The serine-threonine kinase Akt/PKB is activated downstream of phosphatidylinositol 3-kinase in response to several growth factor stimuli and has been implicated in the promotion of cell survival. Although both phosphatidylinositol 3,4,5-trisphosphate (PIP_{3}) and phosphatidylinositol 3,4-bisphosphate (PI_{3,4}-P_{2}) have been implicated in the regulation of Akt activity in vivo, the relative roles of these two phospholipids in vivo are not well understood. Co-ligation of the B cell receptor (BCR) and the inhibitory F_{c}RIIB1 on B cells results in the recruitment of the 5'-inositol phosphatase SHIP to the signaling complex. Since SHIP is known to cleave PIP_{3} to generate PI_{3,4-P_{2}} both in vivo and in vitro, and Akt activity has been reported to be regulated by either PIP_{3} or PI_{3,4-P_{2}}, we hypothesized that recruitment of SHIP through F_{c}RIIB1 cross-linking to the BCR in B cells might regulate Akt activity. The nature of this regulation, positive or negative, might also reveal the relative contribution of PIP_{3} and PI_{3,4-P_{2}} to Akt activation in vivo. Here we report that Akt is activated by stimulation through the BCR in a phosphatidylinositol 3-kinase-dependent manner and that this activation is inhibited by co-cross-linking of the BCR to F_{c}RIIB1. Using mutants of F_{c}RIIB1 and SHIP-deficient B cells, we demonstrate that inhibition of Akt activity is mediated by the immune cell tyrosine-based inhibitory motif within F_{c}RIIB1 as well as SHIP. The SHIP-dependent inhibition of Akt activation also suggests that PIP_{3} plays a greater role in Akt activation than PI_{3,4-P_{2}} in vivo.

Akt (also called protein kinase B) is a serine/threonine kinase that is activated upon ligation of several cell surface receptors, including the receptors for insulin and platelet-derived growth factor (1–4). The biological significance of Akt has been demonstrated by its ability to protect a variety of cell types from apoptosis (5–8). Akt, in addition to its kinase domain, contains an amino-terminal pleckstrin homology (PH) domain, which can bind the phospholipids phosphatidylinositol 3,4,5-trisphosphate (PIP_{3}) or phosphatidylinositol 3,4-bisphosphate (PI_{3,4-P_{2}}) (9). Phosphatidylinositol-3 kinase (PI3K), which phosphorylates the 3'-position of the inositol ring in phosphatidylinositol 4,5-bisphosphate to generate PI_{3,4-P_{2}}, has been shown to function upstream of Akt (10), since the specific PI3K inhibitor wortmannin can block Akt activation, and platelet-derived growth factor receptor mutants that fail to activate PI3K also fail to activate Akt (1–3).

Recent studies have established the importance of both 3'-phosphorylated inositol phosphates (PI_{3,4-P_{2}} and PIP_{3}) and Akt phosphorylation in activation of Akt. PI_{3,4-P_{2}} has been shown to directly activate Akt in vitro via interaction with the Akt PH domain, while PIP_{3} was inhibitory in this experimental system (11–13). Additionally, Akt enzymatic activity was shown to be dependent on phosphorylation of Akt on a specific serine (Ser^{473}) and a specific threonine (Thr^{308}) (14, 15). Akt is phosphorylated by at least two serine-threonine kinases only in the presence of 3'-phosphorylated phospholipids (16, 17). One of the kinases that phosphorylates Akt, PDK1, has recently been cloned (18–20). The studies mentioned above demonstrating direct activation of Akt by PI_{3,4-P_{2}} and its inhibition by PIP_{3} were performed with immunoprecipitated Akt in the absence of PDK1 (11–13). In contrast, when purified PDK1 was present, activation of Akt was dependent on the presence of 3'-phosphorylated inositol lipids, with PIP_{3} being at least 2–3-fold more effective than PI_{3,4-P_{2}} in allowing Akt activation (18, 19, 21, 22). Studies utilizing PH mutants of Akt and PDK1 revealed that the regulatory action of PIP_{3} in the PDK-mediated activation of Akt is primarily directed toward Akt rather than PDK1 (17, 18, 20, 22). Based on these data, the current model of Akt activation is that Akt is recruited to the membrane by its PH domain binding to 3-OH-phosphorylated phosphatidylinositol phosphates, which causes a conformational change in Akt, which allows phosphorylation and activation by PDK1 and at least one other kinase. Although there is some indication that PIP_{3} may be more permissive in allowing PDK1-mediated activation of Akt than PI_{3,4-P_{2}} in vitro (18, 19), the relative roles of PIP_{3} and PI_{3,4-P_{2}} in Akt activation in vivo remain to be determined.

Antigen-mediated activation of B cells through the B cell receptor (BCR) initiates a cascade of intracellular biochemical events including activation of tyrosine kinases, activation of PI3K and PLCγ, subsequent generation of phospholipid and inositol phosphate second messengers, and calcium flux (23). The significance of PI3K activation in BCR signaling has been demonstrated by the ability of the PI3K inhibitor wortmannin receptor, SH2, Src homology 2; ITIM, immune cell tyrosine-based inhibitory motif; GSK-3, glycogen synthase kinase-3.
to inhibit BCR-induced calcium flux (24) and anti-Ig-induced proliferation of the human B cell line RL (25). BCR-mediated signals are selectively initiated by co-ligation of the FcγRIIB1 receptor to the BCR (26). This FcγRIIB1-mediated inhibition is dependent upon a 13-amino acid immune cell tyrosine-based inhibitory motif (ITIM) in the cytoplasmic tail of FcγRIIB1 (27, 28) and the interaction of the Src homology 2 (SH2) domain of the inositol phosphatase SHIP with the ITIM (24, 29–31). The enzymatic activity of SHIP has also been shown to be critical for this inhibitory effect (32). SHIP has been shown in vitro (33, 34) and in vivo (35) to have 5′-phosphatase activity toward PIP₃, resulting in dephosphorylation of PIP₃ and production of PI 3,4-P₂ (33, 34) and PI 3,4,5-P₃. Consistent with the requirement for enzymatically active SHIP for FcγRIIB1-mediated inhibition, FcγRIIB1 co-cross-linking to the BCR diminishes the BCR-induced levels of PIP₃ (35) in vivo. The failure of recruitment by PIP₃ of the kinase Btk (via its PH domain) under FcγRIIB1 cross-linking conditions has been shown to be responsible for the FcγRIIB1–mediated inhibition of BCR-mediated calcium entry (35–37). A similar mechanism could be involved in regulation of other downstream effectors whose activity is dependent on phospholipids.

Although BCR stimulation results in PI3K activation and Akt is activated downstream of PI3K in several cell types, BCR regulation of Akt has not been reported. In this report, we show that Akt is activated by BCR-cross-linking in a PI3K-dependent manner. Since there is conflicting evidence on the relative importance of PIP₃ and PI 3,4-P₂ in Akt activation (11–13, 16, 18, 19, 21, 22) and FcγRIIB1-associated SHIP is known to dephosphorylate PIP₃, to generate PI 3,4-P₂ (33, 34), we hypothesized that recruitment of SHIP through FcγRIIB1 cross-linking to the BCR diminishes the BCR-induced levels of PIP₃ (35) in vivo. The failure of recruitment by PIP₃ of the kinase Btk (via its PH domain) under FcγRIIB1 cross-linking conditions has been shown to be responsible for the FcγRIIB1–mediated inhibition of BCR-mediated calcium entry (35–37). A similar mechanism could be involved in regulation of other downstream effectors whose activity is dependent on phospholipids.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—Murine A20 and IIA1.6 cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, 2 mM l-glutamine, and 20 μM 2-mercaptoethanol, at 37 °C and 5% CO₂. IIA1.6 cells reconstituted with either wild type or CT53 mutant of FcγRIIB1 have been previously described (27). The DT40 cell line with deletion of the SHIP gene has been previously described (32). DT40 cells were cultured in the same medium as above additionally supplemented with 1% chicken serum (Sigma) and 50 μM 2-mercaptoethanol. Histone-H2B was purchased from Boehringer Mannheim, and wortmannin was obtained from Calbiochem. Polyclonal goat anti-Akt antibody (catalog no. sc-1618) and fluorescein isothiocyanate-labeled goat anti-Akt antibody (catalog no. sc-1618) for 2 min at 37 °C. Primary antibody (1 μg/ml murine anti-chicken IgG (murine IgM class antibody)) was then added, and cells were incubated at 37 °C for the indicated times. For experiments involving wortmannin, cells were incubated for 15 min in the presence of 50 nM wortmannin prior to adding antibodies for stimulation as described above. After stimulation, cells were lysed in lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40; 10 mM sodium pyrophosphate; 10 μg/ml each of aprotinin, leupeptin, and pepstatin; 10 mM NaF, 1 mM NaVO₄; and 2 mM phenylmethylsulfonyl fluoride. Cellular debris was cleared by centrifugation, and lysates were pre-cleared with protein A-Sepharose for 30 min at 4 °C. In assays performed using H2B as substrate, Akt was immunoprecipitated using 2 μg of goat anti-Akt antibody and protein A-Sepharose. Beads were washed twice with lysis buffer and twice with a solution containing 0.5 M LiCl, 100 mM Tris, pH 7.5, 1 mM EDTA followed by two washes in the kinase buffer (50 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol). After the last wash, 15 μl of the reaction buffer (2.5 μg of histone-H2B, 50 μM cold ATP, 5 mM protein kinase A and protein kinase C inhibitors, 3 μCi of [γ⁻³²P]ATP, 50 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol) was added, and the kinase reaction was performed for 30 min at room temperature. The reaction was stopped by adding 15 μl of 2X SDS sample buffer, and proteins were analyzed on an 8–15% SDS-polyacrylamide gel electrophoresis gradient gel. After electrotransfer to nitrocellulose, the gel was stained and immunoblotted with primary antibody (recognizing phosphorylated serine 473) was from New England Biolabs. Protein kinase A and protein kinase C inhibitors were obtained from Santa Cruz Biotechnology. Akt kinase assay kit using glycogen synthase kinase-3 peptide as substrate was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Sheep polyclonal anti-Akt antibody used for immunoprecipitation and immunoblotting was included in this kit.

**Generation of Stably Transfected DT40 Cells**—10⁷ cells were suspended in 0.5 ml of phosphate-buffered saline and transfected with 25 μg of linearized pPurom-FcγRIIB1 plasmid (directing expression of FcγRIIB1 under the actin promoter) at 250 V and 960 microfarads in a Gene Pulser II electroporator (Bio-Rad). Cells were selected in 0.5 μg/ml puromycin (Sigma). Surface expression of FcγRIIB1 on transfected cells was determined by fluorescence-activated cell sorting analysis (see below).

**Akt Kinase Assay**—Cells were suspended at 2 × 10⁷ cells/ml in plain RPMI 1640 medium, and 0.5-ml aliquots were incubated for 10 min at 37 °C prior to stimulation. A20 and IIA1.6 cells were stimulated with 2.5 μg/ml F(ab')₂ anti-mouse IgG (to cross-link the BCR) or 5 μg/ml intact anti-mouse IgG (to cross-link the BCR to FcγRIIB1) for the indicated times at 37 °C. DT40 cells were stimulated by first incubating with secondary antibody (either 1.5 μg/ml F(ab')₂ anti-mouse IgM or 3 μg/ml intact anti-mouse IgM) for 2 min at 37 °C. Primary antibody (1 μg/ml murine anti-chicken IgG (murine IgM class antibody)) was then added, and cells were incubated at 37 °C for the indicated times. For experiments involving wortmannin, cells were incubated for 15 min in the presence of 50 nM wortmannin prior to adding antibodies for stimulation as described above. After stimulation, cells were lysed in lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40; 10 mM sodium pyrophosphate; 10 μg/ml each of aprotinin, leupeptin, and pepstatin; 10 mM NaF, 1 mM NaVO₄; and 2 mM phenylmethylsulfonyl fluoride. Cellular debris was cleared by centrifugation, and lysates were pre-cleared with protein A-Sepharose for 30 min at 4 °C. In assays performed using H2B as substrate, Akt was immunoprecipitated using 2 μg of goat anti-Akt antibody and protein A-Sepharose. Beads were washed twice with lysis buffer and twice with a solution containing 0.5 M LiCl, 100 mM Tris, pH 7.5, 1 mM EDTA followed by two washes in the kinase buffer (50 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol). After the last wash, 15 μl of the reaction buffer (2.5 μg of histone-H2B, 50 μM cold ATP, 5 mM protein kinase A and protein kinase C inhibitors, 3 μCi of [γ⁻³²P]ATP, 50 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol) was added, and the kinase reaction was performed for 30 min at room temperature. The reaction was stopped by adding 15 μl of 2X SDS sample buffer, and proteins were analyzed on an 8–15% SDS-polyacrylamide gel electrophoresis gradient gel. After electrotransfer to nitrocellulose, the gel was stained and immunoblotted with primary antibody (recognizing phosphorylated serine 473) as a substrate (after stimulating and lysing cells as described above) using the Akt kinase assay kit from Upstate Biotechnology following the manufacturer’s protocol. Akt immunoblots for the experiments using GSK-3 peptide as substrate were performed using sheep anti-Akt antibody.

**Fluorescence-activated Cell Sorting Analysis**—Cells were incubated at 5 × 10⁶ cells/ml in phosphate-buffered saline with 1 μg/ml 2.4G2 (anti-FcγRIIB1) antibody for 20 min at 4 °C. Cells were washed twice in phosphate-buffered saline and stained with fluorescein isothiocyanate-conjugated anti-mouse IgM (Serotec, Oxford, U.K.). Finally, stained cells were sorted by FACS using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). As a negative control, cells were stained with fluorescein isothiocyanate-conjugated anti-rat IgG with no primary antibody.

**RESULTS**

**Stimulation through the B Cell Receptor Induces Akt Kinase Activity**—We used murine A20 cells to test whether BCR stimulation leads to Akt activation. Cells were either left unstimulated or stimulated by cross-linking the BCR with the F(ab')₂ fragment of anti-mouse IgG. Cells were then lysed, Akt was immunoprecipitated, and in vitro kinase assays were performed using histone-H2B as a substrate, as described under “Experimental Procedures.” As shown in Fig. 1, BCR stimulation led to an increase in Akt kinase activity (lanes 1 and 2). It has been shown that Akt-mediated activation of Akt occurs downstream of PI3K and is inhibited by the specific PI3K inhibitor wortmannin (1–3). Since BCR stimulation leads to PI3K activation (23, 24), we examined whether Akt activation in B cells is also downstream of PI3K. BCR activation of Akt was inhibited by preincubation of the cells with the PI3K inhibitor wortmannin prior to BCR cross-linking (Fig. 1, top panel, lane 3). These results show that
BCR cross-linking activates Akt in a PI3K-dependent manner. BCR-mediated Activation of Akt Is Inhibited by Co-cross-linking FcγRIIB1—FcγRIIB1 can inhibit several BCR-mediated downstream signals when co-cross-linked to the BCR, including the PI3K-dependent calcium entry into cells (23, 26). Since Akt activity can be modulated in vitro by 3'-phosphorylated phosphoinositides (16) and FcγRIIB1 recruits SHIP (26), which convertsPIP₃ to PI 3,4-P₂ in vivo (35), we hypothesized that FcγRIIB1 recruitment would affect BCR-induced activation of Akt in vivo. However, since Akt has been reported to be activated in vitro by both the substrate (PIP₃) and the product (PI 3,4-P₂) of SHIP’s enzymatic activity depending on the experimental system used (11–13, 16, 18, 21, 22), FcγRIIB1 co-cross-linking could either positively or negatively regulate Akt activity. To address this question, A20 B cells were left unstimulated, stimulated by cross-linking the BCR only (with F(ab')₂ fragment of anti-mouse IgG), or cross-linking BCR with FcγRIIB1 (using intact anti-mouse IgG) in a time course of activation. Akt was immunoprecipitated, and its activity was analyzed by an in vitro kinase assay using histone–H2B as a substrate. As shown in Fig. 2A, FcγRIIB1 co-cross-linking to the BCR resulted in a decrease in Akt activity as compared with BCR cross-linking alone (compare lanes 2–5 with lanes 6–9). The decrease in Akt activation observed upon FcγRIIB1 co-cross-linking was not simply due to delayed kinetics of activation, since a time course of activation extended to 60 min ruled out this possibility (data not shown). The kinase activity at these time points was correlated to phosphorylation of Akt on serine 473 as assayed by immunoblotting with phosphospecific anti Akt antibody (Fig. 2B). As controls, Akt and H2B levels are shown in Fig. 2, C and D, respectively. The decrease in Akt activity is not due to a general lack of activation in the BCR plus FcγRIIB1 cross-linked cells, since several proteins become tyrosine-phosphorylated in the stimulated cells as compared with the unstimulated cells (Fig. 2E).

To further confirm these results, we also assayed Akt kinase activity following stimulation performed in triplicate using a different anti-Akt antibody for immunoprecipitation (Sheep anti-Akt, Upstate Biotechnology) and using a peptide whose sequence was derived from GSK-3 (a known substrate of Akt) as a substrate. As shown in Fig. 3A, stimulation of A20 cells by co-cross-linking FcγRIIB1 to the BCR resulted in a substantial decrease in Akt activity as compared with BCR cross-linking alone. Immunoprecipitation of proteins from BCR-activated A20 lysates with control anti-Akt antibodies, and in vitro kinase assays were performed as described under “Experimental Procedures.” A, autoradiogram of phosphorylated H2B. B, Akt was assayed for phosphorylation on serine 473 by immunoblotting with phosphospecific anti-Akt antibody. C, level of immunoprecipitated Akt was determined by α-Akt immunoblotting; D, the level of H2B was determined by Coomassie staining; E, anti-phosphotyrosine blot of the total cell lysates. Molecular mass standards (in kDa) are indicated to the right.

FcγRIIB1-mediated Inhibition of Akt Activation Is Dependent upon the ITIM in the Cytoplasmic Tail of FcγRIIB1—Previous work has demonstrated that the inhibitory effects of FcγRIIB1 can be recapitulated by a 13-amino acid sequence within the cytoplasmic tail of FcγRIIB1, the ITIM (27, 28). We therefore tested the role of the ITIM in the FcγRIIB1–mediated inhibition of Akt activation. The IIA1.6 cell line, an FcγRIIB1-deficient derivative of the A20 B cell line, reconstituted with the wild type FcγRIIB1, or a truncation mutant of this receptor lacking the ITIM (CT53), was used to address this question. As expected, IIA1.6 cells stably transfected with the wild type FcγRIIB1 exhibited a pattern of Akt activation similar to the parental A20 cells, both in response to BCR cross-linking alone and following BCR plus FcγRIIB1 cross-linking (Fig. 4, left panels). In IIA1.6 cells reconstituted with the CT53 mutant of FcγRIIB1 (which lacks the ITIM), co-cross-linking of FcγRIIB1 to the BCR failed to show inhibition of BCR-mediated Akt activation (Fig. 4, right panels). These data indicate that FcγRIIB1-mediated inhibition of BCR-induced Akt activation is dependent upon the ITIM of FcγRIIB1.
SHIP Inhibits Akt in B Cells

ITIM and the inositol phosphatase SHIP (26). Using SHIP-deficient DT40 B cells, Ono et al. (32) demonstrated that FγRIIB1-mediated inhibition of calcium flux and NFAT activation are dependent on the recruitment of enzymatically active SHIP. Since we have shown that FγRIIB1-mediated inhibition of Akt activation is dependent upon the ITIM, we sought to test whether it was also dependent on SHIP. To evaluate this, we utilized parental DT40 cells (SHIP-expressing) and a DT40 line in which the SHIP genes were deleted by homologous recombination (32). SHIP-expressing and SHIP-deficient DT40 cells were stably transfected with plasmids encoding wild type FγRIIB1, and clones expressing FγRIIB1 on the surface were selected. Calcium flux patterns in these clones following BCR and BCR plus FγRIIB1 cross-linking were similar to what has been previously reported (data not shown) (32). Next, we assessed the state of Akt activation in these cells following BCR or BCR plus FγRIIB1 cross-linking. Co-cross-linking of FγRIIB1 to the BCR in SHIP-expressing DT40 cells resulted in inhibition of Akt activation in a pattern similar to A20 cells (Fig. 5A). In contrast, when SHIP-deficient DT40 cells were used, FγRIIB1 co-cross-linking to the BCR did not result in a strong inhibition of Akt activation (Fig. 5B). Akt activation by the BCR could be inhibited by the PI3K inhibitor wortmannin in the SHIP-deficient DT40 cells, indicating that Akt activation in these cells still occurs in a PI3K-dependent manner (Fig. 5C).

We also confirmed these results in DT40 cells by assaying Akt kinase activity following stimulation in three independent replicates toward GSK-3-derived peptide. Co-cross-linking of FγRIIB1 to the BCR in SHIP-expressing DT40 cells resulted in inhibition of Akt activation in a pattern similar to A20 cells (Fig. 6A). In contrast, when SHIP-deficient DT40 cells were used, FγRIIB1 co-cross-linking to the BCR did not result in a significant inhibition of Akt activation (Fig. 6B). Immunoblotting for Akt indicated that the same level of Akt was precipitated in all lanes (Fig. 6, bottom panels). In this immunoblotting we also observed a shift in Akt mobility upon BCR stimulation, which was inhibited by FγRIIB1 cross-linking in SHIP-expressing but not in SHIP-deficient DT40 cells (Fig. 6, bottom panels). In addition, we observed in several experiments higher levels of Akt activation in SHIP-deficient cells as compared with SHIP-expressing cells upon BCR stimulation (Fig. 6).

**DISCUSSION**

Co-ligation of FγRIIB1 and the BCR results in a potent inhibitory signal, which leads to a selective attenuation of BCR-mediated signals (26). This phenomenon represents a negative feedback mechanism triggered by immune complex or anti-idiotypic antibodies to suppress excessive B cell immune response (23, 26). The negative regulation by FγRIIB1 requires the recruitment and the enzymatic activity of the inositol phosphatase SHIP (32). The mechanism of this FγRIIB1/SHIP-mediated inhibitory action is not fully understood. Recently, it has been suggested that SHIP-mediated dephosphorylation of PIP3 and consequently the inhibition of Btk membrane localization and activation plays a central role in inhibition of calcium flux by FγRIIB1 (35–37).

It has been shown that co-ligation of FγRIIB1 and BCR induces apoptosis in mouse splenocytes (38, 39). The serine threonine kinase Akt, an enzyme activated downstream of PI3K, is a major signaling protein involved in protection from apoptosis (5–8). Although BCR stimulation activates PI3K, as demonstrated by the ability of wortmannin to inhibit BCR signaling (23, 24), activation of Akt by BCR engagement has not been reported previously. Here, we show the activation of Akt by BCR stimulation in a PI3K-dependent manner. Fur-
SHIP Inhibits Akt in B Cells

Fig. 5. Inhibition of Akt activation by FcγRIIB1 requires the inositol phosphatase SHIP. Parental DT40 (SHIP-expressing) cells (A) or SHIP-deficient DT40 cells (B) stably transduced with wild type FcγRIIB1 were stimulated by BCR cross-linking only or BCR plus FcγRIIB1 co-cross-linking as described under “Experimental Procedures,” and Akt kinase assays were performed. The level of immunoprecipitated Akt was determined by immunoblotting with goat anti-Akt antibody (middle panels), and the level of H2B was determined by Coomassie staining (bottom panels). The expression of transfected FcγRIIB1 was analyzed by flow cytometry using anti-FcγRIIB1. C, SHIP-deficient DT40 cells were left unstimulated (lane 1) or activated for 5 min by cross-linking the BCR in the absence (lane 2) or presence (lane 3) of wortmannin. Akt was immunoprecipitated and assayed for its kinase activity toward the substrate H2B.

There have been conflicting data in the literature on the role of PIP3 versus PI 3,4-P2 in the activation of Akt. In the absence of PDK1, one of the kinases that phosphorylates Akt, the addition of PI 3,4-P2 containing micelles to immunoprecipitated Akt increased its enzymatic activity, whereas PIP3 either had no effect or was inhibitory (11–13). In contrast, when purified PDK1 was present, activation of Akt was dependent on the presence of 3'-phosphorylated inositol lipids, with PIP3 being at least 2–3-fold more effective than PI 3,4-P2 in allowing Akt activation (18, 19, 21, 22). Studies utilizing PH mutants of Akt and PDK1 revealed that the regulatory action of PIP3 in the PDK-mediated activation of Akt is primarily directed toward Akt rather than PDK1 (41). Alternatively, there could be some redundancy for SHIP, since a second SH2-containing inositol phosphatase (SHIP2) has been recently described (42, 43).

Others have suggested a biphasic pattern of Akt activation, with PIP3 being responsible for the early phase and PI 3,4-P2 mediating the late activation of Akt in a calcium- and calpain-dependent manner in platelets (44). Since a rise in calcium levels is an important event in BCR-mediated signaling, we tested a possible role for calcium flux upstream of Akt activation in B cells. However, we did not observe any effect of either EGTA or the calpain inhibitor calpeptin on BCR-induced Akt activation in A20 cells (data not shown). Additionally, in several experiments, we observed a greater level of BCR-mediated activation of Akt in SHIP-deficient DT40 cells compared with SHIP-expressing cells, most likely due to the absence of PIP3 dephosphorylation. This suggests that SHIP also directly regulates the BCR-mediated rise in PIP3 levels. This is consistent with the reported increase in Btk activation and calcium flux (another PIP3-dependent event) in SHIP-deficient cells as compared with SHIP-expressing cells (37). Thus, SHIP, through its effects on PIP3, could set the threshold for the magnitude of Akt activation and the downstream consequences.

There have been conflicting data in the literature on the role of PIP3 versus PI 3,4-P2 in the activation of Akt. In the absence of PDK1, one of the kinases that phosphorylates Akt, the addition of PI 3,4-P2 containing micelles to immunoprecipitated Akt increased its enzymatic activity, whereas PIP3 either had no effect or was inhibitory (11–13). In contrast, when purified PDK1 was present, activation of Akt was dependent on the presence of 3'-phosphorylated inositol lipids, with PIP3 being at least 2–3-fold more effective than PI 3,4-P2 in allowing Akt activation (18, 19, 21, 22). Studies utilizing PH mutants of Akt and PDK1 revealed that the regulatory action of PIP3 in the PDK-mediated activation of Akt is primarily directed toward Akt rather than PDK1 (17, 18, 20, 22). Thus, it has not been clear whether PIP3 or PI 3,4-P2 mediates the activation of Akt in vivo. SHIP is known to dephosphorylate PIP3 both in vitro (33, 34) and in vivo (35) to generate PI 3,4-P2. It has been proposed that SHIP functions upstream of Akt and that the generation of PI 3,4-P2 positively regulates Akt activation (10–13). Inhibition of BCR-induced signals by FcγRIIB1 involves recruitment of SHIP to the BCR signaling complex (26). Recently, Scharenberg et al. (35) have demonstrated in A20 cells that FcγRIIB1 co-cross-linking to the BCR decreases cellular PIP3 levels generated by BCR stimulation. Therefore, this system provides a useful tool to indirectly address the role of PIP3 versus PI 3,4-P2 in vivo activation of Akt. Our data strongly suggest that, in vivo, dephosphorylation of PIP3 inhibits Akt activation and that SHIP-mediated generation of PI 3,4-P2 does not positively regulate Akt. Therefore, in this system, PIP3 appears to be a more potent activator of Akt than PI 3,4-P2.

Additionally, in several experiments, we observed a greater level of BCR-mediated activation of Akt in SHIP-deficient DT40 cells compared with SHIP-expressing cells, most likely due to the absence of PIP3 dephosphorylation. This suggests that SHIP also directly regulates the BCR-mediated rise in PIP3 levels. This is consistent with the reported increase in Btk activation and calcium flux (another PIP3-dependent event) in SHIP-deficient cells as compared with SHIP-expressing cells (37). Thus, SHIP, through its effects on PIP3, could set the threshold for the magnitude of Akt activation and the downstream consequences.

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FcγRIIB1 cross-linking to the BCR results in an increased sensitivity to apoptosis in mouse splenocytes (38, 39). Since
activation of Akt leads to protection from apoptosis in several cell types (40), it is reasonable to hypothesize that FcγRIIB1/SHIP-mediated inhibition of Akt might be responsible for this increased sensitivity to apoptosis. However, Ono et al. (32) reported that in the DT40 cell line, the absence of SHIP correlates with an increased sensitivity to FcγRIIB1-mediated apoptosis. This increased sensitivity to apoptosis may reflect differences in cell types and/or experimental conditions. Our results show that SHIP-deficient DT40 cells do not exhibit FcγRIIB1-mediated inhibition of Akt, suggesting that the increased sensitivity to apoptosis in these cells cannot be explained by lack of Akt activation. The reason for this inconsistency at present is not clear. The correlation between FcγRIIB1-mediated inhibition of Akt activation and its relevance to survival/apoptosis of B cells during an immune response is currently under investigation.

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Fig. 6. SHIP-dependent inhibition of Akt kinase activity toward GSK-3-derived peptide. Parental DT40 (SHIP-expressing) cells expressing wild type FcγRIIB1 (lanes 1–4) or SHIP-deficient DT40 cells expressing wild type FcγRIIB1 (lanes 5–8) were stimulated by BCR cross-linking only or BCR plus FcγRII co–cross-linking for 5 min as described under "Experimental procedures." Proteins were precipitated with α-Akt antibodies (sheep polyclonal antibodies from a kit as described under "Experimental Procedures") (lanes 1–3 and 5–7) or with control antibodies (lanes 4 and 8). Akt kinase assays were performed using glycogen synthase kinase-3-derived peptide as a substrate. Data from SHIP-expressing and SHIP-deficient DT40 cells are shown in A and B, respectively. Top panels, cpm of incorporated radioactivity were measured in a scintillation counter. Background levels (360 cpm for these experiments) were subtracted as described in the legend to Fig. 3. Each value represents the average of three independent replicates, with S.D. indicated by the error bars. Bottom panels, level of immunoprecipitated Akt was determined by immunoblotting with sheep α-Akt antibody. Akt and IgH are indicated by arrows. This immunoblot represents one of the three replicate experiments. The other two immunoblots yielded similar results. Ipt, immunoprecipitation.
SHIP Inhibits Akt in B Cells

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