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SHIP Modulates Immune Receptor Responses by Regulating Membrane Association of Btk

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Summary

Membrane recruitment of SHIP is responsible for the inhibitory signal generated by FcγRIIB coligation to the BCR. By reducing the level of PIP_3, SHIP regulates the association of the tyrosine kinase Btk with the membrane through PH domain–phosphoinositol lipid interactions. Inhibition of BCR signaling by either FcγRIIB coligation, membrane expression of SHIP, or inhibition of PI3K, conditions which result in decreased levels of PIP_3, is suppressed by the expression of Btk as a membrane-associated chimera. Conversely, increasing PIP_3 levels by deletion of SHIP results in increased Btk association with the membrane and hyperresponsive BCR signaling. These results suggest a central role for PIP_3 in regulating the B cell stimulatory state by modulating Btk localization and thereby calcium fluxes.

Introduction

Aggregation of immune receptors such as the B cell receptor (BCR), T cell receptor (TCR), or Fc receptor (FcR) on a variety of cells results in their activation through the sequential actions of the Src and Syk families of tyrosine kinases (reviewed by Weiss and Littman, 1994; DeFranco, 1997; Kurosaki, 1997). Substrates of these kinases include enzymes involved in the regulation of both membrane-associated and soluble inositol polyphosphates. Thus, tyrosine phosphorylation of phospolipase C-γ leads to its activation and the generation of IP_3 (inositol [1,4,5]trisphosphate) and diacylglycerol, stimulating calcium mobilization from intracellular stores (Bijsterbosch et al., 1985; Takata et al., 1995), while the activation of PI3K (phosphatidylinositol 3-kinase) catalyzes the conversion of $PI(4,5)P_2$ (phosphatidylinositol [4,5]bisphosphate) to $PI(3,4,5)P_3$ (phosphatidylinositol [3,4,5]trisphosphate), which is correlated with increased calcium fluxes (Tuveson et al., 1993; Yano et al., 1993; Buhl et al., 1997).

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Balancing these stimulatory pathways are the inhibitory receptors and their associated signaling molecules, which are responsible for setting threshold levels for activation signals as well as terminating activation responses. These inhibitory receptors share several common features (Vély and Vivier, 1997). They are inert until coligated to a stimulatory receptor, at which time they undergo tyrosine phosphorylation of a conserved inhibitory motif in their cytoplasmic domains. This phosphorylation leads to the recruitment of distinct signaling molecules capable of inhibiting calcium fluxes in the stimulated cell. Distinctions among the inhibitory receptors have led to their segregation into two general classes, based on their effects on calcium fluxes and recruitment of signaling molecules (Gupta et al., 1997; Ono et al., 1997). CD22 and natural killer inhibitory receptors exemplify a class of molecules in which the inhibitory response occurs early in the signaling pathway through the recruitment of the SH1 (Src homology domain-1)–containing tyrosine phosphatase SHP-1 (reviewed by Long et al., 1997). The action of this phosphatase results in the inhibition of IP_3 generation and hence the loss of calcium release from intracellular stores (Kaufman et al., 1995). In contrast, the Fc receptor FcγRIIB inhibitory signaling is representative of a class of molecules in which the inhibitory response occurs late in the signaling pathway. This receptor recruits a novel SH2–containing inositol polyphosphate phosphatase (SHIP), causing a block in calcium influx (Muta et al., 1994; Ono et al., 1996). SHIP catalyzes the hydrolysis of the membrane phospholipid $PI(3,4,5)P_3$ to $PI(3,4)P_2$ and the soluble inositol polyphosphate $Ins(1,3,4,5)P_4$ to $Ins(1,3,4)P_3$ (Damen et al., 1996; Lioubin et al., 1996).

We have investigated the mechanism by which SHIP mediates its inhibitory effect on calcium influx by complementation analysis of $SHIP^{-/-}$ B cells. Since the inhibitory effect of FcγRIIB can be replaced by the expression of a SHIP chimera localized to the cell surface and requires that SHIP have an intact phosphatase domain (Ono et al., 1997), we reasoned that SHIP inhibited calcium influx by regulating the level of the membrane-associated inositol polyphosphate PIP_3 (phosphatidylinositol [3,4,5]trisphosphate), which is hydrolyzed by SHIP to $PI(4,5)P_2$. We therefore sought molecules that interacted with PIP_3 and might influence cellular activation generally and calcium influx specifically. One such candidate is the tyrosine kinase Btk (Bruton's tyrosine kinase), which when mutated results in X-linked agammaglobulinemia in humans and X-linked immunodeficiency in mice (reviewed by Rawlings and Witte, 1994; Smith et al., 1994). Btk is thus necessary for B cell development and activation. The activity of this kinase in turn is dependent upon its ability to interact with PIP_3 through its plextrin homology or PH domain (Salim et al., 1996; Rameh et al., 1997). B cells in which the Btk PH domain is unable to bind to PIP_3 are compromised in mediating BCR-triggered cellular activation (Bradley et al., 1994; de Weers et al., 1994; Salim et al., 1996; Hyvonen and Saraste, 1997). Conversely, B cells in which mutations in Btk lead to increased membrane association display

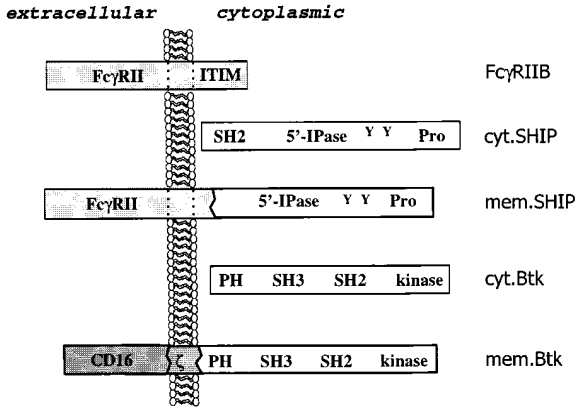

Figure 1. Schematic Representation of the Transfected Proteins

FcγRII contains the complete sequence of murine FcγRIIB1, including an immunoreceptor tyrosine inhibitory motif (ITIM) in the cytoplasmic domain. Cyt. SHIP (cytoplasmic SHIP) is the complete murine SHIP protein, which contains an N-terminal SH2 domain, a 5′-phosphoinositol phosphatase domain (5′-IPase), two PTB binding site motifs (Y Y), and a proline-rich domain (Pro). Cyt. Btk (cytoplasmic Btk) is the complete human Btk protein, which includes SH1 (kinase), SH2, SH3, and an amino-terminal PH domain. Mem. SHIP (membrane SHIP) consists of the murine FcγRIIB1 extracellular and transmembrane domains (amino acids 1–305) fused to a murine SHIP protein that lacks the SH2 domain (amino acids 194–1187). Mem. Btk (membrane Btk) is composed of the extracellular domain of human CD16 (amino acids 1–212), the transmembrane domain of the human T cell receptor ζ chain (amino acids 30–58), and the complete sequence of human Btk in the cytoplasmic tail.

a transformed phenotype, suggestive of unregulated Btk activation (Li et al., 1995; Li et al., 1997a).

We report that the inhibitory effect of either FcγRIIB or SHIP can be suppressed by expression of Btk as a membrane-associated chimera, and that pathways that result in decreased PIP_3 levels reduce Btk membrane association and display reduced calcium mobilization. Conversely, increasing PIP_3 by genetic deletion of SHIP results in increased Btk association with the membrane and renders the cell hyperresponsive to BCR stimulation. These results suggest that Btk, by a regulated association with the cellular membrane mediated by PIP_3, is in turn able to regulate the activity of a calcium influx channel and thereby generate a sustained stimulatory signal.

Results

Expression of Btk as a Membrane Chimera Suppresses FcγRIIB-Mediated Inhibition

We have previously shown that upon coligation to the BCR FcγRIIB becomes tyrosine phosphorylated and triggers the recruitment of the inositol polyphosphate phosphatase SHIP through its SH2 domain (Muta et al., 1994; Ono et al., 1996). Targeted disruption of SHIP in the chicken DT40 B cell line demonstrated that FcγRIIB inhibition of calcium influx was dependent upon SHIP (and not SHP-1) and required an intact phosphatase domain (Ono et al., 1997). These results suggested that PIP_3 or IP_4 hydrolysis was essential to the mechanism by which SHIP mediated its inhibitory effect on calcium influx. A likely candidate to integrate this signal was the PH domain–containing tyrosine kinase Btk. To demonstrate that Btk is involved in the SHIP inhibitory pathway, DT40 cells expressing FcγRIIB were generated in which either a membrane version of Btk (CD16/ζ/Btk) or a wild-type Btk was overexpressed. A schematic representation of the constructs and a summary of the cell lines used in this study are presented in Figure 1 and Table 1, respectively. These cells were stimulated through BCR cross-linking (Figure 2A) or coengagement of BCR and FcγRIIB. FcγRIIB coengagement results in an inhibition of calcium influx (Figure 2A, left), as previously reported

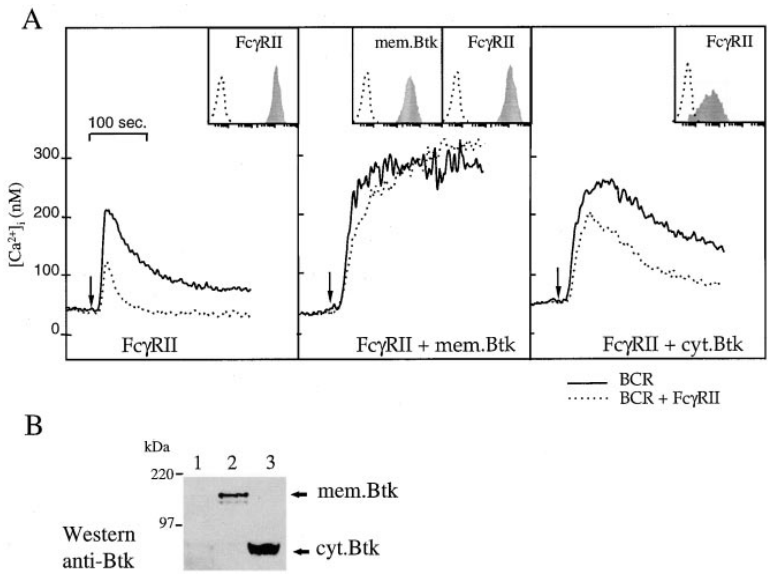

Figure 2. Membrane-Localized Btk Suppresses FcγRII Inhibition

(A) Intracellular calcium^{2+} mobilization triggered by BCR-FcγRII coligation compared with BCR stimulation alone. The chicken BCR (surface IgM) was stimulated by cross-linking with an mAb (mouse IgM) against chicken IgM. For coligation of murine FcγRII with the BCR, rabbit antibody against mouse IgM was added prior to stimulation with the anti-chicken antibody. Arrows indicate the addition of anti-chicken IgM. DT40 cells were transfected with the murine FcγRII construct alone, or FcγRII together with the membrane Btk chimera or FcγRII together with the cytoplasmic Btk construct. Histograms show surface expression levels of transfected constructs as determined by FACS staining, where dotted lines indicate staining of untransfected cells. The calcium^{2+} traces shown are representative of at least two different independent clones and three different measurements for each case.

(B) Expression level of endogenous and transfected Btk protein detected by immunoblotting with anti-Btk antibodies. Extracts from 5×10^4 cells were loaded. Lane 1 is DT40 transfected with FcγRII; lane 2 is DT40 transfected with FcγRII and membrane Btk; and lane 3 is DT40 transfected with FcγRII and cytoplasmic Btk.

Table 1. Expression Level of Endogenous BCR and Transfected Proteins in the DT40 Clones

Cell Line	Transfected Constructs				
	BCR	FcγRII	Membrane SHIP	Membrane Btk	Cytoplasmic Btk
DT40	++	−	−	−	−
DT40 (FcγRII)	++	+++	−	−	−
DT40 (FcγRII + mem. Btk)	++	+++	−	++	−
DT40 (FcγRII + cyt. Btk)	++	+	−	−	+++
DT40 (mem. SHIP)	++	−	+	−	−
DT40 (mem. SHIP + mem. Btk)	++	−	+	++	−
DT40 (mem. SHIP + cyt. Btk)	++	−	+	−	+++
DT40 (mem. Btk)	++	−	−	++	−
DT40 *ship*$^{-/-}$	++	−	−	−	−
DT40 *ship*$^{-/-}$ (mem. SHIP)	++	−	+	−	−

mem., membrane; cyt., cytoplasmic.

(Choquet et al., 1993; Muta et al., 1994). This inhibitory effect of FcγRIIB on BCR-induced calcium influx was abrogated by the expression of Btk as a membrane chimera (Figure 2A, middle). In addition, these cells display a sustained calcium elevation upon stimulation, suggesting that expression of Btk as a transmembrane molecule leads to a prolongation of calcium influx. Overexpression of a wild-type Btk construct (Figure 2A, right) did not reverse the inhibitory effect of FcγRIIB coligation, despite a markedly increased level of Btk in those cells (Figure 2B). These results indicate that membrane-associated Btk acts as a suppressor of FcγRIIB-mediated inhibition and leads to a sustained elevation of intracellular calcium.

Membrane Btk Expression Is Sufficient To Bypass the Inhibitory Effect of SHIP

FcγRIIB mediates its inhibitory effect through its recruitment of SHIP to the membrane, because replacement of the cytoplasmic domain of FcγRIIB with SHIP is sufficient to inhibit calcium influx in BCR-stimulated cells (Ono et al., 1997). We therefore formally sought to demonstrate that membrane expression of Btk directly overcomes the inhibition mediated by SHIP on calcium influx. DT40 cell lines expressing an FcγRIIB/SHIP chimera and either membrane or cytoplasmic versions of Btk were constructed (Figure 1 and Table 1). Membrane expression of Btk suppresses the inhibition by the FcγRIIB/SHIP chimera (Figure 3A, left), again displaying a sustained increase in intracellular calcium, while cytoplasmic overexpression of Btk cannot overcome the inhibitory effect of SHIP localization to the membrane (Figure 3A, right). We conclude therefore that membrane expression of Btk alone is sufficient to overcome the inhibition of calcium influx observed when SHIP is associated with the membrane.

The Effect of Inhibiting PI3K Is Reversed by Membrane Btk

The above results suggest that SHIP, when associated with the membrane, gains access to its substrate $PI(3,4,5)P_3$, hydrolyzing it to $PI(3,4)P_2$, which in turn limits the association of the PH domain of Btk with the membrane. This hypothesis predicts that inhibition of other pathways, which lead to the production of PIP_3, should also be suppressible by membrane expression of Btk. Wortmannin is known to inhibit the enzyme PI3K, which is responsible for the conversion of $PI(4,5)P_2$ to $PI(3,4,5)P_3$ (Yano et al., 1993). Wortmannin treatment of DT40 cells inhibits the influx of calcium stimulated by BCR aggregation (Figure 4, top). This inhibition is reversed if the membrane version of Btk is expressed in these cells (middle) but retained if the wild-type Btk is overexpressed (bottom). Thus, reduction in PIP_3 levels by decreased synthesis, rather than increased degradation, results in a decrease in calcium mobilization, which is suppressed by membrane-associated Btk.

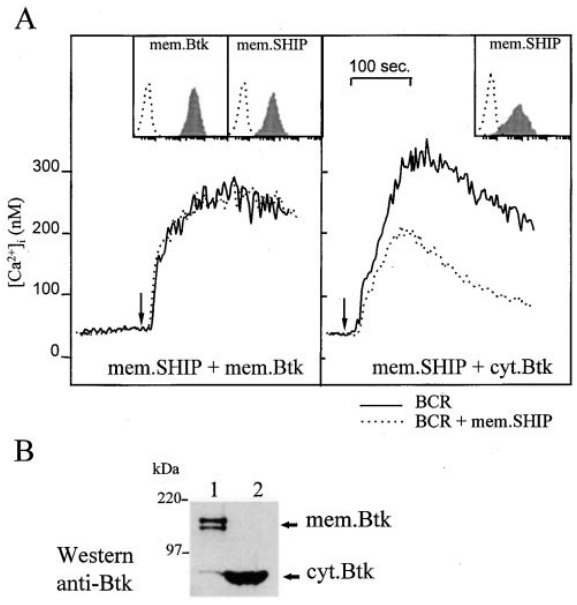

Figure 3. Expression of Membrane-Localized Btk Suppresses SHIP Inhibition

(A) Intracellular Ca^{2+} mobilization triggered by the BCR when coligated to membrane SHIP compared with BCR stimulation alone. DT40 cells were transfected with membrane SHIP together with the membrane Btk chimera or with the cytoplasmic Btk construct. Cells were stimulated as in Figure 2.

(B) Expression level of endogenous and transfected Btk protein detected by immunoblot with anti-Btk antibodies. Lane 1 is an extract from DT40 cells transfected with membrane SHIP and membrane Btk; lane 2 is an extract from DT40 cells transfected with membrane SHIP and cytoplasmic Btk.

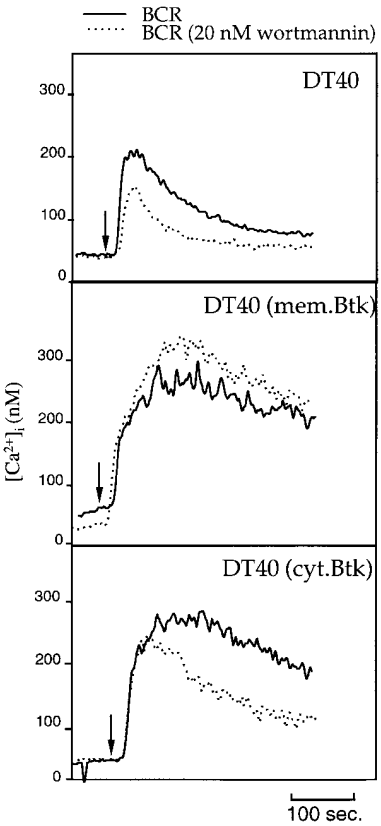

Figure 4. Membrane-Localized Btk Overcomes Wortmannin Inhibition

Intracellular Ca^{2+} mobilization was measured after BCR engagement with anti-chicken IgM antibodies. Untransfected DT40 cells, cells transfected with the membrane Btk construct, or the cytoplasmic Btk construct were stimulated at the time point indicated (arrow). Dotted traces indicate that the stimulation was performed after 30 min incubation with 20 mM wortmannin.

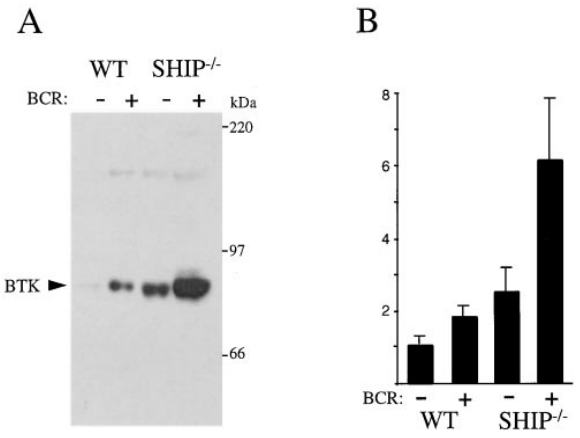

Figure 5. Increased Membrane Localization of Endogenous Btk in DT40 *ship*$^{-/-}$ Cells

(A) Wild-type DT40 and SHIP$^{-/-}$ DT40 cells were stimulated by cross-linking the BCR with anti-chicken IgM antibodies (+) or were left unstimulated (−). Cells were lysed in hypotonic buffer as described (Kawakami et al., 1994) 2 min after stimulation. Membrane fractions were analyzed by immunoblotting with anti-Btk.

(B) Quantification of Btk enrichment in membrane fractions. The intensity of the Btk bands revealed in immunoblots of membrane fractions was measured by phosphorimager analysis (BioRad). The BCR expression level, detected on the same blot by incubation with anti-chicken IgM, was used to normalize values for the amount of membrane extract. Bars represent the average of four experiments in arbitrary units; standard errors are indicated.

Native Btk Association with the Membrane Is Increased in SHIP$^{-/-}$ Cells

Direct demonstration of an increased association of endogenous Btk with the membrane of SHIP$^{-/-}$ cells is presented in Figure 5. Wild-type DT40 cells display minimal Btk in the membrane fraction of unstimulated cells and a significant increase in membrane-associated Btk upon BCR aggregation. To demonstrate that the level of PIP_3 in the membrane regulates this association, we turned to the SHIP$^{-/-}$ DT40 cell line we had generated. Unstimulated DT40 SHIP$^{-/-}$ cells have a higher constitutive level of membrane-associated Btk, which is further increased by BCR aggregation. This increase in Btk membrane association is consistent with our expectation that decreasing PIP_3 hydrolysis by eliminating SHIP, with the resulting increase in PIP_3, will produce a shift in the partitioning of Btk to the membrane fraction.

SHIP$^{-/-}$ Cells Are Hyperresponsive to BCR Aggregation

The above results demonstrate that basal levels of membrane-associated Btk are increased in SHIP$^{-/-}$ DT40 cells (Figure 5). This finding raises the possibility that these cells will be hyperresponsive to BCR cross-linking, since increased membrane localization of Btk is associated with a sustained calcium signal (Figure 2). A direct comparison of the levels of intracellular calcium in response to BCR aggregation in SHIP$^{+/+}$ and SHIP$^{-/-}$ cells is shown in Figure 6. Deletion of SHIP results in an approximately 200% increase in peak intracellular calcium levels, with a sustained increase in these levels, as compared to wild-type cells (left). Inhibition of PI3K by wortmannin or complementation of the deficient cells by expression of membrane SHIP abrogates the hyperresponsive phenotype, restoring these cells to a wild-type level of intracellular calcium induction in response to BCR aggregation (right). Mutation of the phosphatase domain of SHIP results in a molecule that is unable to complement the deficient cells (data not shown). These results support the conclusion that the hyperresponsive phenotype results from increased levels of PIP_3 due to the absence of SHIP hydrolytic activity. Thus, in wild-type DT40 cells SHIP appears to be involved in determining the threshold for BCR-induced calcium influx by regulating the level of PIP_3, which in turn regulates Btk association with the membrane.

Discussion

The immune system is characterized by its ability to generate graded responses under differing ligand conditions. This discrimination is believed to underlie the mechanisms of positive and negative selection and affinity maturation (Goodnow, 1992; Nossal, 1994; Cornall

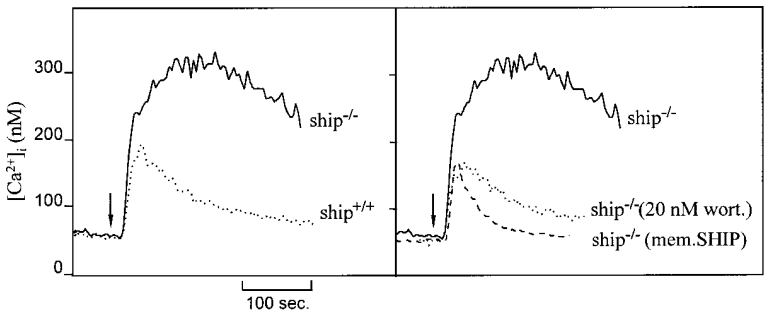

Figure 6. Enhanced Ca^{2+} Mobilization in DT40 *ship*$^{-/-}$ Cells upon BCR Stimulation

Intracellular Ca^{2+} mobilization was measured upon cross-linking of the BCR with anti-chicken IgM antibodies. Left, wild-type DT40 cells (*ship*$^{+/+}$) were compared with SHIP$^{-/-}$ DT40 cells (*ship*$^{-/-}$). Right, the response of SHIP$^{-/-}$ DT40 cells was compared with SHIP$^{-/-}$ DT40 cells transfected with membrane SHIP (mem.SHIP) or SHIP$^{-/-}$ DT40 cells preincubated for 30 min with 20 nM wortmannin (wort.).

et al., 1995; Rajewsky, 1996). Mechanistically then, immune receptors must display both threshold requirements and modulated activation responses upon stimulation. The recent recognition that both inhibitory and stimulatory pathways are simultaneously present upon receptor aggregation offers a basis for this fine-tuning. We propose that SHIP is a general regulator of immunoreceptor activation. It is involved both in terminating activation signals from the BCR and FcR on inflammatory cells and in setting threshold levels for immunoreceptor activation, as demonstrated here and in other work (Okada et al., unpublished data). It performs these two functions through a common mechanism, in which the association of Btk with the membrane is regulated by modulating the availability of PIP_3. A general model for this mechanism of SHIP-regulated association of Btk, based on the data presented here, is shown in Figure 7.

Several lines of evidence support this model. $PI(3,4,5)P_3$ is generated from $PI(4,5)P_2$ by PI3K, an ubiquitous mediator of immune receptor activation necessary for BCR, TCR, and FcR stimulation (Yamanashi et al., 1992; Yano et al., 1993; Gold and Aebersold, 1994; von Willebrand et al., 1994). One role of PIP_3 is the membrane recruitment of the tyrosine kinase Btk through its PH domain. Previous studies have shown that Btk is essential for B cell activation, since Btk-deficient DT40 cells fail to mobilize calcium in response to BCR cross-linking (Takata and Kurosaki, 1996), while B cells from x-linked immunodeficient or Btk-deficient mice have attenuated levels of calcium mobilization in response to BCR stimulation (Rigley et al., 1989). The consequence then of regulating the level of PIP_3 is to regulate the association of Btk with the membrane, an interaction that is critical to the activity of this molecule and to its role in cellular activation (Li et al., 1995, 1997b). Indeed, PI3K has been shown to activate Btk in cotransfection studies, measured by enhanced cell transformation and hyperphosphorylation of Btk (Li et al., 1997b). Mutations that compromise the ability of Btk to interact with the membrane render the molecule inactive and result in cells that are nonresponsive to receptor-induced activation (Rawlings et al., 1993; Thomas et al., 1993; Takata et al., 1995).

Recruitment of the cytosolic enzyme SHIP to the membrane results in its ability to regulate intracellular calcium (Ono et al., 1996, 1997). While localization to the membrane is accomplished through an SH2-mediated interaction for FcγRIIB inhibitory signaling (Ono et al., 1996), different domains of SHIP may be involved in its recruitment for BCR responses independent of FcγRIIB. Regardless of how SHIP is recruited to the membrane, its ability to regulate intracellular calcium is dependent upon an active phosphatase domain, suggesting that a common mechanism involving inositol hydrolysis is required. Indeed, once associated with the membrane SHIP hydrolyzes $PI(3,4,5)P_3$ to $PI(3,4)P_2$ (Damen et al., 1996). Although SHIP is also able to catalyze the hydrolysis of IP_4 (Damen et al., 1996; Lioubin et al., 1996), a molecule that has been proposed to interact with Btk (Fukuda et al., 1996) and may also regulate its activity, its role is likely to be minimal. IP_4 levels upon BCR activation are identical in SHIP$^{-/-}$ and wild-type DT40 cells (Okada et al., unpublished data). Furthermore, the hyperresponsive phenotype seen in DT40 SHIP$^{-/-}$ cells

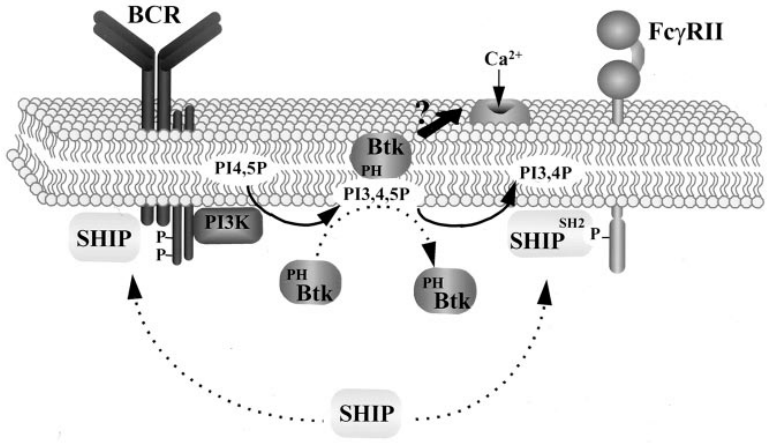

Figure 7. Model for the Mechanism of Inhibition of Ca^{2+} Mobilization by SHIP

Coligation of FcγRII to the BCR results in SHIP recruitment to the membrane via an SH2 domain interaction. Once in the membrane, SHIP hydrolyzes $PI(3,4,5)P_3$ to $PI(3,4)P_2$, thereby interfering with PH domain–mediated Btk membrane localization induced by BCR signaling. Membrane-localized Btk increases Ca^{2+} influx by an unknown mechanism. Additionally, SHIP can regulate BCR activating signals independent of FcγRII coligation by its phosphoinositol hydrolyzing activity, via interactions yet to be described.

can be reverted by inhibition of PI3K, through reduction in the elevated level of PIP_3 (Figure 6). Thus, these data in aggregate suggest that PIP_3 is the more relevant substrate to account for the effect of SHIP on calcium mobilization. This model predicts that regulating levels of PIP_3 may be a general mechanism for modulating receptor responses through regulated association of PH domain molecules with the plasma membrane.

There are several possible mechanisms by which Btk-regulated association with the membrane could account for its effect on calcium responses. In Btk-deficient DT40 cells, a reduction in tyrosine phosphorylation of phospolipase C-γ is observed, and an abrogation of IP_3 generation is seen despite normal activity of Syk, indicating that among its roles Btk is a regulator of phospolipase C-γ activation in a Syk-independent pathway (Takata and Kurosaki, 1996). However, this effect of Btk on calcium release from intracellular stores is unlikely to account for the effects of SHIP recruitment since this arm of the calcium mobilization pathway is unperturbed in FcγRIIB- or SHIP-deficient cells, indicating that this aspect of calcium regulation is not likely to be a target for membrane-associated Btk. Our data favor the interpretation that Btk association with the membrane affects a later phase of calcium mobilization, which results from influx of calcium through a plasma membrane channel. This effect may be a direct one on the channel or an indirect one by affecting the capacitative calcium entry pathway.

SHIP, together with the Btk family (Itk and Tec), has been found in a wide variety of cells of the immune system, including lymphocytes and cells of the myelomonocytic lineage, and is conserved across chickens, mice, and humans (Damen et al., 1996; Kavanaugh et al., 1996; Desiderio, 1997; Geier et al., 1997). In addition, Btk is activated by a wide variety of receptors, including BCR, FcR, CD28, interleukin-3, interleukin-5, and interleukin-6 (Wahl et al., 1997 and references therein). Thus, the mechanism we have described for chicken B cells is likely to be generally applicable in other species and cell types. The relative lack of redundancy in BCR-induced phospholipid hydrolysis in DT40 cells (Takata et al., 1995; Takata and Kurosaki, 1996) has enabled us to isolate the connection between SHIP and Btk, which can now be extrapolated to other cells and species. However, whether SHIP functionally interacts with other immune receptors, such as TCR and FcR, to set threshold responses as it does for the BCR remains to be determined.

Experimental Procedures

Expression Constructs and Antibodies

The cytoplasmic Btk construct consists of the human Btk sequence cloned in the pApuro expression vector (Takata et al., 1994). The membrane Btk chimera (CD16/ζ/Btk), provided by Minoru Takata, was constructed in the pApuro vector by fusing the entire sequence of human Btk to the cytoplasmic domain of the CD16/ζ construct described in Salcedo et al. (1993). The FcγRII construct has been described previously (Muta et al., 1994). The cytoplasmic SHIP construct contains the entire sequence of murine SHIP cloned in the pRc/cytomegalovirus expression vector (InVitrogen). The construction of the FcγRII-SHIP chimera (membrane SHIP) in pRc/cytomegalovirus has been described previously (Ono et al., 1997).

Cell surface expression of BCR was analyzed by fluorescence-activated cell sorter (FACS) using fluorescein isothiocyanate (FITC)–conjugated anti-chicken immunoglobulin M (IgM) (Bethyl). Surface expression of FcγRII or the FcγRII chimera (membrane SHIP) was detected by FACS staining with FITC-conjugated 2.4G2 antibodies (Pharmingen). FITC-labeled 3G8 antibodies (Pharmingen) were used to stain cells expressing the CD16 chimera (membrane Btk).

BCR cross-linking for calcium measurement experiments was performed with anti-chicken IgM monoclonal antibody (mAb) M4 at 1 μg/ml as described previously (Ono et al., 1997). Mouse FcγRII was coligated to the endogenous chicken BCR by addition of rabbit anti-mouse IgM as secondary antibody at 10 μg/ml. This antibody specifically recognizes the Fc portion of M4 mAb and is bound with low affinity by FcγRII (Phillips and Parker, 1984). The secondary antibody was added 1 min prior to cell stimulation with anti-chicken IgM antibodies.

Western blots were incubated with polyclonal anti-human Btk (Pharmingen) followed by horseradish peroxidase–conjugated goat anti-rabbit antibody (Amersham). Proteins were visualized by enhanced chemiluminescence technique (Amersham).

DT40 Cell Culture and Transfections

Wild-type or SHIP-deficient chicken DT40 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% chicken serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics. The construction of $SHIP^{-/-}$ DT40 cells was performed according to the method of Okada et al. (unpublished data). Cells were transfected by electroporation at 250 V and 960 μF in phosphate-buffered saline (10^7 cells in 0.5 ml). Twenty micrograms of expression constructs were cotransfected with 2 μg of pBabe-puro' vector (Morgenstern and Land, 1990). Transfectants were selected in 0.5 mg/ml puromycin 24 hr after electroporation. The presence of FcγRII or membrane SHIP was determined by FACS analysis with FITC-conjugated 2.4G2 antibody (Pharmingen). The presence of membrane Btk was verified by FACS analysis with FITC-conjugated 3G8 antibody (Pharmingen). Cotransfected cytoplasmic constructs were detected by Western blot analysis with anti-SHIP (Ono et al., 1997) (cytoplasmic SHIP transfectants) or anti-Btk antibodies (cytoplasmic Btk transfectants) (Pharmingen).

Calcium Measurements

Cells (5×10^6) were loaded with 2 μM Fura-2 AM (Molecular Probes) in 3 ml of medium at 36°C for 30 min with intermittent shaking. After washing the cells three times with Hanks' balanced salt solution buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, cells were resuspended in the same buffer at 2×10^6 cells/ml. Next, 0.5 ml of cell suspension was diluted with 1 ml of phosphate-buffered saline containing 1mM $CaCl_2$ and 1 mM $MgCl_2$, and cytosolic calcium concentration was recorded at room temperature at 510 nm emission wavelength excited by 340 and 360 nm using a fluorescence spectrophotometer (LB50B, Perkin Elmer). Calculation of calcium concentration was performed using FL WinLab software (Perkin Elmer).

Membrane Localization of Btk

First, 10^8 DT40 cells, either wild-type or $SHIP^{-/-}$, were serum-starved for 12 hr and then stimulated for 2 min at 37°C with 10 μg/ml anti-chicken IgM mAb M4 (Ono et al., 1997). Subcellular fractionation was then performed at 4°C as described by Kawakami et al. (1994). Cells were resuspended in 5 ml of hypotonic lysis buffer (1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 1 mM sodium vanadate, 2 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 μM microcystin, 2 mM benzamidine, 5 mM sodium pyrophosphate, 50 mM NaF, and 40 ng/ml PMSF) and Dounce homogenized. Cell lysates were centrifuged at 1600 $\times$ g for 10 min over a 1 M sucrose cushion. The resulting supernatants were centrifuged at 100,000 $\times$ g for 30 min. Precipitates were washed twice with lysis buffer and resuspended in lysis buffer containing 1% Triton X-100. These resuspensions were then centrifuged at 20,000 $\times$ g and the resulting supernatants assayed for protein concentration (BioRad). Equivalent amounts of protein were subjected to 10% SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. Membranes were then sequentially blotted with anti-Btk (Pharmingen,

1:500 dilution) and horseradish peroxidase–conjugated goat anti-rabbit IgG (BioRad, 1:5000 dilution). Equivalent loading was confirmed by blotting with anti-chicken IgM (Bethyl, 1:1000 dilution) followed by horseradish peroxidase–conjugated rabbit anti-goat IgG (Pierce, 1:5000 dilution). Proteins were visualized by enhanced chemiluminescence (Amersham). Signals were quantified by densitometric analysis.

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