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# SHIP Negatively Regulates IgE + Antigen-Induced IL-6 Production in Mast Cells by Inhibiting NF-κB Activity[1]

**Janet Kalesnikoff,* Nicole Baur,* Michael Leitges,† Michael R. Hughes,* Jacqueline E. Damen,* Michael Huber,‡ and Gerald Krystal[2]***

**We demonstrate in this study that IgE + Ag-induced proinflammatory cytokine production is substantially higher in Src homology-2-containing inositol 5′-phosphatase (SHIP)$^{-/-}$ than in SHIP$^{+/+}$ bone marrow-derived mast cells (BMMCs). Focusing on IL-6, we found that the repression of IL-6 mRNA and protein production in SHIP$^{+/+}$ BMMCs requires the enzymatic activity of SHIP, because SHIP$^{-/-}$ BMMCs expressing wild-type, but not phosphatase-deficient (D675G), SHIP revert the IgE + Ag-induced increase in IL-6 mRNA and protein down to levels seen in SHIP$^{+/+}$ BMMCs. Comparing the activation of various signaling pathways to determine which ones might be responsible for the elevated IL-6 production in SHIP$^{-/-}$ BMMCs, we found the phosphatidylinositol 3-kinase/protein kinase B (PKB), extracellular signal-related kinase (Erk), p38, c-Jun N-terminal kinase, and protein kinase C (PKC) pathways are all elevated in IgE + Ag-induced SHIP$^{-/-}$ cells. Moreover, inhibitor studies suggested that all these pathways play an essential role in IL-6 production. Looking downstream, we found that IgE + Ag-induced IL-6 production is dependent on the activity of NF-κB and that IκB phosphorylation/degradation and NF-κB translocation, DNA binding and transactivation are much higher in SHIP$^{-/-}$ BMMCs. Interestingly, using various pathway inhibitors, it appears that the phosphatidylinositol 3-kinase/PKB and PKC pathways elevate IL-6 mRNA synthesis, at least in part, by enhancing the phosphorylation of IκB and NF-κB DNA binding while the Erk and p38 pathways enhance IL-6 mRNA synthesis by increasing the transactivation potential of NF-κB. Taken together, our data are consistent with a model in which SHIP negatively regulates NF-κB activity and IL-6 synthesis by reducing IgE + Ag-induced phosphatidylinositol-3,4,5-trisphosphate levels and thus PKB, PKC, Erk, and p38 activation.** ***The Journal of Immunology,*** **2002, 168: 4737–4746.**

Immunoglobulin E + Ag-induced mast cell activation is responsible for both allergic disorders, such as hay fever and asthma, and host resistance to parasites via the secretion of proinflammatory mediators (1–3). The three major classes of proinflammatory mediators released by activated mast cells are preformed granule-associated chemical mediators, newly synthesized arachidonic acid metabolites, and proinflammatory vasoactive cytokines (e.g., TNF-α and IL-6) (2, 4). IgE initiates these processes by binding to mast cells via the high-affinity IgER, FcεRI, which belongs to the immune receptor superfamily and exists as a tetramer of one α-, one β-, and two identical disulfide-linked γ-chains. The α subunit binds the Fc portion of a single IgE molecule. Following crosslinking of IgE-bound FcεRIs by a multivalent Ag, the β- and γ-chains mediate signal transduction events via their immunoreceptor tyrosine-based activation motifs, which, upon phosphorylation by members of the Src kinase family, attract Src homology-2-containing signaling proteins (5, 6). One of the proteins that becomes tyrosine phosphorylated in mast cells in response to IgE + Ag is the hemopoietic-specific Src homology-2-containing inositol 5′-phosphatase (SHIP)[3] (7–9). Although this phosphorylation event does not appear to increase the enzymatic activity of SHIP (8), it may be involved in the localization of SHIP at the plasma membrane (10) where it cleaves the 5′-phosphate from the phosphatidylinositol 3-kinase (PI-3K)-generated product phosphatidylinositol-3,4,5-trisphosphate ($PIP_3$) to yield PI-3,4-$P_2$. This in turn reduces the ability of certain pleckstrin homology (PH)-containing proteins (e.g., protein kinase B (PKB)/Akt, phosphoinositide-dependent protein kinase (PDK)-1, Bruton's tyrosine kinase) to target to the plasma membrane and be activated (11–13).

Using bone marrow-derived mast cells (BMMCs), we recently demonstrated that SHIP is a key negative regulator of IgE + Ag-induced mast cell degranulation (7). To further explore the role that SHIP plays in regulating IgE + Ag-induced mast cell activation, we have now compared cytokine production in murine BMMCs from SHIP$^{+/+}$ and SHIP$^{-/-}$ littermates. We demonstrate in this study that SHIP negatively regulates IgE + Ag-induced IL-6 mRNA and protein levels and requires its phosphatase activity to exert this negative effect. Moreover, we show that SHIP represses IL-6 mRNA levels, at least in part, by reducing PKB,

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[3] Abbreviations used in this paper: SHIP, Src homology-2 containing inositol 5′-phosphatase; PI-3K, phosphatidylinositol 3-kinase; $PIP_3$, phosphatidylinositol-3,4,5-trisphosphate; PH, pleckstrin homology; PKB, protein kinase B; PKC, protein kinase C; PDK, phosphoinositide-dependent protein kinase; cPKC, classical PKC; BMMC, bone marrow-derived mast cells; Erk, extracellular signal-related kinase; HA, hemagglutinin; WT, wild type; RPA, RNase protection assay; HSA, human serum albumin; Mek, mitogen-activated/extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; pRL-TK, thymidine kinase promoter-dependent renilla luciferase construct; IKK, IκB kinase; Bay11, NF-κB inhibitor Bay11-7082; Shc, Src homology and collagen; PTEN, phosphatase and tensin homolog deleted on chromosome 10.



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protein kinase C (PKC), p38, and extracellular signal-related kinase (Erk) activity. This culminates in a marked reduction in NF-κB activity and, thus, IL-6 mRNA and protein synthesis.

## Materials and Methods

### *Mast cell isolation*

Bone marrow cells aspirated from 4- to 8-wk-old SHIP$^{+/+}$ and SHIP$^{-/-}$ C57B6 or PKCδ$^{+/+}$ and PKCδ$^{-/-}$ 129/SV mice were cultured as described previously (7, 14). By 8 wk in culture, greater than 98% of the cells were c-*kit*- and FcεRI-positive, as assessed by FITC-labeled anti-c-*kit* Abs (BD PharMingen, Mississaga, Canada) and FITC-labeled IgE (anti-erythropoietin 26), respectively (7).

### *Introduction of SHIP constructs into SHIP$^{-/-}$ BMMCs*

The hemagglutinin (HA)-tagged murine SHIP cDNA in a Bluescript KS$^{+}$ vector, was the starting material for the PCR-based point mutation in the D675G SHIP construct using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) (15). SHIP$^{-/-}$ bone marrow cells were infected with the wild-type (WT) or D675G SHIP construct and BMMCs derived as described in (15).

### *BMMC stimulation and Western blotting*

To stimulate with IgE + Ag, BMMCs were preloaded with 1–5 μg/ml anti-DNP IgE (clone SPE-7 IgE; Sigma-Aldrich, St. Louis, MO) for 4 h or overnight in IMDM + 10% FCS + 150 μM monothioglycerol, washed three times to remove unbound IgE, and then resuspended in IMDM + 0.1% BSA for 5 min at 37°C before adding 20 ng/ml DNP-human serum albumin (HSA) (30–40 moles DNP/mole HSA; Sigma-Aldrich) for the indicated times. For inhibitor studies, inhibitors were added 15 min before the addition of DNP-HSA at the indicated concentrations. The inhibitors used were the PI-3K inhibitor LY294002, the mitogen-activated/extracellular signal-regulated kinase kinase (Mek) inhibitor PD98059, the p38 inhibitor SB203580, the NF-κB inhibitor Bay11-7082 (Bay11), the PKC inhibitor compound 3 (bisindolymaleimide I), and the classical PKC (cPKC) inhibitor GÖ6976, all obtained from Calbiochem (La Jolla, CA), and wortmannin (Sigma-Aldrich) and cycloheximide (Boehringer Mannheim, Mannheim, Germany). The cells were solubilized by boiling for 1 min with SDS-sample buffer (using 1 × 10$^{6}$ BMMCs/sample for total cell lysates). The phospho-PKB (Ser$^{473}$), phospho-IκB (Ser$^{32}$), IκB, phospho-Erk 1/2, phospho-p38, phospho-c-Jun N-terminal kinase (JNK), and JNK Abs were obtained from Cell Signaling (Beverly, MA). Src homology and collagen (Shc) Ab was obtained from BD Transduction Laboratories (Mississauga, Ontario, Canada). The Erk1 and FcεRI β-chain Abs were generous gifts from Drs. S. Pellech (Vancouver, British Columbia, Canada) and R. Siraganian (Bethesda, MD), respectively. The polyclonal anti-SHIP Ab was generated as described in Ref. 15.

### *Plasma membrane preparation*

SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs were stimulated as above and plasma membrane-enriched fractions were prepared as described in Ref. 16. The final Nonidet P-40 solubilized membrane fraction, which was highly enriched for plasma membranes (as assessed by biotinylating the cell surface of intact BMMCs (M. R. Hughes and G. Krystal, unpublished observations), was then subjected to Western blot analysis with anti-PKC Abs (BD Transduction Laboratories).

### *Degranulation assays*

IgE + Ag-induced degranulation was assessed as described in Ref. 7.

### *RNase protection assay (RPA) and ELISA*

For mRNA analysis, BMMCs were stimulated as above and RNA was isolated using TRIzol reagent (Life Technologies, Burlington, Canada). Cytokine mRNA levels were quantitated using a Riboquant MultiProbe RNase protection assay (BD PharMingen) according to the manufacturer's instructions, using [$^{33}$P]dUTP 9NEG-307H from NEN (Boston, MA). Quantitation of cytokine mRNA levels, using a PhosphorImager (Storm 860; Molecular Dynamics, Sunnyvale, CA), was standardized based on levels of the housekeeping gene *GAPDH*. Mouse IL-4, TNF-α, IL-6 (BD PharMingen), and IL-13 (R&D Systems, Minneapolis, MN) ELISAs were performed according to the manufacturer's instructions.

### *EMSAs*

EMSAs were performed as described in (17). Nuclear extracts (5 μg of protein, as determined by BCA Protein Assay kit (Pierce, Rockford, IL)) were incubated with 1 pmol [$^{32}$P]NF-κB consenus oligonucleotide probe (Santa Cruz Biotechnology, Santa Cruz, CA) in binding buffer and 1 μg of poly(dIdC) (BD PharMingen) for 15 min at 23°C and then electrophoresed on 5% polyacrylamide gels in 0.25× Tris-borate-EDTA. Ab supershifts were performed by preincubating with 10 μg of anti-NF-κB Abs (Rel A, Rel B, c-Rel, p50, p52; Santa Cruz Biotechnology) 15 min before the addition of oligonucleotide probe. These Abs were also used for Western blot analysis with nuclear extracts.

### *Cell transfection and luciferase assay*

Cell transfection experiments were conducted as described in Ref. 18. Briefly, BMMCs were incubated for 4 h in fresh growth medium, washed, and resuspended in IMDM + 10% FCS and aliquoted (10$^{7}$/250 μl) into electroporation cuvettes (4-mm gap; Bio-Rad, Hercules, CA). Reporter gene construct (10 μg/ml, pNF-κB-LUC; Stratagene) was added together with 2 μg/ml of thymidine kinase promoter-dependent renilla luciferase construct (pRL-TK; Promega, Madison, WI) to assess transfection efficiency and the cells electroporated (Gene Pulser; Bio-Rad) at 280 V and 960 μF. The cells were then incubated in tissue culture flasks at 1 × 10$^{6}$ cells/ml in IMDM + 10% FCS + 1 μg/ml IgE overnight and then treated ± 20 ng/ml DNP-HSA for 4 h. Luciferase assays were performed according to the manufacturer's instructions (Dual Luciferase Reporter Assay; Promega).

## Results

### *SHIP negatively regulates cytokine production in activated BMMCs*

To explore the role that SHIP plays in regulating the IgE + Ag-induced synthesis and release of proinflammatory cytokines from

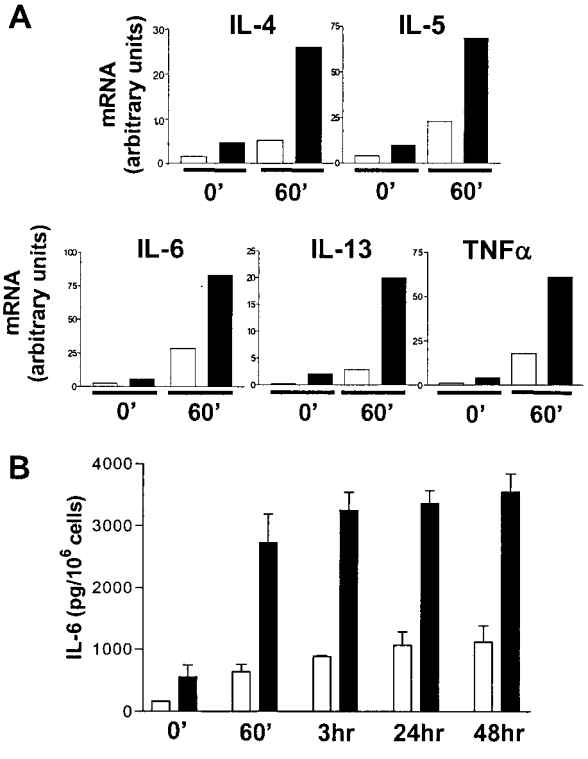


**FIGURE 1.** SHIP negatively regulates cytokine production in BMMCs. *A*, SHIP$^{+/+}$ (□) and SHIP$^{-/-}$ (■) BMMCs were preloaded with 5 μg/ml IgE for 4 h, then stimulated ± 20 ng/ml DNP-HSA for 30 min and subjected to RPAs. The relative band intensities of IL-4, IL-5, IL-6, IL-13, and TNF-α mRNAs were quantified using a phosphorimager and standardized using levels of the housekeeping gene, *GAPDH*. Results are representative of three separate experiments. *B*, SHIP$^{+/+}$ (□) and SHIP$^{-/-}$ (■) BMMCs were preloaded with IgE, washed to remove IL-6 produced in response to IgE alone, and stimulated ± DNP-HSA as in *A* for the indicated times. IL-6 protein levels in the cell supernatants were assessed by ELISA. Each bar represents the mean ± SEM of eight determinations.

BMMCs, we first conducted RPAs with IgE + Ag-stimulated SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs. As shown in Fig. 1*A*, these studies revealed that the mRNA levels of IL-4, IL-5, IL-6, IL-13, and TNF-$\alpha$ were substantially higher in SHIP$^{-/-}$ than in SHIP$^{+/+}$ BMMCs (Fig. 1*A*). This difference in cytokine mRNA levels was observed not only in response to IgE + Ag but in response to steel factor and IL-3 as well (data not shown). To investigate how SHIP negatively regulates cytokine expression at the signal transduction level, we focused our attention on IL-6 because a great deal was already known about the regulation of this cytokine in mast cells (19). As shown in Fig. 1*B*, we found that IL-6 protein levels, as assessed by ELISA, were significantly higher in the conditioned medium from SHIP$^{-/-}$ BMMCs, consistent with the elevated mRNA levels observed in Fig. 1*A*.

### *Addition of WT, but not phosphatase-deficient, SHIP to SHIP$^{-/-}$ BMMCs reduces IL-6 production to that seen in SHIP$^{+/+}$ BMMCs*

To determine whether the observed difference in IL-6 production between SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs was a result of SHIP's enzymatic activity or its ability to serve as an adaptor protein (9, 20), bone marrow cells from SHIP$^{-/-}$ mice were retrovirally infected with N-terminal HA- and C-terminal green fluorescent protein-tagged versions of WT or phosphatase-deficient (D675G) SHIP (Fig. 2*A*, *left panel*) (15). Following 10 days in methylcellulose containing puromycin, mast cell colonies were pooled and put in suspension culture for 8 wk (15). At this time, the two cultures were greater than 98% IgER and c-*kit* positive and Western blot analysis, using anti-SHIP Abs, revealed that the WT and D675G SHIP constructs expressed approximately the same amount of SHIP protein and this level was about half that present in SHIP$^{+/+}$ BMMCs (Fig. 2*A*, *right panel*). Studies with these BMMCs revealed that the introduction of WT SHIP, which reduced IgE + Ag-induced $PIP_3$ (15) and degranulation (Fig. 2*B*) to levels approaching those observed in SHIP$^{+/+}$ BMMCs, reverted the IgE + Ag-induced increase in IL-6 mRNA (as assessed by RPA; Fig. 2*C*) and protein expression (as assessed by ELISA; Fig. 2*D*) to close to those observed in SHIP$^{+/+}$ BMMCs. The D675G SHIP, in contrast, did not revert any of the responses examined (Ref. 15; Fig. 2, *B–D*). These results suggested that the difference in IL-6 production was dependent on the phosphatase activity of SHIP, and thus, likely, $PIP_3$ levels.

### *IgE + Ag activates multiple pathways to a greater extent in SHIP$^{-/-}$ than in SHIP$^{+/+}$ BMMCs*

To explore which $PIP_3$-regulated pathways might be responsible for the elevated IL-6 mRNA and protein levels seen in IgE + Ag-induced SHIP$^{-/-}$ BMMCs, we first compared the activation states of various pathways known to be triggered by IgE + Ag in BMMCs. Specifically, we compared the phosphorylation state of PKB, a PH domain-containing serine/threonine kinase that is recruited to the plasma membrane by the transient IgE + Ag-induced increase in $PIP_3$ and activated via phosphorylation at Thr$^{308}$, by the $PIP_3$ binding PH-containing serine/threonine kinase, PDK1,



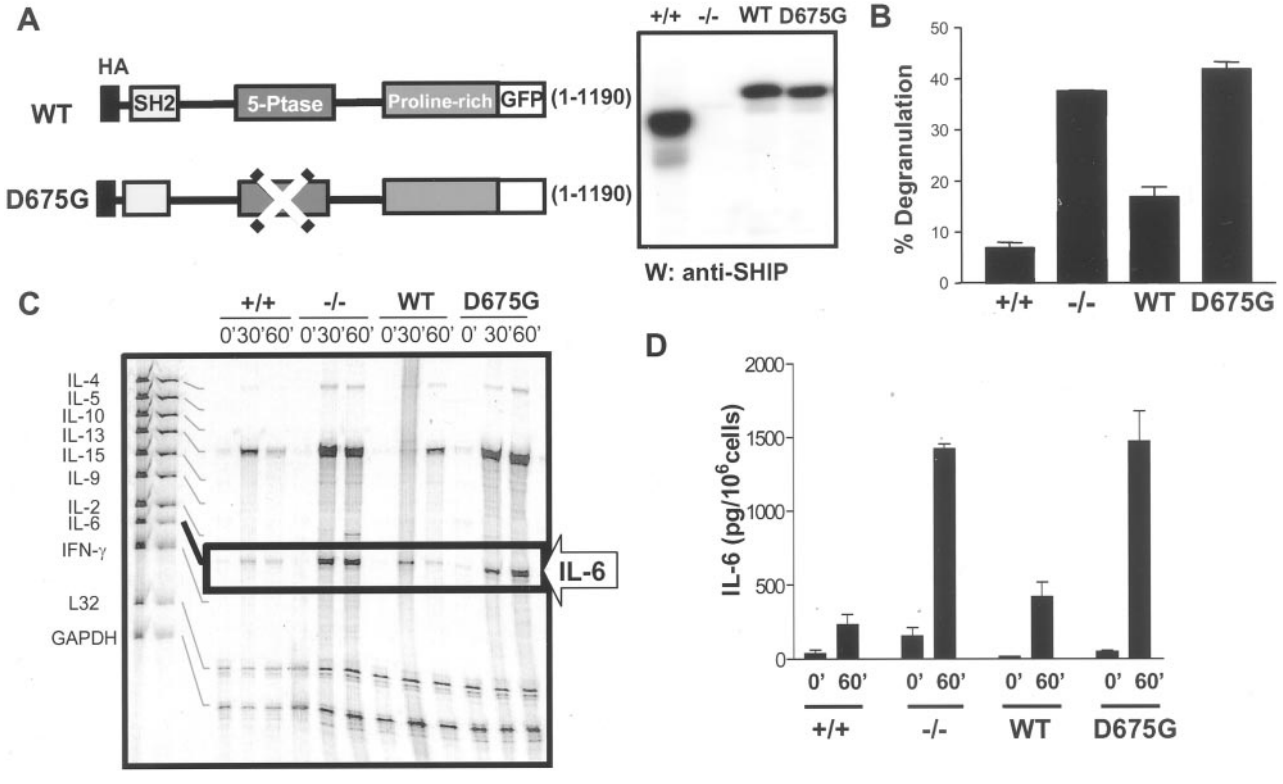


**FIGURE 2.** Addition of WT, but not phosphatase-deficient, SHIP reverts IL-6 production in SHIP$^{-/-}$ to SHIP$^{+/+}$ BMMC levels. *A*, Full length (1190 aa) WT and D675G SHIP constructs HA-tagged at the N terminus (▮) and green fluorescent protein-tagged at the C terminus (▭). Total cell lysates from $1 \times 10^6$ SHIP$^{+/+}$, SHIP$^{-/-}$, WT, and D675G BMMCs were subjected to Western blot analysis using anti-SHIP Abs (*right panel*). *B*, SHIP$^{+/+}$, SHIP$^{-/-}$, WT, and D675G BMMCs were preloaded with 10 $\mu$g/ml IgE for 1 h, then stimulated for 15 min ± 20 ng/ml DNP-HSA; the percentage of degranulation was determined by assaying supernatants and cell pellets for $\beta$-hexosaminidase activity. Each bar represents the mean ± SEM of duplicates after subtracting the percentage of degranulation obtained in the absence of DNP-HSA. *C*, SHIP$^{+/+}$, SHIP$^{-/-}$, WT, and D675G BMMCs were preloaded with 5 $\mu$g/ml IgE for 4 h, treated ± 20 ng/ml DNP-HSA for the indicated times and subjected to RPA (IL-6 mRNA levels are boxed). Results are representative of two separate experiments. *D*, SHIP$^{+/+}$, SHIP$^{-/-}$, WT, and D675G BMMCs were preloaded and stimulated as in *C* and IL-6 protein levels in the supernatants were determined by ELISA. The bars represent the mean ± SEM of four separate experiments.

and at Ser$^{473}$ by either an as yet unidentified PDK2 or by autophosphorylation in a manner similar to PKCs (21–24). As expected, because SHIP down-regulates $PIP_3$ levels and thus the recruitment of PKB (and likely PDK1), we observed more intense and prolonged phosphorylation of PKB in IgE + Ag-induced SHIP$^{-/-}$ BMMCs (Fig. 3*A*, *upper panel*). Reprobing with anti-FcεRI β-subunit Abs demonstrated equal loading (*lower panel*).

We then compared members of the PKC family of serine/threonine kinases because several isoforms of this multigene family may bind to $PIP_3$, in addition to diacylglycerol (25, 26), and subsequently become activated/phosphorylated by PDK1 (27–29). To assess the activation state of various PKC isoforms, we measured their levels in plasma membrane preparations because cell stimulation has been shown to trigger the translocation of PKCs from the cytosol to the plasma membrane where they become activated (30, 31) and gain access to their substrates (32). As seen in the *left panel* of Fig. 3*B*, IgE + Ag treatment resulted in a substantially greater recruitment of PKC α, β, γ, and δ to the plasma membrane of SHIP$^{-/-}$ than SHIP$^{+/+}$ BMMCs. Reprobing with anti-FcεRI β-chain Abs demonstrated equal levels of membrane protein. Importantly, total cell lysates showed comparable levels of these PKC isoforms in SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs (Fig. 3*B*, *right panel*).

Because PKC has been shown to phosphorylate/activate Raf-1 in hemopoietic cells (33), we then compared the IgE + Ag-induced phosphorylation of Erk in SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs and found much more intense and prolonged phosphorylation in SHIP$^{-/-}$ BMMCs (Fig. 3*C*, *top panel*). Reprobing with anti-FcεRI β-subunit Abs demonstrated equal loading (Fig. 3*C*, *bottom panel*). We also looked at the effect of SHIP on IgE + Ag-induced activation of p38 mitogen-activated protein kinase (MAPK), because, depending on the stimulus and cell type, this serine/threonine kinase has been shown to be activated (34) or inhibited (35) by the PI-3K/PKB pathway and also activated by members of the PKC family (36). Interestingly, we found that p38 phosphorylation was both more intense and more prolonged in response to IgE + Ag in SHIP$^{-/-}$ BMMCs (Fig. 3*D*, *top panel*). Reprobing with anti-FcεRI β-subunit Abs demonstrated equal loading (Fig. 3*D*, *bottom panel*). Lastly, we looked at the effect of SHIP on IgE + Ag-induced phosphorylation/activation of JNK because the activation of this stress-activated protein kinase has been shown to enhance IL-6 production in mast cells (37). As shown in the *top panel* of Fig. 3*E*, JNK phosphorylation was both more intense and more prolonged in response to IgE + Ag in SHIP$^{-/-}$ BMMCs. Reprobing with anti-JNK Abs demonstrated equal loading (Fig. 3*E*, *bottom panel*).

### *IgE + Ag-induced IL-6 production in BMMCs is dependent on the activation of the PI-3K, PKC, Erk, and p38 pathways*

To determine which of these pathways, if any, contributed to the elevated IL-6 production observed in SHIP$^{-/-}$ BMMCs, we added pathway specific inhibitors to SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs and then stimulated the cells with IgE + Ag for 3 h and performed IL-6 ELISAs on the conditioned medium. As seen in Fig. 4*A*, addition of the PI-3K inhibitor LY294002 (25 μM), the PKC inhibitor compound 3 (bisindolylmaleimide; 10 μM), the Mek inhibitor PD98059 (50 μM), or the p38 inhibitor SB203580 (2 μM) completely abrogated IgE + Ag-induced IL-6 production in both SHIP$^{+/+}$ (*left panel*) and SHIP$^{-/-}$ (*right panel*) BMMCs. The PI-3K inhibitor wortmannin (25 nM) also totally inhibited IL-6 production (data not shown). Thus, it appeared that all of these signaling pathways were essential for IL-6 production and, given that they were all elevated in IgE + Ag-induced SHIP$^{-/-}$ BMMCs, could contribute to the elevated IL-6 production observed in these cells. To gain some insight into which PKC isoform(s) were involved, we also examined IL-6 production from IgE + Ag-induced SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs in the presence and absence of the cPKC inhibitor, GÖ6976. As seen in Fig. 4*B* (*left panel*), this inhibitor blocked IL-6 production, indicating a role for a $Ca^{2+}$-dependent PKC in IL-6 regulation. This is consistent with a recent finding showing decreased IL-6 production from PKCβ-deficient BMMCs (38). To determine whether PKC-mediated regulation of IgE + Ag-induced IL-6 production was limited

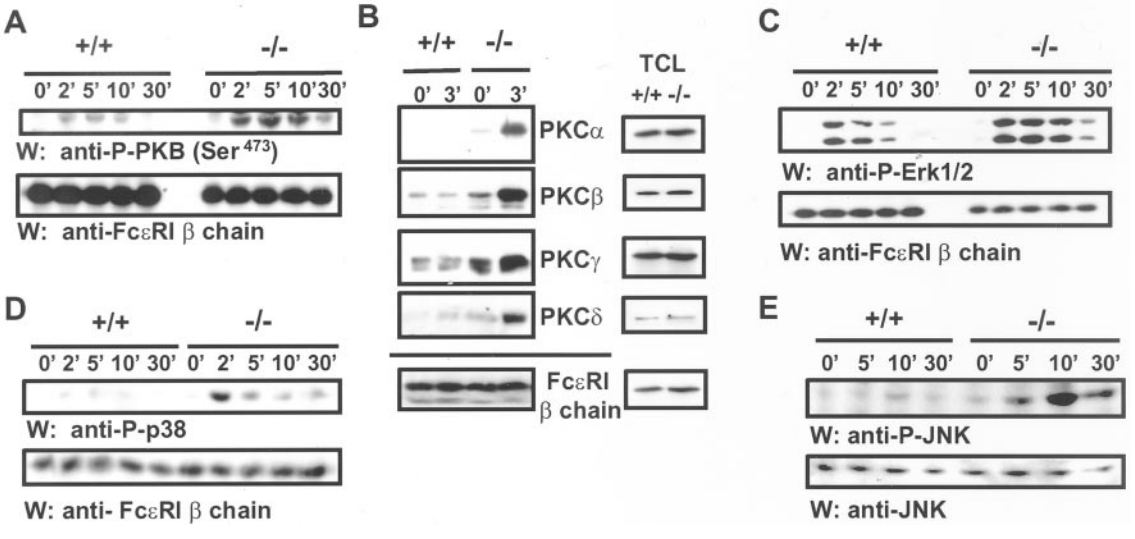


**FIGURE 3.** SHIP represses multiple IgE + Ag-induced signaling pathways in BMMCs. *A*, SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs were preloaded with 5 μg/ml IgE for 4 h and stimulated ± 20 ng/ml DNP-HSA for the indicated times. Total cell lysates were subjected to Western blot analysis using anti-phospho-PKB (Ser$^{473}$) Abs (*top panel*). The blot was reprobed with anti-FcεRI β-chain Abs to show equal loading. *B*, SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs were preloaded as in *A*, then treated ± DNP-HSA for 3 min. Western blot analyses were conducted with plasma membrane preparations using anti-PKCα, β, γ, and δ Abs and the blot was reprobed with anti-FcεRI β-chain Abs to show equal loading (*left panels*). Total cell lysates were subjected to Western blot analysis with the same Abs (*right panels*). *C–E*, SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs were preloaded and stimulated as in *A*. Total cell lysates were subjected to Western blot analysis using (*C*) anti-phospho-Erk1/2, (*D*) anti-phospho-p38, and *(E)* anti-phospho-JNK Abs (*top panels*). These blots were reprobed with anti-FcεRI β-chain and anti-JNK Abs to show equal loading. These blots are representative of three separate experiments.

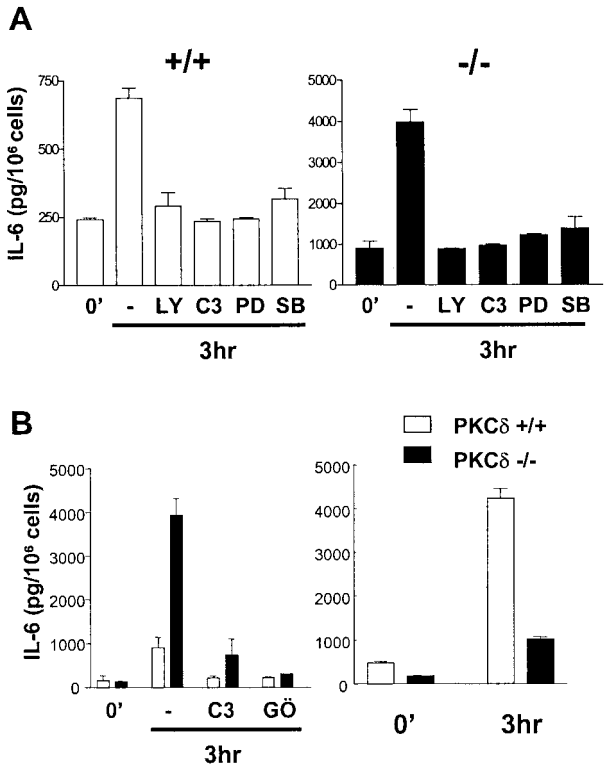


**FIGURE 4.** IgE + Ag-induced IL-6 production in BMMCs is dependent on the activation of the PI-3K, PKC, Erk, and p38 pathways. *A*, $SHIP^{+/+}$ (□; *left panel*) and $SHIP^{-/-}$ (■; *right panel*) BMMCs were preloaded with 5 $\mu$g/ml IgE for 4 h, then stimulated ± 20 ng/ml DNP-HSA for 3 h in the absence (-) or presence of 25 $\mu$M LY294002 (LY), 10 $\mu$M compound 3 (C3), 50 $\mu$M PD98059 (PD), and 2 $\mu$M SB203580 (SB) added 15 min before the DNP-HSA. IL-6 production was assessed by ELISA. Results shown are the mean ± SEM of six determinations. *B*, $SHIP^{+/+}$ (□) and $SHIP^{-/-}$ (■) BMMCs were stimulated as in *A* in the absence (-) or presence of 10 $\mu$M C3 and 1 or 0.1 $\mu$M GÖ6976 (GÖ). IL-6 levels were measured by ELISA (*left panel*). $PKC\delta^{+/+}$ (□) and $PKC\delta^{-/-}$ (■) BMMCs were preloaded as in *A* and then stimulated ± DNP-HSA for 3 h; IL-6 levels in the supernatants were assessed by ELISA (*right panel*). Results shown are the mean ± SEM of four determinations.

to cPKC isoforms, we compared IL-6 production from $PKC\delta^{+/+}$ and $PKC\delta^{-/-}$ BMMCs. As shown in Fig. 4*B* (*right panel*), IL-6 production from $PKC\delta^{-/-}$ BMMCs was significantly reduced compared with their $PKC\delta^{+/+}$ counterparts, suggesting a role for a novel PKC isoform in the regulation of IL-6 production as well.

### *IκB phosphorylation/degradation and NF-κB DNA binding and transactivation are higher in IgE + Ag-induced $SHIP^{-/-}$ BMMCs*

Because it had been shown recently that the transcription factor NF-κB is a key regulator of IgE + Ag-induced IL-6 mRNA in BMMCs by binding directly to κB elements within the IL-6 promoter and activating transcription (19), we next compared the activity of NF-κB in IgE + Ag-induced $SHIP^{+/+}$ and $SHIP^{-/-}$ BMMCs. NF-κB activity is tightly regulated by IκBα (39, 40) which binds and masks the NF-κB nuclear localization signal and thus sequesters NF-κB in the cytoplasm. Following IgE + Ag-stimulation, IκB kinase (IKK) is activated and phosphorylates IκBα, which targets the latter for ubiquitination and degradation by the proteasome, and frees NF-κB to translocate to the nucleus to activate target gene transcription (41, 42). As expected, we found that the Bay11, which irreversibly inhibits the phosphorylation of IκB (43), was a potent inhibitor of IL-6 production in both $SHIP^{+/+}$ and $SHIP^{-/-}$ BMMCs (Fig. 5*A*). Then, using phospho-specific IκBα Abs, we examined IgE + Ag-induced IκB phosphorylation and found much higher phosphorylation in $SHIP^{-/-}$ than $SHIP^{+/+}$ BMMCs (Fig. 5*B*). Moreover, reintroduction of WT SHIP into $SHIP^{-/-}$ cells reduced this phosphorylation close to the levels seen in $SHIP^{+/+}$ BMMCs (Fig. 5*B*). In keeping with this increased IκB phosphorylation in $SHIP^{-/-}$ BMMCs, IκB degradation was significantly greater in these cells, but only when the protein synthesis inhibitor cycloheximide was added (Fig. 5*C*). In the absence of cycloheximide, IgE + Ag-induced degradation of IκB was similar in $SHIP^{+/+}$ and $SHIP^{-/-}$ BMMCs (Fig. 5*D*), most likely because of a compensatory increase in the transcription/translation of the NF-κB target, IκB, in the $SHIP^{-/-}$ cells that masks the increased IκB degradation (39, 40, 42).

We next looked at NF-κB translocation to the nucleus by carrying out anti-NF-κB p65 and p50 immunoblots with nuclear extracts and found, interestingly, that p50, but not p65, levels were substantially higher in IgE + Ag-induced $SHIP^{-/-}$ than $SHIP^{+/+}$ BMMCs (Fig. 6*A*, *left panels*). Total cell levels of NF-κB (p50), in contrast, were comparable (Fig. 6*A*, *right panel*). However, a higher concentration of nuclear NF-κB is not necessarily synonymous with more IL-6 mRNA synthesis (44). To initiate transcription of target genes, NF-κB transcription factors must bind first as dimers to κB sites (40). Therefore, using EMSAs, we investigated whether NF-κB DNA binding was higher in IgE + Ag-induced $SHIP^{-/-}$ than $SHIP^{+/+}$ BMMCs and found, as shown in Fig. 6*B*, that it was. We then conducted supershift studies using Abs to the members of the NF-κB family (p50, p52, p65 (Rel A), Rel B, and c-Rel; Ref. 40) and found that only anti-p50 and anti-p65 supershifted the NF-κB/oligonucleotide complex (data not shown). Thus, a p50/p65 heterodimer was likely the predominant species that bound to κB sites in activated mast cells. As expected, the addition of Bay11 (20 $\mu$M) to $SHIP^{+/+}$ and $SHIP^{-/-}$ BMMCs completely abrogated the nuclear localization of NF-κB, as assessed by EMSA, on nuclear preparations from both cell types (Fig. 6*B*).

Although phosphorylation-induced degradation of IκB and the subsequent nuclear translocation and DNA binding of released NF-κB is regarded as the principle mechanism for activating NF-κB-dependent gene expression, some recent studies have suggested that the transcriptional activity of NF-κB is also highly regulated (34). We thus conducted NF-κB luciferase assays to obtain direct evidence for increased NF-κB transactivation in IgE + Ag-induced $SHIP^{-/-}$ BMMCs. Specifically, $SHIP^{+/+}$ and $SHIP^{-/-}$ BMMCs were electroporated with a NF-κB-firefly luciferase reporter gene construct, together with a pRL-TK to assess transfection efficiency, and the cells were then preloaded with 1 $\mu$g/ml IgE for 18 h and subjected to 20 ng/ml DNP for 4 h. As shown in Fig. 6*C*, the transactivation potential of NF-κB was significantly higher in $SHIP^{-/-}$ BMMCs. Interestingly, the high NF-κB transactivation observed in the $SHIP^{-/-}$ BMMCs in the absence of crosslinker is consistent with our previous data showing that IgE alone is capable of inducing NF-κB transactivation (45) and IL-6 production (46). Both of these processes are negatively regulated by SHIP.

### *PI-3K/PKB and PKC enhance IκB phosphorylation/degradation and NF-κB binding to DNA while Erk and p38 stimulate NF-κB transactivation*

Because we found that the PI-3K/PKB, PKC, Erk and p38 pathways were all elevated in $SHIP^{-/-}$ BMMCs and were all required for IL-6 production, we asked whether and how these pathways elevated NF-κB activity. However, a complication in delineating the relative contributions of these upstream pathways to NF-κB

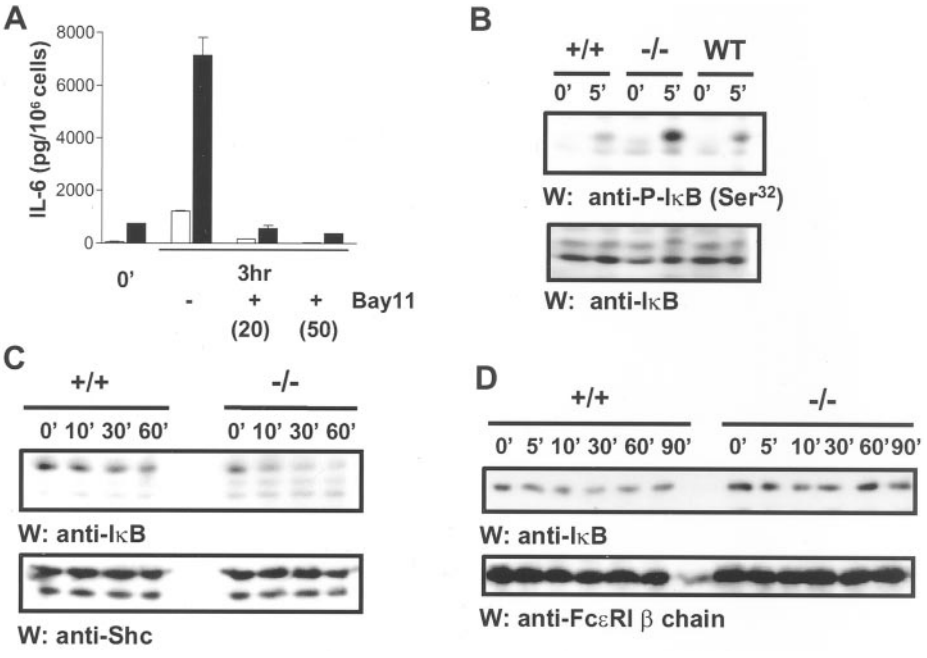


**FIGURE 5.** IκB phosphorylation and degradation are higher in SHIP$^{-/-}$ BMMCs. *A*, SHIP$^{+/+}$ (□) and SHIP$^{-/-}$ (■) BMMCs were preloaded with 5 μg/ml IgE for 4 h, then stimulated ± 20 ng/ml DNP-HSA for 3 h in the presence (+) or absence (-) of Bay11 at 20 or 50 μM added 15 min before the DNP-HSA; IL-6 levels in the supernatants were assessed by ELISA. Results shown are the mean ± SEM of four determinations. *B*, SHIP$^{+/+}$, SHIP$^{-/-}$, and WT BMMCs were preloaded as in *A* followed by 5 min stimulation ± DNP-HSA. Total cell lysates were subjected to Western blot analysis using anti-phospho-IκB (Ser$^{32}$) Abs (*top panel*). The blot was reprobed with anti-IκB Abs to show equal loading (*bottom panel*). *C*, SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs were preloaded as in *A* and stimulated with 50 μg/ml cycloheximide for 15 min before the addition of DNP-HSA for the indicated times. Total cell lysates were subjected to Western blot analysis using anti-IκB Abs (*top panel*). The blot was reprobed with anti-Shc Abs to show equal loading (*bottom panel*). *D*, SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs were preloaded as in *A* and stimulated ± DNP-HSA for the indicated times. Total cell lysates were subjected to Western blot analysis using anti-IκB Abs (*top panel*). The blot was reprobed with anti-FcεRI β-chain Abs to show equal loading (*bottom panel*). All blots are representative of three separate experiments.

activation is that they "cross-talk" with each other. To examine the contribution of cross-talk in IgE + Ag-induced SHIP$^{-/-}$ BMMCs, we first looked at the effect of LY294002 and wortmannin on the activation of PKB, Erk, and p38 and found that these PI-3K inhibitors reduced all three phosphorylation events (Fig. 7*A*). Thus, $PIP_3$ levels affect all three pathways in these cells. We next looked at the effect of compound 3 on these three pathways and found that it not only inhibited Erk phosphorylation, as expected (33) (Fig. 7*B*), but also inhibited PKB (Fig. 7*B*) and, to a lesser extent, p38 phosphorylation as well (Fig. 7*C*). This might be due to nonspecificity of this PKC inhibitor at this concentration (10 μM) or, at least in the case of PKB, a PI-3K-independent, PKC-mediated activation of PKB, as has been reported by Kroner et al. (47). As expected, PD98059 completely inhibited Erk1/2 phosphorylation, but had no effect on PKB (Fig. 7*B*) or p38 phosphorylation (data not shown). SB203580, at a concentration (2 μM) that completely blocked p38 phosphorylation (Fig. 7*C*), had no effect on Erk or PKB phosphorylation (data not shown). Of interest, Bay11 had no effect on Erk, PKB, or p38 phosphorylation (Fig. 7, *B* and *C*). Similar results were obtained with these inhibitors using SHIP$^{+/+}$ BMMCs (data not shown).

With this cross-talk information "in hand", we then asked if IgE + Ag-induced IκB phosphorylation or NF-κB DNA binding was affected by the inhibitors of these upstream pathways. Specifically, we examined the effects of compound 3, Bay11, PD98059, and SB203580 on IκB phosphorylation. As seen in Fig. 7*D* (*top panel*), IκB phosphorylation was markedly inhibited by Bay11, as expected, and by compound 3, but was only slightly inhibited by PD98059 and not at all by SB203580. Reprobing with anti-FcεRI β-chain Abs demonstrated equal loading (Fig. 7*D*, *bottom panel*). Because the concentration of PD98059 used in this study totally abrogated Erk phosphorylation while that of compound 3 only partially inhibited Erk phosphorylation (Fig. 7*B*, *top two panels*), this indicated that the PKC-mediated phosphorylation of IκB likely occurred independent of the Erk pathway. EMSAs of nuclear extracts confirmed and extended these findings by showing that compound 3 as well as LY294002, but neither PD98059 nor SB203580, inhibited NF-κB DNA binding (Fig. 7*E*). The numbers below each lane represent relative band intensities, determined by densitometry.

As mentioned earlier, NF-κB activity can also be regulated at the transactivation step and one of the players involved in this regulation is p38 (34, 48). Therefore, we tested the effects of SB203580, as well as PD98059, compound 3, LY294002, and Bay11 in NF-κB-luciferase assays and found that they all totally abrogated the IgE + Ag-induced increase in luciferase activity (Fig. 7*F*). These results suggested that the p38 and Erk pathways, unlike the PKC and PI-3K pathways, enhance NF-κB activity via increasing NF-κB transactivation independent of IκB degradation and NF-κB DNA binding.

## Discussion

We demonstrate in this study that SHIP negatively regulates IgE + Ag-induced production of proinflammatory cytokines in BMMCs. Focusing on IL-6 production, we show that this repression is dependent on the phosphatase activity of SHIP and thus, most likely, on restraining PI-3K-induced $PIP_3$ levels. This is consistent with our finding that the PI-3K inhibitors, LY294002 and wortmannin, not only block IL-6 production but also inhibit the upstream pathways that we found were both elevated in SHIP$^{-/-}$ BMMCs and

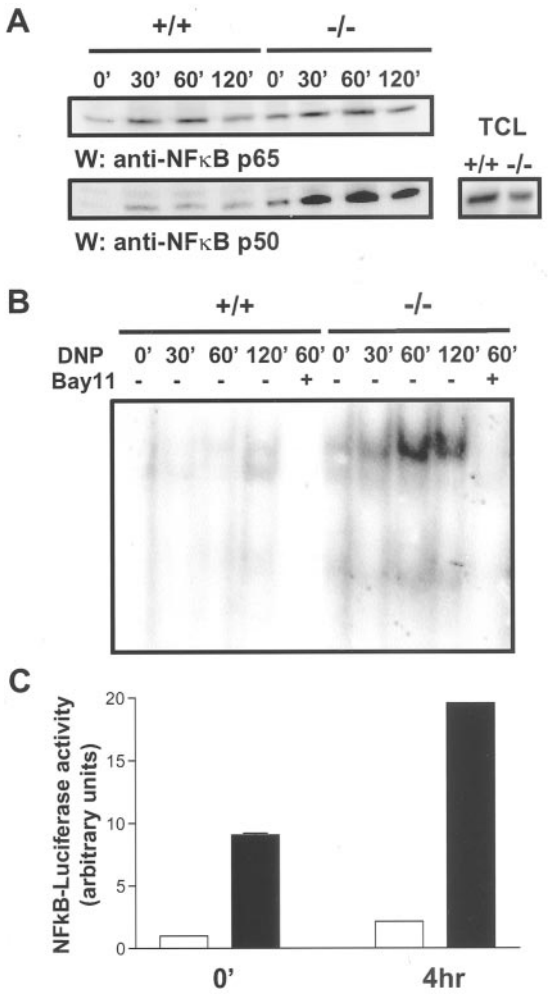


**FIGURE 6.** SHIP negatively regulates IgE + Ag-induced NF-κB DNA binding and transactivation. *A*, Nuclear extracts (5 μg protein/lane) from SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs, preloaded with 5 μg/ml IgE for 4 h then stimulated ± 20 ng/ml DNP-HSA for the indicated times, were subjected to Western blot analysis using anti-p65 (*left top panel*) and anti-p50 NF-κB Abs (*left bottom panel*). Total cell lysates from SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs demonstrated equal levels of p50 (*right panel*). These blots are representative of three separate experiments. *B*, Nuclear extracts from SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs, preloaded as in *A* and stimulated ± DNP-HSA for the indicated times ± 20 μM Bay11, were incubated for 30 min at 23°C with a radiolabeled oligonucleotide probe that contained a NF-κB DNA binding site. Protein/DNA complexes were resolved on 5% polyacrylamide gels and visualized by autoradiography. This autoradiogram is representative of two separate experiments. *C*, SHIP$^{+/+}$ (□) and SHIP$^{-/-}$ (■) BMMCs were electroporated with pNF-κB-Luc (*firefly* luciferase) and pRL-TK (*Renilla* luciferase) constructs, preloaded overnight at 1 μg/ml IgE and stimulated ± DNP-HSA for 4 h. NF-κB activity was determined using a luminometer and normalized to *Renilla* luciferase activity. Results shown are the mean ± SEM of six determinations.

contributed to IL-6 production. These results contrast with a previous study in which wortmannin failed to inhibit IgE + Ag-induced IL-6 production in BMMCs (49) and also contrast with a report that PI-3K may even inhibit IL-1-induced IL-6 production in myeloid cells (50).

To address how SHIP regulates IL-6 production, we honed in on the regulation of IL-6 mRNA synthesis because IL-6 mRNA levels correlated nicely with secreted IL-6 protein levels. Narrowing our window of investigation even further, we focused exclusively on NF-κB activity because this transcription factor has been shown to be a major positive regulator of IgE + Ag-induced IL-6 mRNA synthesis in BMMCs (19). However, we fully appreciate that SHIP may also regulate the activity of other transcription factors involved in IL-6 mRNA synthesis (37) and/or the secretion of the IL-6 protein from these cells (51, 52). As expected, the NF-κB inhibitor, Bay11, blocked IL-6 production in both SHIP$^{+/+}$ and SHIP$^{-/-}$ BMMCs. As a test of the specificity of this inhibitor, we checked its effect on IgE + Ag-induced IL-4 synthesis, since the IL-4 promoter does not possess a κB element (19, 53), and found no inhibition (assessed by RPA and ELISA, data not shown). As a point of interest, we also found that Bay11 had no effect on IL-5 production (assessed by RPA, data not shown) as expected (19), but was capable of inhibiting IL-13 and TNF-α production (assessed by RPA and ELISA, data not shown) (53, 54).

As to how SHIP negatively regulates NF-κB activity, we examined four major levels of NF-κB regulation: IκB phosphorylation/degradation, NF-κB translocation to the nucleus, DNA binding, and transactivation. Our finding that IκB is phosphorylated to a greater extent in SHIP$^{-/-}$ BMMCs is consistent with several recent reports showing that PKB acts as a positive regulator of NF-κB activity by transiently binding and phosphorylating/activating IKK (34, 54–60). Relevant to our finding that IκB degradation is increased in SHIP$^{-/-}$ BMMCs in the presence of cycloheximide, Koul et al. (41) recently reported that the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which also hydrolyses $PIP_3$, inhibits NF-κB DNA binding without affecting IκB degradation. However, they did not examine IκB degradation in the presence of cycloheximide. Gustin et al. (61), in contrast, found, in support of our findings, that PTEN inhibits the activation of IKK and phosphorylation of IκB in response to TNF, but they did not examine IκB degradation.

Interestingly, we observe an increased translocation of the p50, but not the p65, subunit of NF-κB into the nucleus of IgE + Ag-stimulated SHIP$^{-/-}$ BMMCS, as assessed by Western blot analysis of nuclear extracts. Using EMSAs, we show that SHIP negatively regulates NF-κB DNA binding, though this may be explained entirely by the reduced nuclear translocation of p50 in the presence of SHIP. As well, our finding that NF-κB DNA binding is inhibited by LY294002 (confirming the work of Gustin et al. (61)) strengthens the hypothesis that SHIP represses IL-6 production via its hydrolysis of $PIP_3$. Related to this, the 3′-phosphatase, PTEN, has also been shown to negatively regulate NF-κB DNA binding (41, 61).

To gain some insight into the SHIP-regulated upstream pathways that modulate IL-6 mRNA levels in BMMCs and to delineate the contribution of each of these pathways to NF-κB activation, we used specific inhibitors to pathways that were elevated in the absence of SHIP. As mentioned earlier, the elevated PKB activity in SHIP$^{-/-}$ BMMCs likely contributes to the increased NF-κB activity in these cells by directly phosphorylating/activating IKK. Complicating the picture, the $PIP_3$-dependent PDK1, besides playing a critical role in activating PKB, has also been shown to phosphorylate/activate various PKC isoforms (27–29). Related to this, many PKC isoforms, such as α, ε, θ, and ξ, have been shown to positively regulate NF-κB activity in a variety of cell types (36, 62, 63) and the classical PKC isoform, PKCβ, is known to be a positive regulator of IgE + Ag-induced mast cell degranulation and IL-6 production (38). In this study, we show that the novel PKC isoform, PKCδ, also acts as a positive regulator of IL-6 production. As to how PKC isoforms regulate IL-6 production, we show that PKC isoforms regulate IκB phosphorylation/degradation, and thus subsequent NF-κB DNA binding and transactivation, a finding

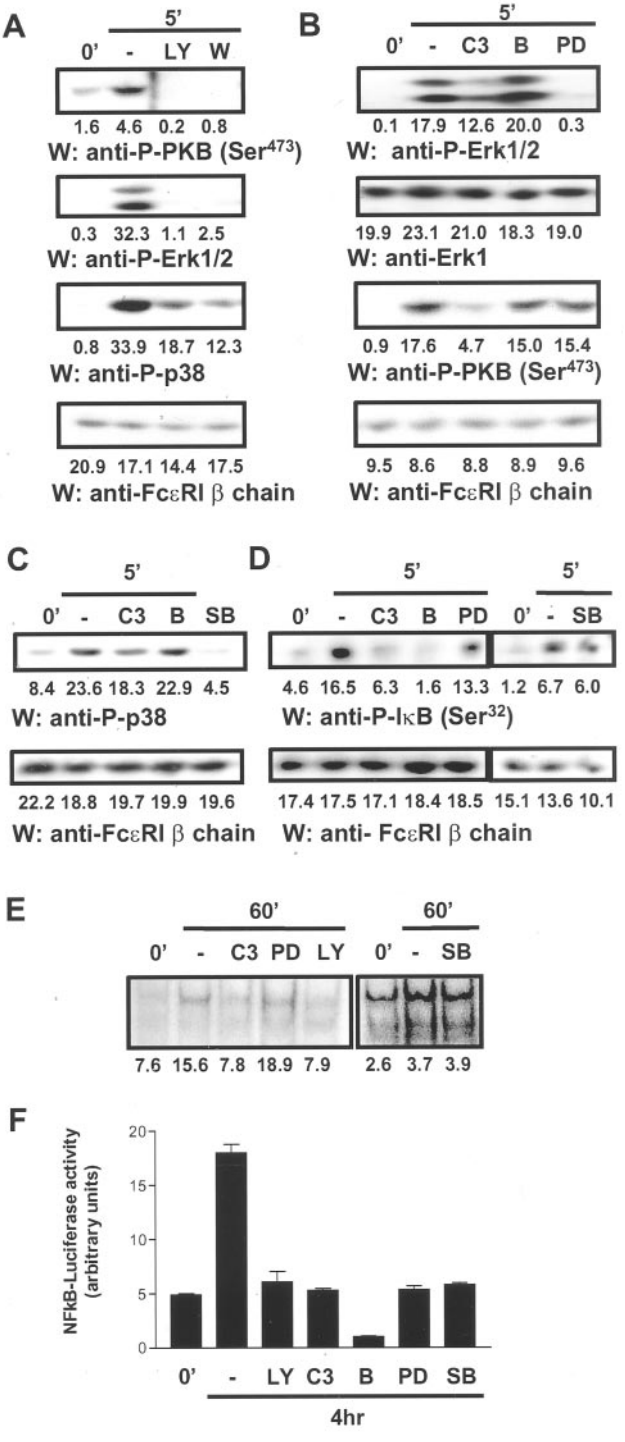


**FIGURE 7.** PI-3K/PKB and PKC enhance IκB phosphorylation/degradation, NF-κB DNA binding, and transactivation, while Erk and p38 only stimulate NF-κB transactivation. *A*, SHIP$^{-/-}$ BMMCs were preloaded with 5 μg/ml IgE for 4 h, then stimulated ± 20 ng/ml DNP-HSA for 5 min in the absence (-) or presence of 25 μM LY294002 (LY) or 25 nM wortmannin (W) added 15 min before DNP-HSA. Total cell lysates were subjected to Western blot analysis using anti-phospho-PKB (Ser$^{473}$) (*top panel*), anti-phospho-Erk1/2 (*second panel*), and anti-phospho-p38 (*third panel*) Abs. The blot was reprobed with anti-FcεRI β-chain Abs to show equal loading. *B* and *C*, SHIP$^{-/-}$ BMMCs were preloaded and stimulated as in *A* in the absence (-) or presence of 10 μM compound 3 (C3), 20 μM Bay11 (B), 50 μM PD98059 (PD), or 2 μM SB203580 (SB). Total cell lysates were subjected to Western blot analysis using (*B*) anti-phospho-Erk1/2 Abs, reprobed with anti-Erk1 Abs (*top panels*) or anti-phospho-PKB (Ser$^{473}$) Abs, then reprobed with anti-FcεRI β-chain Abs (*bottom panels*) or (*C*) anti-phospho-p38 Abs, and then reprobed with anti-FcεRI β-chain Abs. *D*, SHIP$^{-/-}$ BMMCs were stimulated as in *B* and *C* and analyzed using anti-phospho-IκB (Ser$^{32}$) Abs. The blot was reprobed with anti-FcεRI β-chain Abs to show equal loading. *E*, Nuclear extracts from SHIP$^{-/-}$ BMMCs, preloaded as in *A*, were then stimulated ± DNP-HSA for 60 min in the absence (-) or presence of 10 μM C3, 50 μM PD, and 25 μM LY (*left panel*) or 2 μM SB (*right panel*) added 15 min before DNP-HSA, and were incubated for 30 min at 23°C with a radiolabeled oligonucleotide probe that contained an NF-κB DNA binding site. Protein/DNA complexes were resolved on 5% polyacrylamide gels and visualized by autoradiography. The numbers below each lane represent band intensities determined by densitometry. The blots shown in *A–D* and the autoradiogram shown in *E* are representative of at least three separate experiments. *F*, SHIP$^{-/-}$ BMMCs were electroporated with pNF-κB-Luc (*firefly* luciferase) and pRL-TK (*Renilla* luciferase) constructs, preloaded overnight at 1 μg/ml IgE and stimulated ± DNP-HSA for 4 h in the absence (-) or presence of 25 μM LY, 10 μM C3, 20 μM B, 25 μM PD, or 2 μM SB added 15 min before the addition of DNP-HSA. NF-κB activity was determined using a luminometer and normalized to *Renilla* luciferase activity. Results shown in *F* are the mean ± SEM of four determinations.

supported by Lallena et al. (62) who showed that PKCα and atypical PKC isoforms bind to IKKs in vitro and in vivo.

NF-κB activity can also be regulated at the transactivation step and several recent reports support our finding that p38 is capable of up-regulating NF-κB transactivation without affecting IκB degradation, NF-κB nuclear translocation, or NF-κB DNA binding (34, 36, 48, 64). As to how p38 is activated, Madrid et al. (34) found that PKB mediates IL-1-induced activation of NF-κB by activating p38 in an IKK-dependent manner. However, we found that Bay11, which irreversibly inhibits IKK and the subsequent phosphorylation of IκB, had no effect on p38 phosphorylation in response to IgE + Ag. We thus propose that IKK and p38 enhance NF-κB activity by separate pathways; this is supported by studies showing that PKCδ regulates parallel IKK and p38 pathways to enhance NF-κB activity in response to thrombin (36). Because we found that PKC inhibitors partially block IgE + Ag-induced p38 phosphorylation in BMMCs, it is conceivable that p38 activity is regulated in these cells via a PI-3K/PDK1/PKC pathway in response to IgE + Ag. Related to this, the phosphorylation of p38 and Erk are significantly reduced in PKCδ$^{-/-}$ BMMCs (data not shown) and this may contribute to the decreased IL-6 production observed in these cells.

Although p38 is a well-established regulator of NF-κB transactivation (34, 48, 65), the role of Erk in NF-κB regulation is more controversial. Our inhibitor studies suggest that IgE + Ag-induced Erk activation is highly dependent on PI-3K and PKC activation. As well, using the Mek inhibitor, PD98059, we show that Erk activity is required for NF-κB transactivation independent of IκB phosphorylation and NF-κB DNA binding. In support of our finding, Vanden Berghe et al. (65) found that PD98059 inhibited NF-κB transactivation in response to TNF-α. However, Madrid et al. (34) did not find any significant decrease in NF-κB luciferase in 3T3 cells in the presence of PD98059, albeit using very low (2 μM) inhibitor levels. As to how PD98059 inhibits the transactivation of NF-κB, Chen et al. (66) recently showed that NF-κB can be acetylated and that acetylated NF-κB p65 interacts weakly, if at all, with IκBα. Related to this, Vanden Berghe et al. (67) found that both p38 and Erk promoted the acetylation capacity of the enhanceosome and thus stimulated TNF-induced, NF-κB-mediated, *IL-6* gene expression.

Although we also found that SHIP acts as a negative regulator of IgE + Ag-induced JNK phosphorylation, we were unable to study the role of this MAPK family member in the regulation of NF-κB activity and IL-6 production due to the lack of JNK-specific inhibitors. However, it has been reported that the $PIP_3$-dependent tyrosine phosphorylation of the PH-containing Rac guanine nucleotide exchange factor,

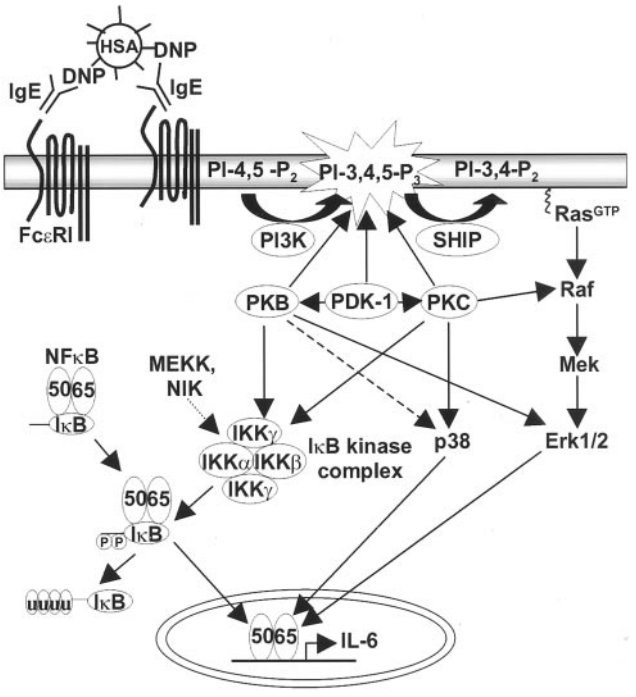


**FIGURE 8.** A model of IgE + Ag-induced IL-6 mRNA synthesis. PKB and PKC enhance IKK-mediated phosphorylation/degradation of IκB and the nuclear translocation/DNA binding of NF-κB, while p38 and Erk, which are activated by PKB and PKC, enhance NF-κB transactivation without increasing IκB phosphorylation or NF-κB DNA binding.

Vav, stimulates IL-6 production in mast cells by a Rac/JNK-dependent pathway, and we observe increased membrane recruitment and phosphorylation of Vav in SHIP$^{-/-}$ BMMCs (M. Ware and G. Krystal, unpublished observations).

Taken together our results suggest a model, shown in Fig. 8, in which SHIP represses IL-6 production in BMMCs, at least in part, by reducing PI-3K-generated $PIP_3$ levels induced by IgE + Ag. This in turn inhibits PKB- and PKC-mediated IκB phosphorylation/degradation and the nuclear translocation and DNA binding of released NF-κB, as well as p38- and Erk-mediated NF-κB transactivation. Thus, these upstream pathways which synergize to stimulate IL-6 mRNA synthesis, are all negatively regulated by SHIP.

## Acknowledgments

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