We examined activation of the serine/threonine kinase Akt in the murine B cell line A20. Akt is activated in a phosphoinositide 3-kinase (PtdIns 3-kinase)-dependent manner upon stimulation of the antigen receptor, surface immunoglobulin (sIg). In contrast, Akt induction is reduced upon co-clustering of sIg with the B cell IgG receptor, FcγRIIB. Co-clustering of sIg-FcγRIIB transmits a dominant negative signal and is associated with reduced accumulation of the PtdIns 3-kinase product phosphatidylinositol 3,4,5-trisphosphate (PtdIns 3,4,5-P3), known to be a potent activator of Akt. PtdIns 3-kinase is activated to the same extent with and without FcγRIIB co-ligation, indicating conditions supporting the generation of PtdIns 3,4,5-P3. We hypothesized that the decreased Akt activity arises from the consumption of PtdIns 3,4,5-P3 by the inositol-5-phosphatase Src homology 2-containing inositol 5-phosphatase (SHIP), which has been shown by us to be tyrosine-phosphorylated and associated with FcγRIIB when the latter is co-ligated. In direct support of this hypothesis, we report here that Akt induction is greatly reduced in fibroblasts expressing catalytically active but not inactive SHIP. Likewise, the reduction in Akt activity upon sIg-FcγRIIB co-clustering is absent from avian B cells lacking expression of SHIP. These findings indicate that SHIP acts as a negative regulator of Akt activation.

Cellular survival in many systems is regulated by the serine/threonine kinase Akt, also known as protein kinase B (reviewed in Ref. 1). Akt prevents apoptosis in growth factor-responsive cells (1, 2) by phosphorylation of the protein Bad (3, 4), which causes the latter to dissociate from Bcl-2 and Bcl-xL (5, 6). Akt activation in most systems studied to date is dependent on the activity of the lipid kinase phosphoinositide 3-kinase (PtdIns 3-kinase), which generates 3-phosphorylated inositol phospholipids. The PtdIns 3-kinase class IA enzymes, which are regulated by tyrosine kinases, appear to be responsible for Akt activation (7). This class of PtdIns 3-kinase contains two distinct and constitutively associated subunits, an 85-kDa regulatory protein, which contains two Src homology 2 (SH2) domains, and a catalytic 110-kDa subunit.

Which PtdIns 3-kinase products are most important for Akt activity is currently a matter of debate. Both phosphatidylinositol 3,4-bisphosphate (PtdIns 3,4-P2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns 3,4,5-P3) bind to the Akt pleckstrin homology domain, but with different affinities, so that PtdIns-3,4,5-P3 binding is considerably more avid (8). Both 3-phosphoinositide lipids were reported to promote Akt activation, but whether this is a direct effect is unclear (9–12). Besides regulation by 3-phosphoinositides, Akt must also be phosphorylated at conserved serine and threonine residues for maximal activation (reviewed in Ref. 13). At least one kinase responsible for phosphorylating and activating Akt has been isolated (9, 10). The activity of this kinase, PtdIns 3,4,5-P3-dependent kinase-1, is greatly increased by PtdIns 3,4,5-P3 and to a lesser extent by PtdIns 3,4-P2. Intriguingly, membrane localization of Akt causes its maximal activation even in resting cells (14, 15). Based on these findings, 3-phosphoinositides probably regulate Akt by a dual mechanism, first by bringing Akt into close proximity with the serine/threonine kinase PtdIns 3,4,5-P3-dependent kinase-1 and second by stimulating PtdIns 3,4,5-P3-dependent kinase-1 activity (9, 10, 16). PtdIns 3,4,5-P3 is possibly a more effective mediator of Akt induction than PtdIns 3,4-P2; however, there is no direct comparison of these two lipids in this regard.

Stimulation of B cells by the antigen receptor or surface immunoglobulin (sIg) activates protein tyrosine kinases of the Src and Syk family, which in turn induce several biochemical pathways that cause B cell proliferation and Ig secretion (reviewed in Ref. 17). These pathways proceed largely through tyrosine phosphorylation of the conserved immunoreceptor tyrosine-based activation motifs within the two proteins Igα and Igβ, which are associated with sIg. Immunoreceptor tyrosine-based activation motif phosphorylation promotes recruitment of proteins and enzymes via their SH2 domains.

Earlier experiments established that sIg stimulation of B lymphocytes leads to PtdIns 3-kinase association with tyrosine-phosphorylated proteins (18) and the accumulation of 3-phosphoinositide products (19). Tyrosine phosphorylation of the p85 and p110 subunits themselves varies from system to system, but phosphorylation itself does not appear to regulate PtdIns 3-kinase enzymatic activity (20). Recent studies indicated that the B cell membrane protein CD19 engages the SH2 domains of the p85 adapter subunit of PtdIns 3-kinase (21, 22). Presumably, the p85 adapter associates with PtdIns 3-kinase, which in turn interacts with the Src homology 2 domain of SH2-containing inositol 5-phosphatase (SHIP); SH2-containing inositol 5-phosphatase; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; HA, peptide fragment from hemagglutinin; IgH, immunoglobulin heavy chain; PAGE, polyacrylamide gel electrophoresis.
ably, plasma membrane translocation of PtdIns 3-kinase by association of the SH2 domains of the p85 subunit with surface receptors like CD19 promotes production of the 3-phosphoinositides, since membrane targeting of the enzyme in other cells stimulated distal biological effects of PtdIns 3-kinase (23–26).

In contrast to activation by sIg triggering, co-clustering of the B cell IgG receptor FcγRIIB with sIg provokes a dominant-negative inhibitory or negative signal that abolishes the proliferative effect of sIg stimulation. Co-clustering of sIg and FcγRIIB (negative signaling) probably occurs in vivo in the later stages of the immune response, when antigen is coated with specific IgG (reviewed in Ref. 27). Such complexes engage both sIg and FcγRIIB on naïve B cells by binding uncoated antigenic epitopes through sIg and the Fc portions of the coating antibodies through FcγRIIB.

Recent experiments revealed that B cells exposed to negative signaling conditions do not accumulate PtdIns 3,4,5-P3 and that the induction of Btk, a PtdIns 3,4,5-P3-responsive tyrosine kinase, was reduced (28). Inhibition of these signaling events may be due either to deficient PtdIns 3-kinase activation or to increased consumption of nascent PtdIns 3,4,5-P3. The former possibility is raised in experiments examining the tyrosine phosphorylation of CD19 and its subsequent engagement of the p85 SH2 domain; results indicated that both events are reduced under negative signaling conditions (21, 22). The latter possibility was raised by recent studies indicating that negative signaling conditions promote tyrosine phosphorylation of the polyphosphoinositide 5-phosphatase, SHIP (29, 30), through SHIP recruitment to tyrosine-phosphorylated FcγRIIB (31). SHIP recognizes only 3-phosphoinositides (including PtdIns 3,4,5-P3) and hydrolyzes at the D5-position of the inositol ring (32, 33). How these various events regulate SHIP activity is not clear; nevertheless, there is evidence that the catalytic activity of this enzyme is enhanced under conditions of negative signaling in B cells (28).

We investigated the induction of Akt under positive and negative signaling in the murine B cell line A20. We found that Akt was rapidly induced under both conditions; however, the level of Akt activity under negative signaling conditions was only half that under positive signaling. The reduced activity was not due to deficient activation of PtdIns 3-kinase itself and was dependent on the presence of the SHIP docking protein FcγRIIB. Furthermore, COS-7 fibroblasts co-transfected with Akt and wild-type SHIP exhibited reduced Akt induction upon serum stimulation, whereas those co-transfected with Akt and catalytically deficient SHIP did not. Last, the avian B cell line DT40 stably transfected with murine FcγRII exhibited a similar decrease in Akt induction upon sIgL-FcγRII co-clustering, and this effect was absent in the SHIP ((−/−) DT40 derivative, previously described (34). The evidence provided here suggests a model of Akt regulation whereby both PtdIns 3,4-P2 and PtdIns 3,4,5-P3 contribute to Akt activation, PtdIns 3,4,5-P3 being more potent in this regard. SHIP negatively regulates Akt by reducing the available PtdIns 3,4,5-P3.

**EXPERIMENTAL PROCEDURES**

**Antibodies, Cells, Stimulation, and Reagents**—The murine B cell line A20 and its FcγRIIB-deficient derivative IIA1.6 were maintained at 37 °C in RPMI with 10% fetal bovine serum (FBS; both from Life Technologies, Inc.). COS-7 fibroblasts were grown in DMEM with 10% FBS (both from Life Technologies, Inc.). Wild-type and SHIP ((−/−) DT40 chicken B cells stably transfected with murine FcγRIIB were kindly provided by Dr. Jeffrey V. Ravetch (Rockefeller University, New York) and have been described (34).

Anti-Akt used for both immunoprecipitation and immunoblotting was a sheep polyclonal antibody from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-p85 antiserum was generated to a glutathione S-transferase fusion protein containing the N-terminal SH2 domain of p85, as described previously (31). Anti-HA was purchased from Roche Molecular Biochemicals. Stimulating antibodies were from Pierce, and 2.4G2 was purchased from Pharmingen (San Diego, CA); these were used as described earlier (35). Mouse anti-chicken IgM monoclonal IgG antibody M1 was kindly provided by Dr. Max Cooper (University of Alabama, Birmingham). DT40 transfectants were stimulated as follows. 5 × 105 cells were stimulated with 2 μg/ml M1 for 5 min at 37 °C. For most other experiments, 10 × 105 B cells were stimulated with 20–30 μg/ml anti-Ig reagents as described (29). In some experiments, some samples were preincubated for 10 min at 37 °C with 10 μg/ml (for DT40s) or 30 μg/ml (for A20s) rat anti-mouse FcγRII/III monoclonal antibody 2.4G2, a blocking antibody to FcγRII or for 10 min at 37 °C with 100 nm wortmannin (a concentration known to specifically inhibit PtdIns 3-kinase (36)) or with an equal volume of the wortmannin solvent, MeSO. For pervanadate stimulation, a solution of 1:145 of 100 nm activated sodium orthovanadate:30% H2O2:deionized water was left at room temperature for 20 min; 5% by volume of this solution was added to cells at 37 °C for 5 min.

Plasmas were obtained from the following sources. HA-Akt in pSG5 was kindly provided by Dr. David Stokoe (University of California at San Francisco) and has been described in Ref. 9. pCDNA3.1/His B vector was purchased from Invitrogen (Carlsbad, CA). Wild type SHIP cDNA was obtained from Dr. G. Krystal (Terry Fox Laboratory, British Columbia Cancer Research Center, Vancouver, Canada) and subcloned into pcDNA3.1/His B (Invitrogen). The SHIP D672A mutant was made using a QuikChange Site-Directed Mutagenesis kit (Stratagene (La Jolla, CA)). This mutant is catalytically inactive (37).

**Immunoprecipitation and Immunoblotting—**Akt was immunoprecipitated as described below; other proteins (p85, phosphotyrosine, HA-Akt) were obtained by overnight incubation with 1–5 μg of purified antibodies and processed and analyzed for immunoblotting as described (31). Akt migrates at 59 kDa, very close to the heavy chain of the immunoprecipitating antibody (~55 kDa). However, the two proteins could be distinguished by running in parallel an anti-sheep immunoglobulin immunoprecipitate (to detect IgH) and A20 murine B cell whole cell lysate (to detect Akt). For CD19 immunodepletion, samples were incubated with 25 μg of anti-CD19 followed by protein A-Sepharose. The supernatants were found to contain no detectable CD19 (not shown) and were then subjected to immunoprecipitation with anti-phosphotyrosine. Western blots were developed by chemiluminescence and quantitated using a Roche Molecular Biochemicals Lumi-Imager with LumiAnalyzer software supplied by the manufacturer or by NIH Image software.

**Akt in Vitro Kinase Assay—**Lysates of resting or anti-Ig-activated B cells were immunoprecipitated with anti-Akt antibody and protein G-Sepharose. After washing extensively in 1 Triton lysis buffer, the pellets were subjected to in vitro kinase assays using histone H2B, essentially as described elsewhere (38). The reaction was stopped with 5x SDS sample buffer (0.6 x Tris, pH 6.8, 50% glycerol, 12% SDS), separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Phosphorylation of histone H2B was quantitated either by autoradiography using Imaging software by liquid scintillation counting. Values are expressed as -fold increase over the nonstimulated samples after subtracting background. Filters were stained with Coomassie Blue stain (0.2% Coomassie Blue in 30% methanol, 10% glacial acetic acid, 60% water) to show equal amounts of substrate.

**Isolation of Membranes and PI 3-Kinase Assay—**B cells were stimulated and lysed by sonication in TES (20 mM Tris, pH 7.4, 5 mM EDTA, 250 mM sucrose, 3 mM sodium orthovanadate, 1 μg each of aprotinin and leupeptin, and 1 mM phenylmethyl sulfonyl fluoride). Cell fractions were prepared as described previously (39), with minor modifications. Cytosol was defined as the supernatant of the initial 13,000 × g, 20-min centrifugation. The resulting pellet was washed, resuspended a second time, and centrifuged for 1 h at 30,000 × g onto a 1.12 M sucrose cushion. The material above the sucrose cushion contained plasma membrane markers and no detectable cytoplasmic markers (not shown) and was resuspended in TES buffer. The membrane fractions from each sample were normalized for protein content, and PtdIns 3-kinase was immunoprecipitated with 5 μl of rabbit polyclonal anti-p85 antiserum. The resulting immunoprecipitates were applied to a PtdIns 3-kinase assay (31; the PtdIns 3-kinases were pretreated with a Molecular Dynamics Storm system, and the amount of radioactivity in each case is taken to represent the amount of PtdIns 3-kinase present.

**Transient Transfections—**COS-7 fibroblasts were transfected using a protocol kindly supplied by Dr. David Stokoe. In brief, cells were seeded into 10-cm culture dishes and grown at 37 °C until they were 60–70% confluent (1 or 2 days) and then incubated for 3.5 h with premixed DNA.
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FIG. 1. Akt activation by B cell antigen receptor stimulation is downstream of PtdIns 3-kinase and reduced under negative signaling. 5 × 10⁶ A20 B cells were stimulated with the indicated agents, and anti-Akt immunoprecipitates were assayed for Akt activity. A, autoradiogram of a representative experiment; the position of the Akt in vitro substrate, histone H2B, is shown. B, quantitation of the same experiment. Numbers above the bars represent -fold increase over the activity in the unstimulated sample. NS, no stimulus; I + 2.4G2, preincubated with 2.4G2 and stimulated with intact anti-Ig for 1 min; I, stimulated for 1 min with intact anti-Ig; 2.4G2, incubated with 2.4G2 alone; I + 2.4G2 + Wt, preincubated with 100 nM wortmannin and then treated exactly as in lane 2; PtdI, stimulated with pervanadate as described under “Experimental Procedures”; NSIg, treated as in lane 2 and immunoprecipitated with normal sheep immunoglobulin. These results are representative of four separate experiments.

and lipofectamine added in serum-free DMEM. 4 µg of HA-Akt DNA was added with 20 µg of wild-type SHIP or SHIP D672A or equal moles of the vector pcDNA3.1/His B. The cells were grown in DMEM with 10% FBS for 48 h and then starved in serum-free DMEM for 6 h prior to harvesting. Stimulation was done by the addition of 20% FBS for 10 min. The Akt assay was performed on anti-HA immunoprecipitates as described above.

RESULTS

Reduced Activation of the PtdIns 3,4,5-P₃-responsive Enzyme Akt under Negative Signaling Conditions—We examined the induction of Akt in the murine B cell line A20 pretreated with the PtdIns 3-kinase inhibitor wortmannin or an equal volume of Me₂SO and then stimulated under positive or negative signaling conditions using intact anti-Ig antibody in the presence or absence, respectively, of the FcyRIIb-blocking monoclonal antibody 2.4G2, as described earlier (29, 31, 35, 41). Anti-Akt immunoprecipitates were subjected to an in vitro kinase assay with histone H2B as an in vitro substrate, as described earlier (38). As a positive control, B cells were stimulated with pervanadate, previously shown to induce Akt activation through induction of PtdIns 3-kinase (42, 43). The results (Fig. 1, A and B) demonstrate potent Akt induction by pervanadate (lane 6) and positive signaling conditions of sIg triggering alone (lane 2). Akt induction under positive signaling conditions was blocked by wortmannin (lane 5), indicating that, as in other systems, stimulation of Akt by sIg is dependent on PtdIns 3-kinase activity. However, the induction of Akt by stimulation with intact anti-Ig (lane 3) was 50% of that triggered by sIg stimulation alone. In a total of four similar experiments, Akt activity under negative signaling was 40.1 ± 4.4% less than Akt activity under positive signaling. We examined the kinetics of Akt induction in lysates derived from A20 B cells activated under positive or negative signaling conditions, using F(ab’₂)₂ or intact anti-Ig antibodies. As shown in Fig. 2, A and B, the induction of Akt is rapid, peaking less than 1 min after stimulation. However, at all time points, Akt induction under negative signaling conditions was reduced to about half the levels seen under positive signaling conditions.

Similar Association and Activation of PtdIns 3-Kinase with Tyrosine-phosphorylated Proteins and with the Membrane Fraction under Positive and Negative Signaling—The reduced induction of Akt could conceivably result from lower PtdIns 3-kinase activity, since it has been demonstrated that PtdIns 3,4,5-P₃ levels are much lower under negative signaling (28) and the association of the p85 subunit of PtdIns 3-kinase with CD19 is reduced due to decreased tyrosine phosphorylation of CD19 (21, 22).

To compare the activity of PtdIns 3-kinase under positive and negative signaling, we measured the amount of PtdIns 3-kinase associated with tyrosine-phosphorylated proteins in lysates of A20 B cells stimulated under either condition. First, anti-phosphotyrosine immunoprecipitates were analyzed by immunoblotting with antibody to the p85 subunit of PtdIns 3-kinase. Results, shown in Fig. 3A, indicated that the amount of p85 associated with the phosphotyrosine fraction was equivalent at all time points examined under both signaling conditions. However, in confirmation of the earlier reports (21, 22), we observed that the amount of p85 associated with CD19 was reduced by approximately 60% in B cells after stimulation with intact anti-Ig compared with stimulation with F(ab’₂)₂ fragments (Fig. 3B). Nevertheless, anti-phosphotyrosine immunoprecipitates from samples immunodepleted with anti-CD19 still contained the p85 subunit (Fig. 3C), indicating that p85 was associated with phosphotyrosine-containing proteins other than CD19.

The above procedures measure the amount of the p85 regulatory subunit associated with tyrosine-phosphorylated proteins. Conceivably, association of p85 and the catalytic p110 subunit might be reduced under negative signaling conditions, and thus measurements of p85 association may not reflect PtdIns 3-kinase activity. To test this possibility, we measured PtdIns 3-kinase enzymatic activity in anti-phosphotyrosine immunoprecipitates. These measurements (Fig. 3D) consistently revealed approximately 2-fold greater PtdIns 3-kinase activity derived from B cells stimulated under negative signaling conditions.

Membrane translocation of PtdIns 3-kinase is perhaps most relevant to the activation of the enzyme, since genetic manipulations of either PtdIns 3-kinase subunit that induce membrane targeting stimulate distal biochemical and biological activation events (23–26). To examine membrane translocation of PtdIns 3-kinase, we generated membrane and cytosolic frac-

FIG. 2. Kinetics of Akt activation under positive and negative signaling conditions. Anti-Akt immunoprecipitates from 5 × 10⁶ A20 B cells treated with either F(ab’₂)₂ or intact rabbit anti-mouse Ig for the indicated times were assayed for Akt activity using histone H2B as a substrate. In A, the upper image shows the autoradiograph, and the lower image shows a Coomassie Blue stain; the position of histone H2B is shown in both. In B, the labeled histone H2B bands were quantitated and are graphed according to the time and condition of stimulation. These results are representative of three separate experiments.
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**Fig. 3.** Association of PtdIns 3-kinase with tyrosine-phosphorylated proteins under positive and negative signaling. A, amount of p85 in anti-phosphotyrosine immunoprecipitates under positive and negative signaling. Lysates from A20 B cells, resting (NS) or stimulated with F(ab')2 or intact rabbit anti-mouse IgG for the indicated times were immunoprecipitated with anti-phosphotyrosine antibody, and the samples were probed with anti-p85 antibody after SDS-PAGE separation and transfer to nitrocellulose. The position of p85 is shown, -fold increase, shown below each lane, was calculated using NIH Image software. These results are representative of four separate experiments. B, amount of p85 associated with CD19 under positive and negative signaling conditions. A20 B cells were left unstimulated (NS) or stimulated with F(ab')2 (F) or intact (I) rabbit anti-mouse IgG. Samples were lysed and immunoprecipitated with anti-CD19 antibody. After SDS-PAGE and transfer to nitrocellulose, the samples were probed with anti-p85 antibody. NRS, immunoprecipitated with nonspecific immunoglobulin; lysate, whole lysate of resting B cells. The position of p85 is indicated, and quantitation as -fold increase is shown below each lane. These results are representative of four separate experiments. C, presence of p85 in CD-19-depleted phosphotyrosine fraction under positive and negative signaling. A20 B cells were stimulated as above, lysed, and immunodepleted with 25 mg of anti-CD19 followed by Protein G-agarose beads. The resulting supernatants were immunoprecipitated with anti-phosphotyrosine antibody, and samples were analyzed by SDS-PAGE and transfer to nitrocellulose and probed with anti-p85 antibody. NRS, immunoprecipitated with nonspecific immunoglobulin; lysate, whole lysate of resting B cells. The position of p85 is indicated, and quantitation as -fold increase is shown below each lane. These results are representative of two similar experiments. D, amount of PtdIns 3-kinase activity associated with the phosphotyrosine fraction under positive and negative signaling. 4 × 10^6 A20 B cells were stimulated for the indicated times with either F(ab')2 (open symbols) or intact (closed symbols) anti-Ig, lysed, and immunoprecipitated with anti-phosphotyrosine antibodies. The PtdIns 3-kinase activity in the immunoprecipitates was assayed using phosphatidylinositol and [γ-32P]ATP as a substrate; the reaction products were analyzed by thin layer chromatography. The radioactivity of the PtdIns 3-phosphate product was quantitated with a Storm imager, and expressed as a multiple of the amount in the nonstimulated sample. The averages of two duplicate sets are plotted; error bars are plus or minus one S.D. These results are representative of three similar experiments.

**The Role of SHIP in the Negative Signaling-induced Reduction in Akt Activation**—Previous experiments in our laboratory indicated that FcγRIIb-deficient B cells are incapable of promoting SHIP tyrosine phosphorylation (31). We examined the extent of Akt stimulation using the same B cell lines to more rigorously test the role of SHIP in blocking Akt induction under negative signaling conditions. Since SHIP tyrosine phosphorylation and/or FcγRIIb recruitment of SHIP is associated with reduced PtdIns 3,4,5-P_3, we predicted that the FcγRIIb-deficient B cells would not display the reduction in Akt activity after exposure to negative signaling conditions. Accordingly, Akt immunoprecipitates derived from resting or activated A20 B cells (FcγRIIb-) or from the A20 derivative IAI.6 (FcγRIIb-) were measured for Akt activity as described above. Consistent with the notion that SHIP negatively influences Akt activation, FcγRIIb- B cells (Fig. 5) displayed reduced Akt activity when stimulated under negative signaling conditions (57.4 ± 4.7% that under positive signaling in two similar experiments). In contrast, the FcγRIIb+ B cells displayed similar induction of Akt activity under both signaling conditions; in the same two experiments, activity under negative signaling was 114.6 ± 27.4% that under positive signaling.

To directly test the effect of SHIP on Akt in *vivo*, COS-7 fibroblasts were transfected with HA-tagged Akt and co-transfected with excess wild-type SHIP or a catalytically deficient SHIP mutant (SHIP D672A) or the empty vector (pDNA3.1/His B). After 40 h to permit expression of the transfected genes, Akt activity was examined in serum-starved cells upon serum stimulation, which potently activates Akt (42, 43). The results, shown in Fig. 6, indicate that co-transfection of wild-type SHIP reduces the basal level of Akt activity by 70% and the serum-stimulated level by 64%, compared with the activity in the cells co-transfected with vector alone. In contrast, co-transfection of catalytically deficient SHIP caused a reduction of only 23% of serum-stimulated Akt activity.

Finally, the activation of Akt under positive and negative
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**Fig. 4.** PtdIns 3-kinase activity in membrane fractions of B cells under positive and negative signaling. 18–25 × 10^6 A20 B cells were stimulated with 20 μg/ml rabbit anti-mouse Ig, either with or without preincubation with 2.4G2 blocking anti-FcγRIIb antibodies, as indicated. The cells were lysed by sonication, and the membrane and cytosol fractions were isolated by ultracentrifugation. PtdIns 3-kinase indicated. The cells were lysed, Akt was immunoprecipitated with anti-p85 antibodies, and the immunoprecipitates were assayed for PtdIns 3-kinase activity using phosphatidylinositol 3-phosphate (PtdIns 3-P) as substrate. The radioactivity of the product phosphatidylinositol 3-phosphate (PtdIns 3-P) was measured with a Molecular Dynamics Storm imager. C, cytosol fraction; PM, plasma membrane fraction. Amounts of radioactive PtdIns 3-P in the plasma membrane fractions relative to the nonstimulated sample are shown above the respective lanes. The results are representative of two separate experiments.

**Fig. 5.** Akt activation in FcγRIIb-negative IIA1.6 cells is similar under positive and negative signaling. A, 1 × 10^7 A20 (FcγRIIb) or IIA1.6 (FcγRIIb) B cells were treated with intact or F(ab′)_2 fragments of anti-Ig for 60 s. Anti-Akt immunoprecipitates were assayed for Akt activity by in vitro phosphorylation of histone H2B. The autoradiograph of a representative experiment is shown; the position of the histone H2B substrate band is indicated. The sample in the last lane on the right labeled Control was derived from A20 B cells stimulated with F(ab′)_2 anti-Ig and immunoprecipitated with normal sheep immunoglobulin. These results are representative of two separate experiments. B, anti-Akt immunoblot of the same membrane; the 59-kDa Akt band migrates slightly slower than the 55-kDa heavy chain of anti-Akt (IgH), shown clearly in the normal sheep immunoglobulin lane (Control). Lanes in the Akt immunoblot are aligned with the corresponding lanes in the Akt autoradiograph. A20 and IIA1.6 WCL, whole lysate from 2 × 10^6 nonstimulated A20 or IIA1.6 B cells, used to indicate the position of Akt relative to IgH in the Control lane. C, the phosphorylation of histone H2B in a similar experiment was quantitated and graphed according to the cellular source and condition of stimulation.

**Fig. 6.** Serum-stimulated Akt activity is reduced by transiently co-transfected wild-type SHIP. COS-7 fibroblasts were transfected with lipofectamine alone (Mock) or combinations of the following: HA-tagged wild-type Akt (HA-Akt), pcDNA3.1/His B (pcDNA); wild-type SHIP (wt SHIP); and catalytically deficient SHIP (SHIP D672A), as indicated above the lanes. Cells were either stimulated with 20% FBS (+ serum) or not (NS), and anti-HA immunoprecipitates were assayed for activity by in vitro phosphorylation of histone H2B. A, autoradiogram of radioactive histone H2B bands. B, quantitation of histone phosphorylation in A. The radioactivity was quantitated by cutting the H2B band from the gel and measured by liquid scintillation counting. These results are representative of two separate experiments.

**Fig. 7.** Akt activation is not depressed under negative signaling in SHIP (−/−) DT40 cells. 5 × 10^6 wild-type or SHIP (−/−) DT40 chicken B cells stably transfected with murine FcγRIIb were stimulated for 5 min with the mouse anti-chicken Ig monoclonal antibody M1 either without (M1) or with (2.4G2 + M1) preincubation with 2.4G2 for 10 min, as described under “Experimental Procedures.” The cells were lysed, Akt was immunoprecipitated, and Akt activity was assayed as before. Results are expressed as -fold increase over the nonstimulated samples and are representative of two similar experiments.

**DISCUSSION**

The serine/threonine kinase Akt operates distal to PtdIns 3-kinase in growth factor receptor-stimulated fibroblasts (2, 23, 25, 44–46) and acts to protect cells from apoptosis (47–50). While neither induction of Akt activity nor its relationship to apoptosis has been investigated in a B cell model, studies have indicated that negative signaling conditions promote apoptotic cell death in B cells (51, 52).

Results in Figs. 1 and 2 indicate that sIg stimulation induces Akt, and this induction is completely blocked by the PtdIns 3-kinase inhibitor wortmannin. Since PtdIns 3-kinase is known to be recruited and activated under after sIg triggering (18, 19), Akt activation in this system, like others, is downstream of PtdIns 3-kinase. It is worthwhile noting that the kinetics of Akt activation (Fig. 2) closely parallel the reported formation of
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PTDIns 3,4-P₂ and PtdIns 3,4,5-P₃ synthesis, which accumulate to high levels in less than 1 min after sIg triggering (19, 28).

While Akt induction depends on activation of PtdIns 3-kinase and the formation of its 3-phosphoinositide products, it is unclear which 3-phosphoinositide stimulates Akt. Some findings indicate that Akt activity is directly stimulated by the SHIP product, PtdIns 3,4-P₂ (12), while others show that Akt activity is indirectly induced by the SHIP substrate, PtdIns 3,4,5-P₃ via PtdIns 3,4,5-P₃-dependent kinase-1 (9, 10). Our model of positive and negative signaling in B cells may be ideal to resolve this issue, since the influence of SHIP can be turned on or off by small changes in the stimulating reagent (29) or by known genetic manipulations of the SHIP docking protein FcγRIIB (31). Our findings demonstrate that Akt induction in B cells inversely correlates with SHIP induction, in that positive signaling conditions are more efficacious in the stimulation of Akt than negative signaling conditions. Furthermore, we found that the reduction in Akt activation under negative signaling conditions required expression of FcγRIIB, since FcγRIIB-deficient A20 derivative IIA1.6 responds equally upon stimulation with F(ab')₂ or intact anti-Ig reagents (Fig. 5). Last, we specifically tested the effect of SHIP on Akt in transiently co-transfected COS-7 cells. Transfection of Akt with wild-type SHIP inhibited Akt activation, whereas catalytically inactive D672ASHIP did not. These findings directly indicate that SHIP inhibited Akt activation, whereas catalytically inactive SHIP docking protein FcγRIIB did not. These findings directly indicate that SHIP 5-phosphatase activity has a negative effect on Akt induction and suggest that the reduced Akt activity seen after sIg triggering of FcγRIIB co-clustering is due to the recruitment of SHIP to FcγRIIB (described in Refs. 30 and 31). The observations in the wild type and SHIP (−/−) DT40 FcγRIIB transfectants (Fig. 7) establish that in the absence of SHIP, the inhibition of Akt activation under negative signaling is relieved, confirming that SHIP is indeed responsible for down-regulating Akt activation under negative signaling. Similar results of Akt suppression have been recently reported in studies of the tumor suppressor gene and inositol polyphosphate phosphatase, PTEN (53).

The decreased Akt activation under negative signaling does not appear to be due to decreased PtdIns 3-kinase activity, since equivalent amounts of the enzyme are translocated to the membrane and associated with the phosphotyrosine fraction under both signaling conditions. This finding, together with the observation that p85 is present in CD19-depleted lysates (Fig. 3), suggests that PtdIns 3-kinase is translocated to the membrane by other tyrosine-phosphorylated membrane proteins in addition to CD19, such that the reduced association of the enzyme with CD19 under negative signaling (21, 22) does not significantly affect the amount of PtdIns 3-kinase at the membrane.

The equivalent stimulation of PtdIns 3-kinase activity but deficient generation of PtdIns 3,4,5-P₃ (reported in Ref. 28) under negative signaling conditions suggests a role for SHIP-mediated consumption of nascent PtdIns 3,4,5-P₃. It has been shown that the induction of Btk, a protein-tyrosine kinase dependent on formation of 3-phosphoinositides (54), is blocked by overexpression of SHIP (28).

The reduction but not elimination of Akt activity under negative signaling conditions may be due to the reported ability of PtdIns 3,4-P₂ to directly activate the enzyme (11, 12). Negative signaling conditions would promote a large increase in PtdIns 3,4-P₂ through the combined action of PtdIns 3-kinase and SHIP. The PtdIns 3,4-P₂ would then cause Akt activation, albeit with less efficiency than PtdIns 3,4,5-P₃, which also activates Akt indirectly through PtdIns 3,4,5-P₃-dependent kinase-1. An additional possibility is that PtdIns 3,4-P₂ promotes inefficient translocation of Akt, since it has less affinity than PtdIns 3,4,5-P₃ for the pleckstrin homology domain of Akt (8). Thus, reduced Akt membrane translocation would result in reduced Akt activation under negative signaling conditions. We conclude that in the B cell model, both PtdIns 3,4-P₂ and PtdIns 3,4,5-P₃ are capable of Akt activation but that PtdIns 3,4,5-P₃ is a more potent inducer and hence SHIP behaves as a negative regulator for Akt induction.

These findings in antigen receptor-stimulated B lymphocytes are similar to earlier reports of monocyte colony-stimulating factor-induced FcγRIIb monocyte-3-induced DA-ER cells (55), which undergo accelerated apoptosis upon transfection with wild-type but not catalytically deficient SHIP. Likewise, recent experiments investigating survival or apoptosis in cells stimulated with granulocyte colony-stimulating factor revealed SHIP recruitment and tyrosine phosphorylation to the granulocyte colony-stimulating factor receptor (56). Cells expressing granulocyte colony-stimulating factor receptor mutants lacking the fourth cytoplasmic tyrosine failed to recruit and phosphorylate SHIP upon granulocyte colony-stimulating factor stimulation, and the cells displayed a 3-fold increase in cell numbers, compared with those expressing the wild-type receptor. These findings suggest that SHIP recruitment and phosphorylation in response to cytokine stimulation tempers the biological responses to cytokines. There is no molecular or biochemical explanation for the enhanced apoptotic cell death seen in these cases. Based on our findings reported here, we propose that SHIP recruitment and phosphorylation in response to cytokines leads to a reduction in Akt activation, thereby reducing cell survival.

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Note Added in Proof—While this manuscript was in review, two groups (Aman Lamkin, T. D., Okada, H., Kurosaki, T. and Ravichandran, S. (1998) J. Biol. Chem. 273, 33922–33928 and Gupta Scharenberg, A. M., Fruman, D. A., Cantley, L. C., Kinet, J.-P., and Long, E. O. (1999) J. Biol. Chem. 274, 7489–7494) independently reported Akt activation in B cells under sIg triggering and its partial inhibition under co-agglutination of sIg with FcγRIIB.

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