3,4)P2) recruits PKB to the plasma membrane, we would expect more membrane-localized PKB in SHIP−/− cells, whereas if PK(3,4)P2 is the critical second messenger in this process we would expect less. For this study, membranes and cytosol were prepared from cells stimulated for 30 s with 30 ng/ml SF, to examine very early events following addition of SF concentration that stimulated substantially more PKB activity in the SHIP−/− BMMCs. Half the preparations were subjected to immunoblot analysis with an anti-PKB antibody and the other half with anti-phospho-Ser-473-PKB antibody. As can be seen in the anti-PKB blot (top panel of Fig. 4B), following 30 s of stimulation with SF there was a greater decrease in cytosolic PKB and a far greater localization of PKB to the membrane fraction in SHIP−/− BMMCs. This is consistent with PKB binding to PI(3,4,5)P3 (or PI(3,4,5)P3 + PI(3,4)P2) rather than to PI(3,4)P2 alone. The anti-phospho-Ser-473-PKB blot of these samples clearly demonstrates that PKB was substantially more phosphorylated in the membrane fraction of SHIP−/− than in SHIP+/+ cells, following 30 s of exposure to 30 ng/ml SF. Intriguingly, although there was no detectable phosphorylation of PKB at this early time in the cytosol of SHIP+/+ cells, there was phospho-PKB in the cytosol of both SF-stimulated and unstimulated SHIP−/− cells. This is consistent with both the elevated PI(3,4,5)P3 levels and PKB activity in unstimulated SHIP−/− BMMCs and likely reflects a low level of PKB phosphorylation in response to mitogens in the 10% FCS during the 14-h starvation period. At later time points (2 and 5 min with 30 ng/ml SF) cytosolic phospho-PKB increases dramatically in SF-stimulated, but not unstimulated SHIP−/− cells (data not shown). Interestingly, the anti-phospho-PKB blot shown in Fig. 4B reveals that the cytosolic phosphorylated PKB is primarily of the more rapidly migrating form, whereas the plasma membrane-bound phospho-PKB from the SHIP−/− cells is comprised of both bands at approximately equal intensity. Together these results are consistent with higher basal levels of PI(3,4,5)P3 leading to phosphorylation of PKB. An increase in cytosolic phospho-PKB most likely represents the activated form in transit to the nucleus where it is known to phosphorylate transcription factors such as those of the Forkhead family.

**PI(3,4)P2 Is Required for Ser-473 Phosphorylation of PKB**—We next used the SHIP−/− BMMC to determine the sensitivity of PKB translocation and activation to the generation of specific PI3K lipid products. Because the SHIP−/− BMMC accumulates an enormous amount of PI(3,4,5)P3 compared with wild type BMMC, we reasoned that partial inhibition of PI3K with a specific inhibitor, LY294002, could lead to a condition in which the cells still generated a significant amount of PI(3,4,5)P3 but perhaps no PI(3,4)P2. Indeed, this was the case when SHIP−/− BMMC were first treated with 25 μM LY294002 prior to stimulation with SF (Fig. 5A, compare the left and right top panels). In fact, the level of PI(3,4,5)P3 in LY294002-treated SHIP−/− cells was higher than in SF-treated wild type cells, which had not received any LY294002. As expected, wild type BMMCs treated with LY294002 contained no detectable levels of either lipid species. This result shows that loss of SHIP leads to a condition where only a fraction of PI3K activity is needed to generate a substantial amount of PI(3,4,5)P3.

We then compared the levels of cytosolic and membrane associated PKB in these samples. As can be seen in Fig. 5B (upper panel), in the absence of an LY294002 pretreatment there was far more PKB associated with the membrane fraction (and less with the cytosolic fraction) from SHIP−/− than in SHIP+/+ BMMCs following a 2-min exposure to 100 ng/ml SF, consistent with there being far more PI(3,4,5)P3 in these cells at this time point. Most importantly, the level of PKB associated with the membrane fraction from SHIP−/− BMMCs treated with 25 μM LY294002 plus 100 ng/ml SF was still higher than that from SHIP+/+ BMMCs treated with SF. Given that the LY294002-treated SHIP−/− BMMCs treated with 25 μM LY294002 plus 100 ng/ml SF was still higher than that from SHIP+/+ BMMCs treated with SF. Given that the LY294002-treated SHIP−/− BMMCs treated with 25 μM LY294002 plus 100 ng/ml SF was still higher than that from SHIP+/+ BMMCs treated with SF. Given that the LY294002-treated SHIP−/− BMMCs treated with 25 μM LY294002 plus 100 ng/ml SF was still higher than that from SHIP+/+ BMMCs treated with SF.
This suggested that, although membrane localization of PKB is promoted by PI(3,4,5)P3, this lipid does not appear to be sufficient to induce Ser-473 phosphorylation.

Continuing this line of investigation, SHIP+/+ and −/− BMMCs, treated as in Fig. 5 (A and B), were also examined for PKB activity (Fig. 6A). As expected, 25 μM LY294002 pretreatment totally suppressed SF-stimulated PKB activation in the SHIP+/+ cells. Surprisingly, however, SF-induced PKB activity in SHIP−/− cells was also dramatically reduced by 25 μM LY294002. In fact, there was far less PKB activity in SHIP−/− cells treated with 25 μM LY294002 than in non-pretreated SF-stimulated SHIP+/+ cells, even though the previous results showed that PI(3,4,5)P3 levels were higher and there was more PKB associated with the membrane fraction in the SHIP−/− cells.

We investigated this further by using the same cell lysates to examine the phosphorylation of PKB in SHIP+/+ and −/− BMMCs. As can be seen in the top panel of Fig. 6B, the levels of phospho-Thr-308 and phospho-Ser-473 in the SHIP+/+ and −/− BMMCs were about the same following a 2-min exposure to 100 ng/ml SF, as expected. Also as expected, pretreatment of the SHIP+/+ cells with 25 μM LY294002 completely inhibited phosphorylation at both Thr-308 and Ser-473. However, although pretreatment of the SHIP−/− BMMCs with 25 μM LY294002 had no effect on the SF-induced Thr-308 phosphorylation of PKB (left panel), Ser-473 phosphorylation was drastically reduced by the PI3K inhibitor (right panel). As shown above, the level of PI(3,4,5)P3 under these conditions was similar to that in SHIP+/+ BMMC treated with SF, thus accounting for PKB-mediated phosphorylation of PKB at Thr-308. However, lack of PI(3,4)P2 in the SHIP−/− cells treated with 25 μM LY294002 could account for the reduced Ser-473 phosphorylation and the reduced PKB activity.
PI(3,4)P₂ Enhances PKB Phosphorylation at Ser-473

FIG. 7. Addition of PI(3,4)P₂ increases Ser-473 phosphorylation and PKB activity in SHIP⁻/⁻ BMMCs. SHIP⁻/⁻ BMMCs, treated with 25 μM LY294002 at time zero, empty shuttle, or di-C₁₆-PI(3,4)P₂ containing shuttle at 7 min, and 100 ng/ml SF at 10 min, were harvested at 12 min, then subjected to anti-PKB immunoprecipitation and Western analysis with either anti-phospho-Ser-473 or anti-phospho-Thr-308. Blots were re-probed with anti-PKB to show equal loading. Similar results were obtained in two separate experiments.

PI-3,4-P₂ - +
P-S473 PKB + P-T308 PKB +

FIG. 8. Addition of PI(3,4)P₂ increases PKB activity. Cells were prepared and treated as for experiment in Fig. 7. Following treatment with 100 ng/ml SF, or 25 μM LY294002, or lipid shuttles containing the indicated lipids, cell extracts were prepared and PKB assays were performed as described. Results show average ± range from duplicate independent samples and are representative of two separate experiments.

phosphatidylinositol-dependent kinase PDK1 (29–31) phosphorylates PKB in the activation loop of its kinase domain at Thr-308 while a separate kinase phosphorylates PKB in its carboxy-terminal tail at Ser-473. A recent report has described this as Hm kinase due to its phosphorylation at the hydrophobic region of PKB (58). Another possible route for control of Ser-473 phosphorylation could be through autophosphorylation, as suggested by Toker and Newton (32). However, Ser-473 phosphorylation occurs normally in PDK-1-deficient ES cells, which lack Thr-308 phosphorylation and PKB activity (33). Furthermore, a recent study showed that staurosporine can uncouple PKB activity from Ser-473 phosphorylation (34). Thus, PKB autophosphorylation is not likely to be the mechanism by which Ser-473 is regulated. Once phosphorylated at both sites, PKB becomes locked into the fully active conformation, detaches from the plasma membrane, phosphorylates target substrates such as glycogen synthase kinase-3 (35), caspase 9 (36), forkhead transcription factors (37, 38), IκBα (39), eNOS (40, 41), and Raf (42), and it also translocates into the nucleus (43).

One major controversy surrounding this model of PKB activation is whether PI(3,4,5)P₃ or PI(3,4)P₂ is the critical second messenger that attracts PKB and its kinases to the plasma membrane in vivo. There is substantial in vitro data suggesting that PI(3,4)P₂ has a higher affinity than PI(3,4,5)P₃ for PKB (27, 44, 45). Moreover, lipid vesicles containing PI(3,4)P₂ have been shown to modestly activate PKB, perhaps via dimerization (27, 44, 45), whereas vesicles containing PI(3,4,5)P₃ have been reported to either inhibit (27, 44) or have no effect (45). As well, in vivo data indicate that addition of di-C₁₆-PI(3,4)P₂ to serum-starved NIH 3T3 cells stimulates PKB autophosphorylation whereas di-C₁₆-PI(3,4,5)P₃ causes slight inhibition (27).

Finally, platelet studies have shown that PKB activation correlates with PI(3,4)P₂ rather than PI(3,4,5)P₃ production following thrombin stimulation (27), and integrin cross-linking has been reported to generate PI(3,4)P₂ but not PI(3,4,5)P₃ and results in PKB activation (21).

However, in support of PI(3,4,5)P₃ being the critical second messenger, it also binds PKB and in one study examining the
The availability of SHIP−/− BMMCs has given us the unique opportunity to modulate these two phosphoinositides, such that in the presence of 25 μM LY294002 an amount of PI(3,4,5)P3 equivalent to that observed in SHIP+/+ BMMCs is produced in response to SF (Fig. 5A). Under these unique conditions, no PI(3,4)P2 is formed and we were therefore able to investigate the activation of PKB in vivo, in the presence of increased PI(3,4,5)P3 alone. Comparison of PKB levels in the membrane fraction of SF-stimulated SHIP+/+ cells, with LY294002-pretreated SHIP−/− cells, suggests that PI(3,4,5)P3 alone is sufficient in vivo to attract PKB to the plasma membrane. However, our experiments also demonstrated that membrane recruitment via PI(3,4,5)P3 alone is not sufficient to drive Ser-473 phosphorylation, which was prevented by LY294002 pretreatment under these conditions. This led us to consider the possibility that the generation of PI(3,4)P2 is necessary for Ser-473 phosphorylation. To test this, we added exogenous PI(3,4)P2, which restored both Ser-473 phosphorylation and activity. These data, and our results with two structurally unrelated PI3K inhibitors, strongly support the possibility that the Ser-473 phosphorylation of PKB is dependent upon the presence of PI(3,4)P2.

It is also important to note that PDK1-mediated phosphorylation of PKB at Thr-308 in SHIP−/− BMMCs is unaffected by 25 μM LY294002 (Fig. 6B), suggesting that PI(3,4,5)P3 alone is sufficient to impart the conformational change within PKB to allow its phosphorylation by PDK1. In addition, our results demonstrate that little or no phosphorylation of Ser-473 is required to dock PDK-1 and mediate phosphorylation of Thr-308. This could be in contrast to other AGC kinases, such as RSK2, in which phosphorylation of the Ser-473-equivalent residue is thought to provide a docking site for PDK-1 to allow efficient activation loop phosphorylation (48).

We thus propose a model of SF-stimulated PKB activation in BMMCs in which PKB and PDK1 are attracted via PI(3,4,5)P3 whereas a Ser-473 kinase is attracted via PI(3,4)P2 to the plasma membrane (Fig. 9). Another possibility could be that PI(3,4)P2 inactivates a phosphatase responsible for the turnover of Ser-473 phosphorylation, and at this point we cannot rule out this possibility. Hemmings and co-workers (49) have used a membrane-inducible PKB allele to suggest that Ser-473 phosphorylation could be under the control of a PI3K-dependent phosphatase activity, but the mechanism for this is currently unknown. The same group found that hyperosmotic stress caused dephosphorylation of PKB at Ser-473 while also causing elevation of PI(3,4,5)P3 levels and depression of PI(3,4)P2, thus likely affecting 5-phosphatase activity (50). PI(3,4)P2 inhibition of a Ser-473 phosphatase could also allow for the possibility that this residue is a target of autophosphorylation by PKB. In either case, our data show that generation of PI(3,4,5)P3 leads to accumulation of Ser-473 phosphorylation. Of note, it has been proposed that, because PDK1 has such a high affinity for PI(3,4,5)P3 (30), there might be sufficient PI(3,4,5)P3 at the plasma membrane in unstimulated cells to localize significant levels of this enzyme (46). It is therefore possible that there are only two rate-limiting steps in PKB activation: PKB localization and PI(3,4)P2-mediated phosphorylation of Ser-473.

An issue that remains to be resolved is whether the integrin-linked kinase (ILK) may be the Ser-473 kinase. Delcommenne et al. (51) have reported that ILK may act as the putative PDK2. This protein has a PH-like domain that appears to be capable, at least in vitro, of binding to both PI(3,4,5)P3 and, to a lesser extent, PI(3,4)P2. More recent data further supports the role of ILK as a direct regulator of PKB activity (52). Until some insight into the in vivo affinities of ILK for PI(3,4)P2 is obtained, our present findings cannot help in determining whether ILK is serving as the kinase that phosphorylates Ser-473 in BMMCs.

Interestingly, with regards to SHIP, our studies to date as well as others have shown that this inositol-5-phosphatase plays a negative role in proliferation (3, 4, 11), survival (12, 53), and end cell activation (5–8). However, the data presented herein suggest that SHIP may also play a positive role by generating PI(3,4)P2 and thus activating PKB. This raises several interesting points about the regulation of PKB. It seems that nature has set in place the requirement of both PI(3,4,5)P3 and PI(3,4)P2 for its activation. Loss of SHIP may not super-potentiate PKB activity, because PI(3,4,5)P3 levels are reduced, and this may explain why we have not observed any hemopoietic malignancies in mice lacking this hemopoietically expressed gene (11). However, in mice heterozygous for the inositol-3-phosphatase, PTEN, many tumors, including lymphomas, appear (54). Because a reduction in PTEN reduces the hydrolysis of PI(3,4,5)P3 back to PI(4,5)P2, it allows for an increase in both PI(3,4,5)P3 and PI(3,4)P2 (55) and perhaps greater PKB activity than can be obtained in SHIP-depleted cells, and this may facilitate tumor formation. Studies are...
currently underlying to directly compare PKB activity in response to cytokine stimulation of SHIP–/– and PTEN–/– ES cell-derived mast cells to assess this possibility.

In summary, we have shown that SHIP is the primary enzyme responsible for hydrolyzing PI(3,4,5)P_3 and generating PI(3,4)P_2 in SF-stimulated BMMCs. We have also shown that SHIP plays a critical role in regulating PKB activity in these cells and that the overall effect of the large increase in PI(3,4,5)P_3 (and a decrease in PI(3,4)P_2 levels) is increased activation of PKB. This is consistent with reports by Liu et al. (12), Aman et al. (56), and Jacob et al. (57). However, our data reveal that, while the loss of SHIP enhances PKB activity, the generation of PI(3,4)P_2 in SHIP–/– BMMCs, although substantially lower than in SHIP+/+ BMMCs in response to SF, is essential to fully activate PKB by enhancing its phosphorylation at serine 473. Our results also suggest that SP-stimulated SHIP–/– and +/- BMMCs, with their dramatically different PI(3,4,5)P_3/PI(3,4)P_2 ratios, could prove very useful for future studies comparing the potential role of these two phosphoinositides both in recruitment of target proteins and in regulation of cascades of signaling events.

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