

## SIP/SHIP Inhibits *Xenopus* Oocyte Maturation Induced by Insulin and Phosphatidylinositol 3-Kinase

MAJA DEUTER-REINHARD, GERALD APELL, DAVID POT, ANKE KLIPPEL, LEWIS T. WILLIAMS,  
AND W. MICHAEL KAVANAUGH\*

*Chiron Corporation, Emeryville, California 94608*

Received 12 December 1996/Returned for modification 17 January 1997/Accepted 11 February 1997

**SIP (signaling inositol phosphatase) or SHIP (SH2-containing inositol phosphatase) is a recently identified SH2 domain-containing protein which has been implicated as an important signaling molecule. SIP/SHIP becomes tyrosine phosphorylated and binds the phosphotyrosine-binding domain of SHC in response to activation of hematopoietic cells. The signaling pathways and biological responses that may be regulated by SIP have not been demonstrated. SIP is a phosphatidylinositol- and inositol-polyphosphate 5-phosphatase with specificity in vitro for substrates phosphorylated at the 3' position. Phosphatidylinositol 3'-kinase (PI 3-kinase) is an enzyme which is involved in mitogenic signaling and whose phosphorylated lipid products are predicted to be substrates for SIP. We tested the hypothesis that SIP can modulate signaling by PI 3-kinase in vivo by injecting SIP cRNAs into *Xenopus* oocytes. SIP inhibited germinal vesicle breakdown (GVBD) induced by expression of a constitutively activated form of PI 3-kinase (p110\*) and blocked GVBD induced by insulin. SIP had no effect on progesterone-induced GVBD. Catalytically inactive SIP had little effect on insulin- or PI 3-kinase-induced GVBD. Expression of SIP, but not catalytically inactive SIP, also blocked insulin-induced mitogen-activated protein kinase phosphorylation in oocytes. SIP specifically and markedly reduced the level of phosphatidylinositol (3,4,5) triphosphate [PtdIns(3,4,5)P<sub>3</sub>] generated in oocytes in response to insulin. These results demonstrate that a member of the phosphatidylinositol polyphosphate 5-phosphatase family can inhibit signaling in vivo. Further, our data suggest that the generation of PtdIns(3,4,5)P<sub>3</sub> by PI 3-kinase is necessary for insulin-induced GVBD in *Xenopus* oocytes.**

Activation of phosphatidylinositol 3'-kinase (PI 3-kinase) by growth factors and oncogenes has been implicated as a critical step in mitogenic signaling and cellular transformation (for reviews, see references 5, 22 and 38). PI 3-kinase consists of 85- and 110-kDa subunits which associate with receptor tyrosine kinases and intracellular signaling molecules in response to treatment with growth factors or in transformed cells. Blockade of PI 3-kinase function either by mutagenesis or with pharmacological inhibitors prevents mitogenic signaling. Further, two products of PI 3-kinase, phosphatidylinositol (3,4,5) triphosphate [PtdIns(3,4,5)P<sub>3</sub>] and phosphatidylinositol (3,4) bisphosphate [PtdIns(3,4)P<sub>2</sub>], increase in cells treated with mitogenic stimuli (2, 17, 28). The products of PI 3-kinase are presumed to act as second messengers or as regulators of protein-protein interactions. The regulation of PI 3-kinase activity during signaling is less well studied. Changes in subcellular localization, in phosphorylation state and in conformation of the enzyme have been suggested to contribute to activation (3, 6, 7, 24, 28), but little is known about how PI 3-kinase might be downregulated.

Recently, we and others identified a novel, SH2 domain-containing protein, known as SIP (signaling inositol phosphatase) or SHIP (SH2-containing inositol phosphatase), which is likely to be an important signaling molecule but whose function in vivo is unknown (11, 25, 29). SIP/SHIP is tyrosine phosphorylated and binds the phosphotyrosine-binding (PTB) domain of SHC in a variety of activated hematopoietic cell lines but not in quiescent cells. SIP also contains proline-rich motifs which bind to the SH3 domains of GRB2 (25). SIP is a

member of the inositol polyphosphate 5-phosphatase family which hydrolyzes the 5' phosphate from both inositol- and phosphatidylinositol-polyphosphates. SIP has a striking substrate specificity in vitro for those inositol and phosphatidylinositols which are also phosphorylated at the 3' position. Therefore, a known product of PI 3-kinase in vivo, PtdIns(3,4,5)P<sub>3</sub>, would be predicted to be a substrate for the enzymatic activity of SIP. Additional members of the inositol polyphosphate 5-phosphatase family that are specific for PtdIns(3,4,5)P<sub>3</sub> and form signal transduction complexes in cells have been recently identified (19, 30). Thus, hydrolysis of PI 3-kinase products by phosphatidylinositol polyphosphate 5-phosphatases may be a general mechanism for the regulation of PI 3-kinase effects.

It is unknown whether hydrolysis of PtdIns(3,4,5)P<sub>3</sub> by phosphatidylinositol polyphosphate 5-phosphatases would potentiate or inhibit PI 3-kinase effects. SIP hydrolyzes PtdIns(3,4,5)P<sub>3</sub> to PtdIns(3,4)P<sub>2</sub> in vitro; both are produced in vivo upon activation of PI 3-kinase. These two products may activate the same or different signaling pathways, or one may be active and the other inactive. Both PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub> can activate certain isoforms of protein kinase C in vitro (35, 40) and can stimulate pleckstrin phosphorylation in permeabilized platelets (39, 43). However, PtdIns(3,4)P<sub>2</sub> can activate the Akt kinase in vitro, but PtdIns(3,4,5)P<sub>3</sub> has no effect (14, 27). Conversely, PtdIns(3,4,5)P<sub>3</sub> can interact with SH2 domains, but PtdIns(3,4)P<sub>2</sub> does not (37). Therefore, the predicted effect of phosphatidylinositol polyphosphate 5-phosphatase activity on PI 3-kinase signaling is unclear.

We tested the hypothesis that phosphatidylinositol polyphosphate 5-phosphatases like SIP/SHIP may regulate PI 3-kinase activity in vivo by injecting SIP cRNAs into *Xenopus* oocytes. We show that SIP inhibits germinal vesicle breakdown (GVBD) induced by a constitutively activated PI 3-kinase.

\* Corresponding author. Mailing address: Chiron Corporation, 4560 Horton St., M352, Emeryville, CA 94608. Phone: (510) 923-4042. Fax: (510) 923-4115. E-mail: mike\_kavanaugh@cc.chiron.com.

Further, we show that SIP blocks insulin-induced GVBD and phosphorylation of mitogen-activated protein (MAP) kinase, processes which are thought to be mediated by PI 3-kinase in oocytes. Finally, we show directly that SIP reduces levels of PtdIns(3,4,5)P<sub>3</sub> produced in vivo.

#### MATERIALS AND METHODS

**Analysis of proteins expressed in COS cells.** Wild-type human SIP (amino acids 1 to 1188, as numbered in GenBank accession number U57650) tagged with the influenza virus hemagglutinin (HA) epitope at the NH<sub>2</sub> terminus was cloned into the mammalian expression vector pCG (26). Catalytically inactive SIP was generated by deleting amino acid residues 666 to 680 (NLPSWCDRVLWKSYP) within the presumed inositol phosphatase catalytic domain (SIPΔIP) (20, 21, 25) or by substituting an alanine for aspartic acid 672 (D672A SIP) (20). Constructs were expressed by transient transfection in COS 6M cells (15). Forty-eight hours after transfection, the cells were lysed in lysis buffer (20 mM Tris-HCl [pH 8.0] 137 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM sodium orthovanadate, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM EGTA, 1.5 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride, 0.15 U of aprotinin per ml, 20 μM leupeptin) and clarified by centrifugation (15,000 × g, 4°C, 10 min). SIPs were immunoprecipitated with anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim), the pellets were washed, and the immunoprecipitates were analyzed for the ability to hydrolyze inositol (1,3,4,5) tetraphosphate [Ins(1,3,4,5)P<sub>4</sub>] as previously described (25). Aliquots of lysate were also immunoblotted with anti-SIP antiserum 8727 (25) to determine protein expression.

**Xenopus oocytes.** *Xenopus laevis* frogs were maintained as previously described (42). In some experiments, frogs were injected with 25 to 50 IU of pregnant mare serum gonadotropin (PMSG) (Calbiochem) on the day prior to oocyte harvesting. Oocytes were surgically harvested as described elsewhere (42) and manually defolliculated under a dissecting microscope. Stage VI oocytes were selected as described previously (12) and maintained in MRS (modified Ringer's solution; 100 mM NaCl, 4 mM NaHCO<sub>3</sub>, 1.8 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mg of bovine serum albumin per ml) at 18 to 20°C. Small groups of oocytes were tested for insulin responsiveness by incubation in MRS containing 8 μM insulin for 4 to 8 h at 22 to 25°C. If >60% GVBD in response to insulin was observed, fresh oocytes from the same harvest were used for subsequent experiments.

**cRNAs and protein expression.** Wild-type SIP and SIPΔIP (see above) were cloned into the pSp vector, and cRNA was generated by *in vitro* transcription as described previously (33). SIP containing an inactivating point mutation in the SH2 domain (SIPΔSH2) was generated by changing the codon for arginine 31 within the FLVRES motif to a leucine. Constitutively active, farnesylated PI 3-kinase (p110\*) was constructed with a Myc epitope tag, and cRNA was generated as described previously (18, 28). Microinjection of cRNAs into oocytes was performed in MRS essentially as described previously (33). Twenty-five to 50 nl of distilled H<sub>2</sub>O containing 25 to 50 ng of each cRNA or H<sub>2</sub>O alone as a control was injected into groups of 20 to 60 oocytes. The oocytes were then incubated at 18 to 25°C in MRS for 3 to 16 h to allow for translation of the cRNA into protein. The oocytes were then treated with 8 μM insulin (in 50 mM HCl neutralized with an equal volume of 0.5 M NaHCO<sub>3</sub>), progesterone (5 μg/ml), or vehicle for 8 to 16 h at 18°C and scored for GVBD. Each experiment was repeated at least three times. For analysis of SIP and p110\* protein expression, equal numbers of oocytes were lysed in 10 μl of lysis buffer per oocyte by repeated pipetting, and lysates were clarified by centrifugation as described above. Equal volumes of lysates representing equal numbers of oocytes were immunoprecipitated and immunoblotted with anti-SIP serum 8727 (23) or immunoprecipitated with anti-Myc antibodies and immunoblotted with anti-p110 antibodies as described previously (28).

**MAP kinase phosphorylation.** Oocytes were lysed at 8 to 16 h after insulin treatment by repeated pipetting in 10 μl of lysis buffer per oocyte and clarified by centrifugation as described above. Aliquots of lysate containing equal amounts of total protein and representing equal numbers of oocytes were analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and immunoblotted with polyclonal antibodies specific for the phosphorylated form of MAP kinase (New England Biolabs). Immunoblots were then reprobed with polyclonal anti-MAP kinase antibodies which recognize total MAP kinase, as a control for gel loading.

**Analysis of PI 3-kinase products in vivo.** Oocytes were labeled with [<sup>32</sup>P]orthophosphate, and phospholipids were extracted and analyzed by thin-layer chromatography and high-pressure liquid chromatography (HPLC) essentially as described previously (28, 31). Briefly, groups of 20 to 25 oocytes were injected with H<sub>2</sub>O or with 50 ng of SIP cRNA, incubated for 2 to 16 h at 18 to 25°C in MRS, and then incubated for an additional 3 h at room temperature in 1 ml of MRS containing 1 mCi of [<sup>32</sup>P]orthophosphate (9,120 Ci/mmol; NEN). Oocytes were then washed three times in 1 ml of MRS and stimulated with 8 μM insulin in 0.5 ml MRS for 10 min at room temperature. The oocytes were placed on ice, the buffer was aspirated, and the oocytes were lysed in 750 μl of 1:1 (vol/vol) methanol–1 N HCl with vigorous vortexing and repeated pipetting. Chloroform (380 μl) was then added, and the mixture was vortexed for an additional 15 min to extract lipids. The extracts were then centrifuged (15,000 × g, 2 min), the lower, chloroform phase was removed to a new tube, the interface

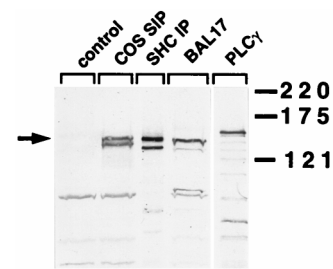


FIG. 1. The full-length form of SIP is 135 kDa. SIP (1,188 residues) was expressed in COS cells as described in Materials and Methods. SHC was immunoprecipitated from activated BAL17 B cells as previously described (25). Untransfected COS cell lysates (control), COS cell lysates containing recombinant SIP (COS SIP), SHC immunoprecipitates (SHC IP), and BAL17 B cell lysates (BAL17) were analyzed by sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis and immunoblotted with anti-SIP antiserum 8727. As a standard for a 145-kDa protein, phospholipase C-γ was immunoprecipitated from BAL17 lysates, analyzed on the same gel, and immunoblotted with monoclonal anti-PLC-γ antibodies (PLCγ). The positions of the largest endogenous form of SIP in B cells and of recombinant SIP in COS cells are shown by the arrow; both are often seen as doublets (see text). Positions of molecular weight standards are indicated in kilodaltons.

material and aqueous layer were reextracted with an equal volume of chloroform, and the organic phases were combined. The extracts were then separated by thin-layer chromatography, deacylated, and analyzed by HPLC as described previously (28, 31). PtdIns(3,4,5)P<sub>3</sub> was identified by using <sup>32</sup>P-labeled standards generated with p110\* as described previously (28).

#### RESULTS

**Characterization of the full-length form of SIP.** We previously reported that the human SIPs represent a family of molecules which are likely to be splice variants of the same gene (25). We postulated that the full-length form of SIP corresponded to a 145-kDa protein, SIP-145, based on the existence of an open reading frame without termination codons 5' to the first methionine codon in the longest SIP cDNA that we cloned (methionine 41 as numbered in reference 25; methionine 1 as numbered in GenBank accession number U57650). Subsequently, additional human SIP/SHIP cDNAs with longer 5' untranslated regions that contain upstream, in-frame stop codons and no additional in-frame methionine codons have been identified (41), suggesting that the postulated SIP-145 does not exist and that full-length SIP/SHIP corresponds to a smaller, 130- to 135-kDa protein translated beginning at methionine 41 in our cDNA. However, SIP protein in cells has been reported by various investigators to have an apparent molecular mass of 130, 140, 145, or 150 kDa (11, 25, 29). To better characterize the full-length form of SIP for subsequent experiments, lysates of unstimulated BAL17 B cells and anti-SHC immunoprecipitates of activated BAL17 cell lysates were immunoblotted with anti-SIP antisera. The largest protein specifically recognized by anti-SIP antisera migrated at an apparent molecular mass of approximately 135 kDa, as measured by quantitative comparison to molecular weight standards (Fig. 1), and migrated significantly faster than a known 145-kDa protein, phospholipase C-γ. This finding suggested that no 145-kDa form of SIP protein exists in these cells. Further, translation starting at methionine 41 in our cDNA would produce a protein with a predicted molecular mass of 133 kDa, which suggested that this methionine is the translation start site for the largest form of endogenous SIP. To investigate further, recombinant SIP protein encoded from methionine 41 to the termination codon (1,188 residues) was expressed in COS 6M cells. This recombinant SIP migrated at the exact same apparent molecular weight as the largest form

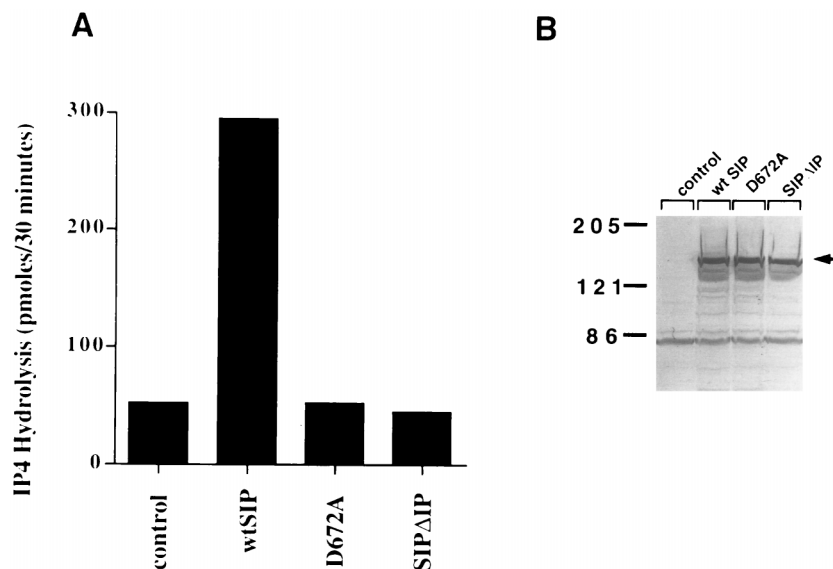


FIG. 2. Expression and activities of wild-type and mutant SIP. HA epitope-tagged wild-type SIP (wtSIP) and SIP containing a point mutation (D672A) or small deletion (SIP $\Delta$ IP) in the presumed catalytic domain were expressed in COS cells as described in Materials and Methods. (A) Anti-HA immunoprecipitates were prepared and assayed for Ins(1,3,4,5)P<sub>4</sub> hydrolyzing activity. (B) Aliquots of the same COS cell lysates were immunoblotted with anti-HA antibody to examine protein expression. SIP proteins are indicated by the arrow. Positions of molecular weight standards are indicated in kilodaltons.

of endogenous SIP present in either lysates or anti-SHC immunoprecipitates from B cells (Fig. 1). These results strongly suggest that translation of full-length endogenous SIP begins at methionine 41 in our cDNA and that full-length SIP has an apparent molecular mass of approximately 135 kDa. These results are consistent with the recently published, longer SIP/SHIP cDNA sequences (41). This form of SIP was therefore used in our experiments, and for clarity, we propose that SIP/SHIP be referred to as a 135-kDa protein in subsequent studies. Attempts to directly sequence the NH<sub>2</sub> terminus of the largest form of SIP protein purified from BAL17 B cells were unsuccessful, presumably because the NH<sub>2</sub> terminus was blocked. We have observed that both recombinant SIP expressed in COS cells and endogenous SIP from B cells is often seen as a doublet on immunoblots (Fig. 1). The significance of the lower form is unknown.

**Catalytically inactive mutant SIPs.** SIP was originally identified as an inositol- and phosphatidylinositol-polyphosphate 5-phosphatase by the presence of sequence motifs homologous to other inositol polyphosphate 5-phosphatases (11, 21, 25). These sequences are presumed to constitute part of the catalytic domain (20, 21), but mutagenesis studies of full-length SIP to demonstrate this directly have not been reported. We wished to generate a mutant SIP protein which is catalytically inactive to use as a control in subsequent experiments. We therefore generated SIP constructs with point mutations or deletions within these conserved motifs and tagged with the influenza virus HA epitope. The mutant proteins were expressed in COS cells, immunoprecipitated with anti-HA antibodies, and tested for inositol polyphosphate 5-phosphatase activity. SIP proteins with a substitution of alanine for aspartic acid 672 (D672A SIP) or with a deletion of amino acids 666 to 680 (SIP $\Delta$ IP) were expressed as well as wild-type SIP (Fig. 2B) but contained no detectable inositol polyphosphate 5-phosphatase activity (Fig. 2A). This experiment demonstrates directly that these residues are necessary for full-length SIP catalytic activity and support the hypothesis (20, 21, 25) that

the PSWCDRVL motif comprises part of the catalytic center of inositol polyphosphate 5-phosphatases.

**SIP inhibits PI 3-kinase-induced *Xenopus* oocyte maturation.** The specificity of SIP in vitro for 3'-phosphorylated phosphatidylinositols suggests that SIP may regulate signaling by PI 3-kinase. We asked whether expression of SIP protein is capable of either activating or inhibiting PI 3-kinase-dependent signaling in vivo. It was previously demonstrated that a constitutively activated form of PI 3-kinase, known as p110\*, induced GVBD in *Xenopus* oocytes in the absence of added growth factors or hormones (18). We therefore studied whether SIP modulated the effects of p110\* in oocytes by coinjecting SIP and p110\* cRNA. As seen in Fig. 3A, injection of p110\* cRNA resulted in GVBD, consistent with previously reported results (18). Injection of SIP cRNA alone did not induce GVBD (data not shown). p110\*-induced GVBD was markedly reduced by coinjection of SIP (Fig. 3A). The inhibition of PI 3-kinase signaling by SIP was not due to alterations in p110\* protein expression (Fig. 3B). Further, catalytically inactive SIP, SIP $\Delta$ IP, had significantly less effect on GVBD induced by p110\* (Fig. 3A), despite expression of SIP $\Delta$ IP protein approximately equivalent to that of wild-type SIP (Fig. 3B). These experiments demonstrate that a biological effect of PI 3-kinase, oocyte maturation, can be inhibited in vivo by the enzymatic activity of SIP.

**SIP specifically blocks insulin-induced GVBD.** Previous studies have demonstrated that insulin action on *Xenopus* oocytes requires activation of PI 3-kinase and the generation of 3'-phosphorylated phosphatidylinositols (8, 9, 16, 31). Because SIP antagonizes PI 3-kinase effects in oocytes (Fig. 3), we postulated that expression of SIP would inhibit insulin signaling in oocytes. Injection of SIP cRNA into oocytes blocked the ability of insulin to induce GVBD (Fig. 4A) in multiple experiments. This effect was not due to a nonspecific toxic effect of SIP, since similar levels of SIP protein expression (Fig. 4B) had no effect on progesterone-induced GVBD in the same experiments (Fig. 4A). Expression of SIP did not affect progester-



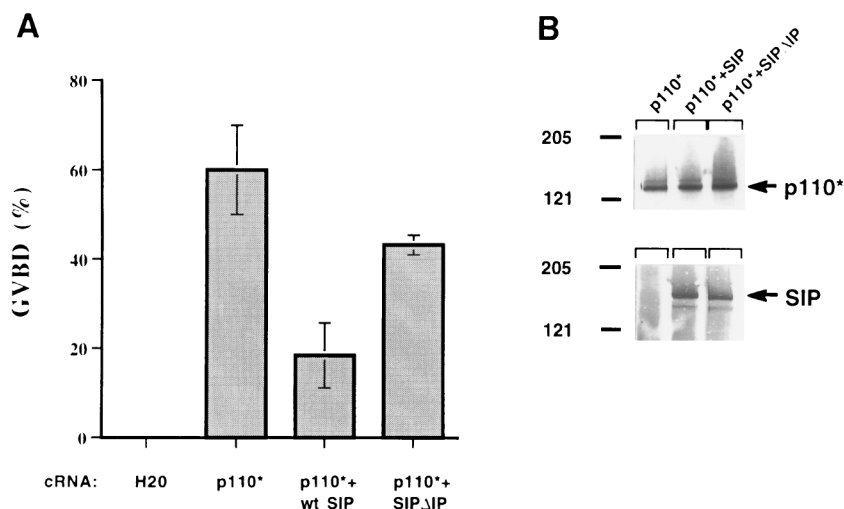


FIG. 3. Effects of SIP on GVBD induced by a constitutively activated PI 3-kinase. *Xenopus* oocytes (30 to 40 per treatment group) were injected with H<sub>2</sub>O or 25 to 50 ng of cRNA of a constitutively activated PI 3-kinase, p110\*, alone, p110\* coinjected with wild-type SIP (wt SIP), or p110\* coinjected with catalytically inactive SIP (SIP $\Delta$ IP). (A) GVBD was scored 12 to 48 h later and is expressed as percentage of the total number of oocytes injected. Results are the means of duplicate experiments  $\pm$  range. Inhibition by SIP was statistically significant ( $P < 0.05$ ); the effect of SIP $\Delta$ IP was not significant ( $P > 0.10$ ). (B) Myc epitope-tagged p110\* was immunoprecipitated from oocyte lysates with anti-Myc antibodies and immunoblotted with anti-p110 antibodies as described in Materials and Methods (top panel). SIP proteins were immunoprecipitated and immunoblotted with antiserum 8727 (bottom panel). The positions of p110\* and SIP are indicated by arrows. Positions of molecular weight standards are indicated in kilodaltons. A representative immunoblot is shown.

one-induced GVBD even when SIP protein was allowed to accumulate to high levels over 16 h prior to treating the oocytes with progesterone (data not shown). Further, the ability of SIP to inhibit insulin-induced GVBD was dependent on the enzymatic activity of SIP, since a catalytically inactive mutant SIP (SIP $\Delta$ IP) expressed at similar levels had little effect (Fig. 4).

The inability of the catalytically inactive SIP $\Delta$ IP to inhibit insulin signaling suggested that expression of the SH2 domain of SIP, which is intact in this construct, is not sufficient for inhibition of insulin effects. We also investigated whether the SH2 domain of SIP is necessary for the ability of SIP to inhibit insulin signaling in this system. We generated a mutant SIP cRNA in which the arginine within the critical FLVRES motif of the SH2 domain was changed to an alanine. Similar mutations have been demonstrated in multiple systems to eliminate the ability of SH2 domains to interact with their tyrosine-phosphorylated targets (4, 23, 32). Injection of SIP cRNA containing the inactivating SH2 domain mutation, but with an intact catalytic domain, inhibited insulin-induced GVBD almost as well as wild-type SIP (Fig. 4). This experiment demonstrates that SIP SH2 domain function is not necessary for inhibition of insulin-induced GVBD in oocytes.

**SIP blocks insulin-induced MAP kinase phosphorylation.** Previous studies have suggested that in *Xenopus* oocytes, activation of PI 3-kinase leads to activation of the Ras pathway, including activation of Ras, Raf kinase, and MAP kinase (8, 18). These results suggest that injection of SIP would block the ability of insulin to activate MAP kinase. To investigate further, oocytes were injected with SIP or mutant SIP cRNAs and treated with insulin for 18 h, and oocyte lysates were analyzed for MAP kinase phosphorylation. As expected, insulin induced MAP kinase phosphorylation (Fig. 5). Phosphorylation of MAP kinase by insulin was blocked by wild-type SIP but not by catalytically inactive SIP (Fig. 5). These results confirm biochemically the observation that SIP enzymatic activity blocks insulin effects in oocytes. Further, these data provide additional support for the hypothesis that SIP inhibits signaling by

PI 3-kinase by showing that SIP inhibits activation of a kinase thought to be downstream of PI 3-kinase in oocytes.

**Effect of SIP on PI 3-kinase products produced in vivo.** To directly determine the effects of SIP expression on the generation of PI 3-kinase products in vivo, oocytes were injected with H<sub>2</sub>O or SIP cRNA and labeled with [<sup>32</sup>P]orthophosphate. The oocytes were then stimulated with insulin for 10 min, and the lipids were extracted and analyzed by thin-layer chromatography and HPLC. Standards for PtdIns(3,4,5)P<sub>3</sub> were generated in vitro with purified p110\*. As shown in Fig. 6, levels of PtdIns(3,4,5)P<sub>3</sub> increase in oocytes treated with insulin, as has been reported previously (10, 31). Expression of SIP significantly reduces the amount of PtdIns(3,4,5)P<sub>3</sub> produced in response to insulin but did not affect levels of PtdIns(4,5)P<sub>2</sub> (Fig. 6). This finding is consistent with the in vitro activity and specificity of SIP and provides the first reported evidence that SIP is a PtdIns(3,4,5)P<sub>3</sub> 5-phosphatase in vivo as well as in vitro. Further, this experiment supports the hypothesis that SIP inhibits insulin signaling by hydrolyzing a product of PI 3-kinase.

## DISCUSSION

The identification of a family of phosphatidylinositol polyphosphate 5-phosphatases which are specific for 3'-phosphorylated substrates in vitro (11, 19, 25, 30) has suggested a novel mechanism for the regulation of PI 3-kinase signaling. However, the activity of these enzymes in vivo has not been demonstrated, nor is it known whether they would potentiate or inhibit signaling by PI 3-kinase. In this report, we have demonstrated that one member of this family, SIP/SHIP, can inhibit PI 3-kinase effects in vivo. Using a constitutively activated form of PI 3-kinase, p110\*, we showed directly that SIP inhibits oocyte maturation induced by PI 3-kinase in the absence of added growth factors or hormones (Fig. 3). Further, SIP blocks insulin-induced phosphorylation of MAP kinase (Fig. 5) and GVBD (Fig. 4), processes which are thought to be mediated by PI 3-kinase in oocytes (8–10, 18, 31). SIP did not

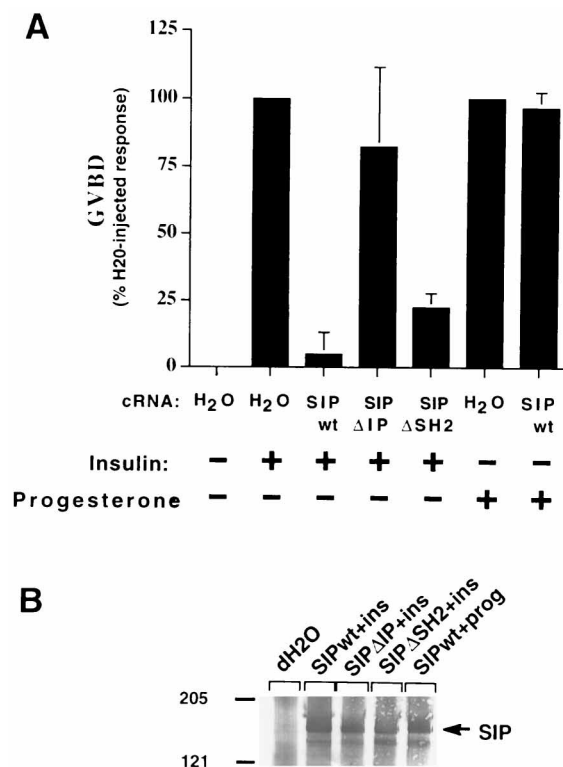


FIG. 4. SIP specifically blocks insulin-induced GVBD. (A) Groups of 20 to 60 oocytes were injected with 25 nl of H<sub>2</sub>O or H<sub>2</sub>O containing 1  $\mu$ g of each cRNA per  $\mu$ l, incubated at 20 to 25°C in MRS for 3 to 16 h, and then treated with 8  $\mu$ M insulin, 5  $\mu$ g of progesterone per ml, or vehicle as described in Materials and Methods. GVBD was scored 16 to 24 h later and is expressed as percentage of the response of insulin- or progesterone-treated, H<sub>2</sub>O-injected oocytes (mean  $\pm$  standard deviation, 2 to 10 experiments). The effects of wild-type SIP (SIP wt) and SIP $\Delta$ SH2 on insulin-induced GVBD were statistically significant ( $P < 0.001$  and  $P < 0.05$ , respectively); the effects of SIP $\Delta$ IP on insulin-induced GVBD and of wild-type SIP on progesterone-induced GVBD were not significant ( $P > 0.10$ ). (B) SIP proteins were immunoprecipitated from oocyte lysates and immunoblotted with antiserum 8727. SIP $\Delta$ SH2 is SIP with an inactivating point mutation in the SH2 domain. dH<sub>2</sub>O, distilled H<sub>2</sub>O; ins, insulin; prog, progesterone. Positions of size markers are indicated in kilodaltons. A representative immunoblot is shown.

affect GVBD induced by progesterone, demonstrating that the effect of SIP is specific. The lack of an effect of SIP on progesterone-induced GVBD is consistent with reports that progesterone-induced oocyte maturation is not inhibited by the PI 3-kinase inhibitor wortmannin and does not require PI 3-kinase activation (9, 31).

Although it is unknown whether the effects of expression of human SIP in a heterologous system such as *Xenopus* oocytes are applicable to other cells, recently published data on the function of SIP in mammalian cells support the hypothesis that SIP is an inhibitor of signaling. Ono et al have reported that SIP associates with the inhibitory Fc $\gamma$  receptor IIB subunit (Fc $\gamma$ RIIB) in B cells and mast cells (36). Coligation of this receptor with Fc $\epsilon$ RI or Fc $\gamma$ RIII on mast cells inhibits degranulation, and coligation with the antigen receptor on B cells inhibits B-cell activation. SIP/SHIP bound to a 13-amino-acid motif in the cytoplasmic domain of Fc $\gamma$ RIIB which has been shown to be necessary for inhibitory signaling by Fc $\gamma$ RIIB (1, 34). It has also been reported that expression of SIP in myeloid cells results in growth inhibition (29). Our data suggest one mechanism by which SIP/SHIP might inhibit signaling in these cells.

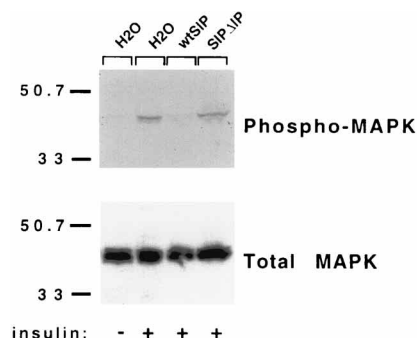


FIG. 5. SIP blocks phosphorylation of MAP kinase induced by insulin. Oocytes were injected with cRNAs, incubated, and treated with insulin for 16 h as described in the legend to Fig. 4 and in Materials and Methods. The oocytes were lysed, and clarified lysates representing equal numbers of oocytes were immunoblotted with polyclonal antibodies specific for the phosphorylated form of MAP kinase (top panel). The same immunoblot was reprobed with antibodies which recognize total MAP kinase protein, to control for loading of samples (bottom panel). wtSIP, wild-type SIP. Positions of molecular weight standards are indicated in kilodaltons. Results are representative of three independent experiments.

The ability of SIP to inhibit insulin and PI 3-kinase signaling is likely to be due to hydrolysis of a biologically active product of PI 3-kinase, PtdIns(3,4,5)P<sub>3</sub>. First, as mentioned above, SIP inhibited processes known to be dependent on PI 3-kinase activity, and we have shown directly by using p110\* that SIP inhibits PI 3-kinase effects. Second, mutant SIP proteins which were demonstrated to be catalytically inactive had little effect on signaling by p110\* (Fig. 3) or by insulin (Fig. 4), demonstrating that the enzymatic activity of SIP is necessary for maximal inhibition. Finally, we have shown directly that expression of SIP results in significant and specific decreases in PtdIns(3,4,5)P<sub>3</sub> generated in vivo in response to insulin (Fig. 6). Hydrolysis in vivo of PtdIns(3,4,5)P<sub>3</sub>, but not PtdIns(4,5)P<sub>2</sub> (Fig. 6), is consistent with the substrate specificity of SIP previously defined in vitro (11, 25). Taken together, these results suggest that generation of PtdIns(3,4,5)P<sub>3</sub> is necessary for GVBD induced by insulin and p110\* and that SIP inhibits signaling by hydrolyzing PtdIns(3,4,5)P<sub>3</sub>. One implication of

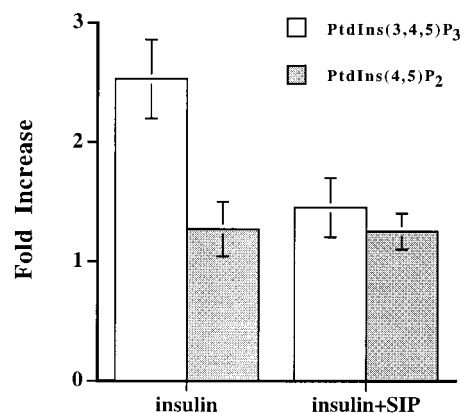


FIG. 6. Effect of SIP on PtdIns(3,4,5)P<sub>3</sub> produced in response to insulin treatment in oocytes. Oocytes were injected with H<sub>2</sub>O or 50 ng of SIP cRNA, labeled with [<sup>32</sup>P]orthophosphate for 3 h, and stimulated with insulin for 10 min, and lipids were extracted as described in Materials and Methods. Lipid extracts were separated by thin-layer chromatography, deacylated, and analyzed by HPLC. Total radioactivity was determined in the HPLC peaks comigrating with deacylated [<sup>32</sup>P]PtdIns(3,4,5)P<sub>3</sub> and [<sup>32</sup>P]PtdIns(4,5)P<sub>2</sub> standards. Results are means of duplicate, independent experiments  $\pm$  range.

these data is that although levels of both PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub> increase in oocytes in response to insulin treatment (31), PtdIns(3,4,5)P<sub>3</sub> is the PI 3-kinase product principally responsible for induction of GVBD. This hypothesis does not exclude a role for PtdIns(3,4)P<sub>2</sub> in other cellular functions; for example, it has been recently suggested that PtdIns(3,4)P<sub>2</sub> is the PI 3-kinase product principally responsible for activation of the Akt/PKB kinase in mammalian cells (13, 27). We have attempted to analyze changes in PtdIns(3,4)P<sub>2</sub> levels in oocytes expressing SIP, but results have been inconclusive. Direction injection into oocytes of either PtdIns(3,4)P<sub>2</sub> or PtdIns(3,4,5)P<sub>3</sub> generated in vitro with p110\* did not induce GVBD (data not shown). Because the amount of PtdIns(3,4,5)P<sub>3</sub> which can be generated in vitro by this method is limited (27), the dose of PtdIns(3,4,5)P<sub>3</sub> injected may have been insufficient to induce GVBD. Alternatively, a single injection of PtdIns(3,4,5)P<sub>3</sub> may not mimic the kinetics of PtdIns(3,4,5)P<sub>3</sub> generated by p110\* or in response to insulin. In vitro, SIP hydrolyzes Ins(1,3,4,5)P<sub>4</sub> as well as PtdIns(3,4,5)P<sub>3</sub>. We cannot exclude a role for hydrolysis of Ins(1,3,4,5)P<sub>4</sub> in the inhibitory action of SIP; however, the ability of SIP to inhibit GVBD induced directly by p110\* demonstrates that SIP can regulate PI 3-kinase signaling in oocytes.

The SH2 domain of SIP was neither necessary nor sufficient for inhibition of insulin signaling. These data exclude the possibility that overexpression of the SH2 domain alone accounted for the inhibitory effect of SIP. In mammalian cells, the SH2 domain is likely to participate in targeting of SIP to signaling complexes and/or to the membrane, where the products of PI 3-kinase are generated. The absence of a requirement for the SH2 domain for SIP activity in oocytes likely reflects either the effect of overexpression of SIP and/or the absence of the endogenous target of the SH2 domain in oocytes. In addition to the SH2 domain, SIP contains binding sites for the PTB domain of SHC and for the SH3 domains of GRB2. In mammalian cells, formation of signal complexes may regulate SIP activity by changing its subcellular localization or by bringing SIP into contact with regulatory molecules.

In conclusion, we have shown that a phosphatidylinositol polyphosphate 5-phosphatase can inhibit biological effects induced by PI 3-kinase and insulin. We propose that this family of enzymes can participate in the downregulation of PI 3-kinase-mediated signaling during mitogenesis, transformation, and immune cell function.

#### ACKNOWLEDGMENTS

We are grateful to Michael Wu for expert assistance and advice and to A. B. Jefferson and Hui Cen.

#### REFERENCES

- Amigorena, S., C. Bonnerot, J. R. Drake, D. Choquet, W. Hunziker, J. G. Guillet, P. Webster, C. Sautes, I. Mellman, and W. H. Fridman. 1992. Cytoplasmic domain heterogeneity and functions of IgG Fc receptors in B lymphocytes. *Science* **256**:1808–1812.
- Auger, K. R., L. A. Serunian, S. P. Soltoff, P. Libby, and L. C. Cantley. 1989. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell* **57**:167–175.
- Backer, J. M., M. G. Myers, Jr., S. E. Shoelson, D. J. Chin, X. J. Sun, M. Miralpeix, P. Hu, B. Margolis, E. Y. Skolnik, J. Schlessinger, and M. F. White. 1992. Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J.* **11**:3469–3479.
- Bibbins, K. B., H. Boeuf, and H. E. Varmus. 1993. Binding of the Src SH2 domain to phosphopeptides is determined by residues in both the SH2 domain and the phosphopeptides. *Mol. Cell. Biol.* **13**:7278–7287.
- Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff. 1991. Oncogenes and signal transduction. *Cell* **64**:281–302.
- Carpenter, C. L., K. R. Auger, M. Chanudhuri, M. Yoakim, B. Schaffhausen, S. Shoelson, and L. C. Cantley. 1993. Phosphoinositide 3-kinase is activated by phosphopeptides that bind to the SH2 domains of the 85-kDa subunit. *J. Biol. Chem.* **268**:9478–9483.
- Carpenter, C. L., K. R. Auger, B. C. Duckworth, W. M. Huo, B. Schaffhausen, and L. C. Cantley. 1993. A tightly associated serine/threonine protein kinase regulates phosphoinositide 3-kinase activity. *Mol. Cell. Biol.* **13**:1657–1665.
- Chuang, L. M., S. F. Hausdorff, M. G. Myers, Jr., M. F. White, M. J. Birnbaum, and C. R. Kahn. 1994. Interactive roles of Ras, insulin receptor substrate-1, and proteins with Src homology-2 domains in insulin signaling in *Xenopus* oocytes. *J. Biol. Chem.* **269**:27645–27649.
- Chuang, L. M., M. G. Myers, Jr., J. M. Backer, S. E. Shoelson, M. F. White, M. J. Birnbaum, and C. R. Kahn. 1993. Insulin-stimulated oocyte maturation requires insulin receptor substrate 1 and interaction with the SH2 domains of phosphatidylinositol 3-kinase. *Mol. Cell. Biol.* **13**:6653–6660.
- Cummings, C., L. Zhu, A. Sorisky, and X. J. Liu. 1996. A peroxovanadium compound induces *Xenopus* oocyte maturation: inhibition by a neutralizing anti-insulin receptor antibody. *Dev. Biol.* **175**:338–346.
- Damen, J. E., L. Liu, P. Rosten, R. K. Humphries, A. B. Jefferson, P. W. Majerus, and G. Krystal. 1996. The 145 kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase. *Proc. Natl. Acad. Sci. USA* **93**:1689–1693.
- Dumont, J. N. 1971. Oogenesis in *Xenopus laevis*. *J. Morphol.* **136**:153–180.
- Franke, T. F., D. R. Kaplan, L. C. Cantley, and A. Toker. 1997. Direct Regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* **275**:665.
- Franke, T. F., S. I. Yang, T. O. Chan, K. Datta, A. Kazlauskas, D. K. Morrison, D. R. Kaplan, and P. N. Tsichlis. 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81**:727–736.
- Gorman, C. 1985. High efficiency gene transfer into mammalian cells, p. 143–190. *In* D. M. Glover (ed.), *DNA cloning, a practical approach*, vol. 2. IRL Press, Oxford, England.
- Gould, G. W., T. J. Jess, G. C. Andrews, J. J. Herbst, R. J. Plevin, and E. M. Gibbs. 1994. Evidence for a role of phosphatidylinositol 3-kinase in the regulation of glucose transport in *Xenopus* oocytes. *J. Biol. Chem.* **269**:26622–26625.
- Hawkins, P. T., T. R. Jackson, and L. R. Stephens. 1992. Platelet-derived growth factor stimulates synthesis of PtdIns(3,4,5)P<sub>3</sub> by activating a PtdIns(4,5)P<sub>2</sub> 3-OH kinase. *Nature* **358**:157–159.
- Hu, Q., A. Klippel, A. J. Muslin, W. J. Fantl, and L. T. Williams. 1995. Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. *Science* **268**:100–102.
- Jackson, S. P., S. M. Schoenwaelder, M. Matzaris, S. Brown, and C. A. Mitchell. 1995. Phosphatidylinositol 3,4,5-trisphosphate is a substrate for the 75 kDa inositol polyphosphate 5-phosphatase and a novel 5-phosphatase which forms a complex with the p85/p110 form of phosphoinositide 3-kinase. *EMBO J.* **14**:4490–4500.
- Jefferson, A. B., and P. W. Majerus. 1996. Mutation of the conserved domains of two inositol polyphosphate 5-phosphatases. *Biochemistry* **35**:7890–7894.
- Jefferson, A. B., and P. W. Majerus. 1995. Properties of type II inositol polyphosphate 5-phosphatase. *J. Biol. Chem.* **270**:9370–9377.
- Kapeller, R., and L. C. Cantley. 1994. Phosphatidylinositol 3-kinase. *Bioessays* **16**:565–576.
- Katzav, S. 1993. Single point mutations in the SH2 domain impair the transforming potential of vav and fail to activate proto-vav. *Oncogene* **8**:1757–1763.
- Kavanaugh, W. M., A. Klippel, J. A. Escobedo, and L. T. Williams. 1992. Modification of the 85-kilodalton subunit of phosphatidylinositol-3 kinase in platelet-derived growth factor-stimulated cells. *Mol. Cell. Biol.* **12**:3415–3424.
- Kavanaugh, W. M., D. A. Pot, S. M. Chin, M. Deuter-Reinhard, A. B. Jefferson, F. A. Norris, F. R. Masiarz, L. S. Cousens, P. W. Majerus, and L. T. Williams. 1996. Multiple forms of an inositol polyphosphate 5-phosphatase form signaling complexes with SHC and GRB2. *Curr. Biol.* **6**:438–445.
- Klippel, A., J. A. Escobedo, M. Hirano, and L. T. Williams. 1994. The interaction of small domains between the subunits of phosphatidylinositol 3-kinase determines enzyme activity. *Mol. Cell. Biol.* **14**:2675–2685.
- Klippel, A., W. M. Kavanaugh, D. Pot, and L. T. Williams. 1997. A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. *Mol. Cell. Biol.* **17**:338–344.
- Klippel, A., C. Reinhard, W. M. Kavanaugh, G. Apell, M.-A. Escobedo, and L. T. Williams. 1996. Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. *Mol. Cell. Biol.* **16**:4117–4127.
- Lioubin, M. N., P. A. Algate, S. Tsai, K. Carlberg, R. Aebersold, and L. R. Rohrschneider. 1996. p150ship, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. *Genes Dev.* **10**:1084–1095.
- Liu, L., A. B. Jefferson, X. Zhang, F. A. Norris, P. W. Majerus, and G. Krystal. 1996. A novel phosphatidylinositol 3,4,5 trisphosphate 5-phospha-

- tase associates with the interleukin-3 receptor. *J. Biol. Chem.* **271**:29729–29733.
31. **Liu, X. J., A. Sorisky, L. Zhu, and T. Pawson.** 1995. Molecular cloning of an amphibian insulin receptor substrate 1-like cDNA and involvement of phosphatidylinositol 3-kinase in insulin-induced *Xenopus* oocyte maturation. *Mol. Cell. Biol.* **15**:3563–3570.
32. **Marengere, L. E., and T. Pawson.** 1992. Identification of residues in GTPase-activating protein Src homology 2 domains that control binding to tyrosine phosphorylated growth factor receptors and p62. *J. Biol. Chem.* **267**:22779–22786.
33. **Muslin, A. J., A. M. MacNicol, and L. T. Williams.** 1993. Raf-1 protein kinase is important for progesterone-induced *Xenopus* oocyte maturation and acts downstream of *mos*. *Mol. Cell. Biol.* **13**:4197–4202.
34. **Muta, T., T. Kurosaki, Z. Misulovin, M. Sanchez, M. C. Nussenzweig, and J. V. Ravetch.** 1994. A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIIB modulates B-cell receptor signalling. *Nature* **368**:70–73. (Erratum, **369**:340.)
35. **Nakanishi, H., K. A. Brewer, and J. H. Exton.** 1993. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-triphosphate. *J. Biol. Chem.* **268**:6–13.
36. **Ono, M., S. Bolland, P. Tempst, and J. V. Ravetch.** 1996. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor FcγRIIB. *Nature* **383**:263–266.
37. **Rameh, L. E., C.-S. Chen, and L. C. Cantley.** 1995. Phosphatidylinositol (3,4,5)P3 interacts with SH2 domains and modulates PI 3-kinase association with tyrosine-phosphorylated proteins. *Cell* **83**:821–830.
38. **Stephens, L. R., T. R. Jackson, and P. T. Hawkins.** 1993. Agonist-stimulated synthesis of phosphatidylinositol(3,4,5)-trisphosphate: a new intracellular signalling system? *Biochim. Biophys. Acta* **1179**:27–75.
39. **Toker, A., C. Bachelot, C. S. Chen, J. R. Falck, J. H. Hartwig, L. C. Cantley, and T. J. Kovacs.** 1995. Phosphorylation of the platelet p47 phosphoprotein is mediated by the lipid products of phosphoinositide 3-kinase. *J. Biol. Chem.* **270**:29525–29531.
40. **Toker, A., M. Meyer, K. K. Reddy, J. R. Falck, R. Aneja, S. Aneja, A. Parra, D. J. Burns, L. M. Ballas, and L. C. Cantley.** 1994. Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P2 and PtdIns-3,4,5-P3. *J. Biol. Chem.* **269**:32358–32367.
41. **Ware, M. D., P. Rosten, J. E. Damen, L. Liu, R. K. Humphries, and G. Krystal.** 1996. Cloning and characterization of human SHIP, the 145 kDa inositol 5-phosphatase that associates with SHC after cytokine stimulation. *Blood* **88**:2833–2840.
42. **Wu, M., and J. Gerhart.** 1991. Raising *Xenopus* in the laboratory. *Methods Cell Biol.* **36**:1–18.
43. **Zhang, J., J. R. Falck, K. K. Reddy, C. S. Abrams, W. Zhao, and S. E. Rittenhouse.** 1995. Phosphatidylinositol (3,4,5)-trisphosphate stimulates phosphorylation of pleckstrin in human platelets. *J. Biol. Chem.* **270**:22807–22810.