Targeted disruption of SHIP leads to Steel factor-induced degranulation of mast cells

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To investigate the role of the src homology 2 (SH2)-containing inositol 5' phosphatase (SHIP) in growth factor-mediated signalling, we compared Steel factor (SF)-induced events in bone marrow-derived mast cells (BMMCs) from SHIP^{-/-} and SHIP^{+/+} littermates. We found SF alone stimulated massive degranulation from SHIP^{-/-} but none from SHIP^{+/+} BMMCs. This SF-induced degranulation, which was not due to higher c-kit levels in SHIP^{-/-} cells, correlated with higher intracellular calcium than that in SHIP^{+/+} cells and was dependent on the influx of extracellular calcium. Both this influx and subsequent degranulation were completely inhibited by PI-3-kinase inhibitors, indicating that SF-induced activation of PI-3-kinase was upstream of extracellular calcium entry. A comparison of phosphatidylinositol-3,4,5-trisphosphate (PIP₃) levels following SF stimulation of SHIP^{+/+} and SHIP^{-/-} BMMCs suggested that SHIP restricted this entry by hydrolyzing PIP₃. Although PI-3-kinase inhibitors blocked the release of intracellular calcium, implicating PIP₃, and PLC γ -2 was slightly more tyrosine phosphorylated in SHIP^{-/-} cells, the increase in inositol-1,4,5-trisphosphate (IP₃) and intracellular calcium levels were identical in SHIP^{-/-} and SHIP^{+/+} BMMCs. These results suggest that SHIP prevents SF from triggering degranulation of normal BMMCs, and does so by hydrolyzing PIP₃, which in turn limits extracellular calcium entry at a step after the release of intracellular calcium.

Keywords: calcium mobilization/IP₃/mast cells/PIP₃/SHIP

Introduction

The src homology 2 (SH2)-containing inositol phosphatase, SHIP, is a recently cloned, hemopoietic-specific signalling intermediate (Damen *et al.*, 1996; Kavanaugh *et al.*, 1996; Lioubin *et al.*, 1996; Liu *et al.*, 1998) that becomes tyrosine phosphorylated in response to both multiple cytokines and to B- and T-cell receptor engagement (reviewed in Liu *et al.*, 1997a). SHIP has been shown to inhibit immune receptor activation in both mast cells and B cells by binding to the tyrosine-phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM)

of the inhibitory coreceptor Fc γ RIIB and inhibiting Fc ϵ R1- and B-cell receptor-induced calcium influx, respectively (Ono *et al.*, 1996, 1997). However, its role in modulating the biological responses of activating receptors has not yet been determined. To gain further insight into the role that this phosphatase plays in regulating the responses elicited by these latter receptors, we recently generated a SHIP knockout mouse by homologous recombination in embryonic stem (ES) cells (Helgason *et al.*, 1998). Although these mice are viable and fertile, they suffer from progressive splenomegaly, massive myeloid infiltration of the lungs, wasting and a shortened lifespan (Helgason *et al.*, 1998). Notably, granulocyte/macrophage progenitors from these mice are substantially more responsive to multiple cytokines, including interleukin-3 (IL-3), granulocyte-macrophage-colony stimulating factor and Steel factor (SF), than their wild-type littermates (Helgason *et al.*, 1998). We have also demonstrated, using mast cells derived from the bone marrow (BMMCs) of SHIP^{-/-} and SHIP^{+/+} littermates, that SHIP plays a vital role in both setting the threshold for and limiting the IgE-induced degranulation process (Huber *et al.*, 1998).

In addition to IgE, SF (also known as mast cell growth factor), has been shown to have dramatic effects on mast cells via its tyrosine kinase-containing cell-surface receptor, c-kit. This cytokine is a potent enhancer not only of the survival, proliferation, chemotaxis and adhesion of mast cells but also of IgE-mediated degranulation (Vosseller *et al.*, 1997). Since we had shown previously that SHIP is tyrosine phosphorylated in response to SF (Liu *et al.*, 1994), we explored the role that SHIP plays in regulating SF-induced events by comparing the responses to SF of mouse SHIP^{+/+} and SHIP^{-/-} BMMCs. The results presented here demonstrate that SHIP prevents SF-induced intracellular signalling in mature mast cells from progressing to degranulation. Moreover, it appears to do so by hydrolyzing phosphatidylinositol-3,4,5-trisphosphate (PIP₃) and this, in turn, inhibits a crucial step between the release of calcium from intracellular stores and the influx of extracellular calcium.

Results

SF, but not IL-3, stimulates the entry of extracellular calcium and the degranulation of SHIP^{-/-} mast cells

In preliminary studies to generate mature mast cells from the bone marrows of SHIP^{+/+} and SHIP^{-/-} mice, we compared the proliferation and maturation rates of suspension cultures supplemented with IL-3 alone versus IL-3 plus SF. Intriguingly, the presence of SF caused the SHIP^{-/-}, but not the SHIP^{+/+} or SHIP^{+/-} cultures, to turn the growth medium yellow, although there were no dead cells in these cultures and the cell densities were not

Fig. 1. SF, but not IL-3, induces degranulation in SHIP^{-/-} but not SHIP^{+/+} or SHIP^{+/-} BMMCs. (A) SHIP^{+/+}, SHIP^{+/-} and SHIP^{-/-} BMMCs were stimulated for 15 min at 37°C with 400 ng/ml SF or 400 ng/ml IL-3 and the percentage of degranulation was determined by assaying supernatants and cell pellets for β -hexosaminidase activity. Each bar is the mean of duplicates $\pm$ SD. Similar results were obtained in three separate experiments. (B) SHIP^{-/-} BMMCs were stimulated for 15 min at 37°C with different concentrations of SF and the percentage of degranulation determined.

significantly different in the two cultures. Cytospins of these preparations suggested that SF might be triggering degranulation in the SHIP^{-/-} cultures and this was investigated further by carrying out degranulation assays with SHIP^{+/+}, SHIP^{+/-} and SHIP^{-/-} mast cell preparations. SF but not IL-3 stimulated a massive degranulation of SHIP^{-/-} BMMCs, while neither cytokine had any effect on SHIP^{+/+} or SHIP^{+/-} cells (Figure 1A). A dose-response study revealed that plateau levels of degranulation were achieved with 100–400 ng/ml of SF (Figure 1B).

To explore the intracellular mechanisms underlying this SF-induced degranulation of SHIP^{-/-} BMMCs, first we compared the expression level of the SF receptor, c-kit, in SHIP^{+/+} and SHIP^{-/-} BMMCs by Western blot analysis using anti-c-kit antibodies. As shown in Figure 2A, the two cell types contained similar levels of c-kit. However, since this does not necessarily reflect cell surface levels we then carried out FACS analysis using fluorescently tagged anti-c-kit and, as shown in Figure 2B, the two cell types were 100% c-kit positive and displayed the same mean fluorescence intensity.

Since IgE-mediated degranulation has been shown to

be dependent on the entry of extracellular calcium (reviewed in Beaven and Cunha-Melo, 1988), we next investigated whether the SF-induced degranulation of SHIP^{-/-} BMMCs was also contingent on the entry of this cation. As shown in Figure 3A, addition of EGTA to the extracellular medium to chelate the calcium ions completely abrogated SF-triggered degranulation in SHIP^{-/-} BMMCs. This result, together with previous reports implicating SHIP as a negative regulator of calcium influx (Ono *et al.*, 1997; Huber *et al.*, 1998), prompted us to explore in greater detail the role of SHIP in SF-induced increases in intracellular calcium by loading SHIP^{+/+} and SHIP^{-/-} BMMCs with fura-2/AM and then stimulating with SF. As shown in Figure 3B, both cell types responded with an increase in intracellular calcium but the cytosolic calcium concentration returned slowly to baseline levels in SHIP^{+/+} BMMCs while it increased in a biphasic fashion and was markedly prolonged in SHIP^{-/-} cells. Interestingly, addition of EGTA dramatically reduced the increase in intracellular calcium to a level that was indistinguishable in SHIP^{+/+} and SHIP^{-/-} mast cells. This suggested that the increase in intracellular calcium in response to SF was predominantly from the extracellular medium. Consistent with the importance of SF-induced calcium influx to subsequent degranulation, IL-3, which did not induce degranulation (Figure 1A), also did not stimulate a calcium influx in SHIP^{-/-} BMMCs (data not shown).

PI-3-kinase inhibitors block both SF-induced degranulation of SHIP^{-/-} BMMCs and calcium entry into SHIP^{-/-} and SHIP^{+/+} BMMCs

Since the 5'-phosphatase activity of SHIP was shown recently to be critical to its ability to reduce calcium entry during Fc γ RIIB mediated inhibition (Ono *et al.*, 1997), we investigated whether it was SHIP's ability to hydrolyze PIP₃ or inositol-1,3,4,5-tetrakisphosphate (IP₄) that was important in restricting SF-induced extracellular calcium entry by testing the effects of PI 3-kinase inhibitors. In preliminary experiments we established that both wortmannin and LY294002 completely inhibited degranulation in SHIP^{-/-} cells induced by SF (Figure 4A). We then explored the effects of these inhibitors on the SF-mediated influx of extracellular calcium. As shown in the right-hand panel of Figure 4B, LY294002 dose-response studies revealed that as little as 10 μ M LY294002 significantly inhibited extracellular calcium influx into SHIP^{-/-} cells and that 25 μ M decreased the intracellular calcium levels to that observed in SF-stimulated SHIP^{+/+} BMMCs (left-hand panel of Figure 4B). At 100 μ M LY294002, no significant elevation of SF-induced intracellular calcium was observed for either cell type. Interestingly, as the concentration of this PI-3-kinase inhibitor was increased, the delay between SF-stimulation and the rise in intracellular calcium increased, consistent with a threshold level of PIP₃ being required to trigger increased calcium levels and that reducing the number of active PI-3-kinase molecules lengthened this process. Similar results were obtained with wortmannin (data not shown). These results demonstrate that PI-3-kinase acts upstream of increases in intracellular calcium during SF stimulation of both SHIP^{+/+} and SHIP^{-/-} BMMCs.

Fig. 2. SF-induced degranulation in SHIP^{-/-} BMMCs is not due to differences in cell surface c-kit numbers. (A) Total cell lysates from 2×10^6 SHIP^{+/+} and SHIP^{-/-} BMMCs were solubilized and subjected to Western blotting with anti-c-kit antibodies (Santa Cruz). (B) Cell surface expression of c-kit in BMMCs from SHIP^{+/+} and SHIP^{-/-} mice were assessed by FACS with FITC-labeled anti-c-kit antibodies (Pharmingen). Background staining was with propidium iodide.

SF stimulation leads to substantially higher PIP₃ levels in SHIP^{-/-} than in SHIP^{+/+} BMMCs and this enhances extracellular calcium entry at a step after intracellular calcium release

Our results suggested that the primary function of SHIP might be to hydrolyze PIP₃ and that, in the absence of SHIP, PIP₃ would increase the intracellular calcium concentration sufficiently for degranulation. To test this, we measured PIP₃ levels in SHIP^{+/+} and SHIP^{-/-} cells in response to SF. As predicted, levels of this phospholipid increased far higher and remained elevated substantially longer following SF-stimulation in SHIP^{-/-} BMMCs (Figure 5A). Anti-c-kit immunoprecipitates from SF-stimulated SHIP^{+/+} and SHIP^{-/-} BMMCs possessed the same level of PI-3-kinase activity, demonstrating that the elevation of PIP₃ levels in the SHIP^{-/-} cells was not due to a difference in PI-3-kinase activity in the two cell types (data not shown). One question remained, however. How do elevated PIP₃ levels lead to an increase in the entry of extracellular calcium in SF-induced BMMCs? Two recent reports involving overexpression of various cDNAs in B-cell lines suggested that PIP₃ was capable of attracting the Btk/Tec family tyrosine kinase, Btk, to the plasma membrane for activation (Fluckiger *et al.*, 1998; Scharenberg *et al.*, 1998). Activation of Btk in turn was postulated to lead to the phosphorylation/activation of PLC-γ2 to generate IP₃ and the sustained emptying of intracellular calcium stores that are critical for activating the store-operated calcium entry (SOC) from the extracellular medium (Fluckiger *et al.*, 1998; Scharenberg *et al.*, 1998). To test whether the elevated PIP₃ levels in the SHIP^{-/-} BMMCs lead to increased entry of extracellular calcium via the same pathway in which we first examined the tyrosine phosphorylation of the PH-containing phospholipases, PLCγ1 and 2. Although both

PLCγ1 and 2 were tyrosine phosphorylated in response to SF, consistent with previous results using antigen-stimulated RBL-2H3 mast cells (Barker *et al.*, 1998), no significant difference in the phosphorylation of PLCγ1 was evident in SF-stimulated SHIP^{+/+} and SHIP^{-/-} BMMCs (data not shown). However, there appeared to be a slight elevation, especially at very early times, in the tyrosine phosphorylation of PLCγ2 in the SHIP^{-/-} cells (Figure 5B). To see whether this translated into a bigger increase in IP₃ levels in the SF-stimulated SHIP^{-/-} BMMCs, we then determined IP₃ levels at different times, following SF-stimulation in SHIP^{+/+} and SHIP^{-/-} cells. As can be seen in Figure 5C, there was no detectable difference in the fold-increase [nor was there any difference in the absolute levels of IP₃ in the two cell types (data not shown)]. We then investigated the possibility that the elevated PIP₃ levels in SHIP^{-/-} cells might be mediating the enhanced entry of extracellular calcium via an enhanced sphingosine kinase activity, since activation of the FcεR1 in RBL-2H3 cells has been shown to activate this enzyme (Choi *et al.*, 1996) and activation of this enzyme and subsequent generation of sphingosine-1-phosphate has been implicated in the release of intracellular calcium in an IP₃-independent manner (Meyer zu Heringdorf *et al.*, 1998; reviewed in Beaven, 1996). However, testing the sphingosine kinase inhibitor, DL-threo-dihydrosphingosine (DHS), over a range of concentrations shown to be effective in a variety of cell systems (i.e. from 10 to 100 μM; Choi *et al.*, 1996; Meyer zu Heringdorf *et al.*, 1998) revealed no significant effect on SF-stimulated SHIP^{-/-} BMMC degranulation. Moreover, at 30 μM DHS, there was no significant effect on SF-induced intracellular calcium release (i.e. fura-2/AM fluorescence studies carried out in the presence of EGTA) with SHIP^{-/-} BMMCs (data not shown).

Fig. 3. SF-induced degranulation in SHIP^{-/-} BMMCs is dependent on extracellular calcium. **(A)** SHIP^{-/-} BMMCs were either incubated with 400 ng/ml SF for 15 min at 37°C or preincubated with 5 mM EGTA for 1 min and then stimulated with 400 ng/ml SF for 15 min. Percentage of degranulation was determined according to Materials and methods. **(B)** Intracellular calcium concentrations were measured in SHIP^{+/+} and SHIP^{-/-} BMMCs in response to SF, with or without EGTA. The arrow indicates the time when 400 ng/ml SF was added. EGTA (5 mM) was added 50 s before. The calcium profile of SHIP^{+/+} BMMCs obtained in the presence of EGTA is similar to that shown with the SHIP^{-/-} BMMCs. Identical results were obtained in four separate experiments.

Fig. 4. PI-3-kinase inhibitors reduce SF-induced degranulation and intracellular calcium levels. **(A)** Wortmannin at 25 nM or LY294002 at 25 μM was added to SHIP^{-/-} BMMCs 25 min before the addition of 400 ng/ml SF and degranulation was measured as described in Materials and methods. Identical results were obtained in three separate experiments. **(B)** LY294002, at the concentrations indicated, or vehicle (DMSO) was added 25 min before the addition of 100 ng/ml SF (indicated by an arrow) to SHIP^{+/+} (left panel) or SHIP^{-/-} (right panel) BMMCs. Calcium was measured as described in Materials and methods. Identical results were obtained in three separate experiments.

We then examined directly the SF-stimulated draining of intracellular calcium stores in SHIP^{+/+} and SHIP^{-/-} BMMCs. For these studies, BMMCs were derived independently from the bone marrows of four SHIP^{+/+} and SHIP^{-/-} mice. As can be seen in Figure 6A, there was substantial variability in the intracellular calcium released from both the SF-stimulated SHIP^{+/+} (Figure 6A, left panel) and SHIP^{-/-} (Figure 6A, right panel) BMMC clones. Importantly, though, the variability and extent of calcium release was similar in SHIP^{+/+} and SHIP^{-/-} BMMCs (consistent with our IP₃ and DHS results). To examine the effect of SF-induced intracellular calcium release on subsequent extracellular calcium entry, the calcium levels of clones displaying high and low intracellular calcium release were compared, in the presence and absence of EGTA. As shown in the left upper and lower panels of Figure 6B, SF-stimulated SHIP^{+/+} clones showing a low

intracellular calcium release (mouse 1^{+/+}) also showed a lower extracellular calcium entry than SF-stimulated SHIP^{+/+} clones showing a high intracellular calcium release (mouse 3^{+/+}). However, no such relationship was observed with the SHIP^{-/-} BMMCs. Here, SF-stimulated BMMCs with a low intracellular calcium release (mouse 2^{-/-}, upper right panel of Figure 6B) showed a similar extracellular calcium entry to that seen with a high intracellular calcium release clone (mouse 3^{-/-}, lower right panel of Figure 6B). Moreover, the SHIP^{-/-} clone displaying a low intracellular calcium release (mouse 2^{-/-}) produced an extracellular calcium entry that was greater than that produced by the SHIP^{+/+} clone showing a high intracellular calcium release (mouse 3^{+/+}). These same clones were also tested for their ability to degranulate and, as shown in Figure 6C, the mouse 3^{+/+} BMMCs, which displayed a higher intracellular calcium release in

Fig. 5. (A) PIP₃ levels are elevated to a substantially higher degree in SF-stimulated SHIP^{-/-} than in SHIP^{+/+} BMMCs. SHIP^{+/+} (□) and SHIP^{-/-} (■) BMMCs were starved for 4 h and then incubated with ³²P-orthophosphate for 90 min. SF (200 ng/ml) was then added for various times and cellular lipids extracted, deacylated, separated by HPLC and PIP₃ levels measured by liquid scintillation counting. Numbers refer to the fold increase and results are representative of four separate experiments. (B) A time course of SF-induced PLC γ -2 tyrosine phosphorylation in SHIP^{+/+} and SHIP^{-/-} BMMCs. SHIP^{+/+} and SHIP^{-/-} BMMCs were starved for 4 h and stimulated for the indicated number of minutes with 400 ng/ml SF. Cell lysates were immunoprecipitated with anti-PLC γ -2 (Santa Cruz). Western blotting was carried out with a monoclonal anti-phosphotyrosine antibody (4G10, UBI). The blot was then reprobed with anti-PLC γ -2 to confirm equal loading. (C) Kinetic analysis of SF-induced IP₃ production. SHIP^{+/+} and SHIP^{-/-} BMMCs were starved for 4 h and stimulated with 400 ng/ml SF for the indicated times. IP₃ levels were determined using a radioreceptor assay and the results are shown as IP₃ fold-increase versus time. Each point is the mean of duplicates $\pm$ SD and similar results were obtained in two separate experiments.

response to SF than the mouse 1^{+/+} BMMCs, also degranulated slightly more in response to this growth factor. However, even though the mouse 3^{+/+} BMMCs displayed a substantially higher intracellular calcium release in response to SF than the mouse 2^{-/-} BMMCs, the latter degranulated to a far greater extent, consistent with SHIP inhibiting a step in degranulation between intracellular calcium release and extracellular entry.

Lastly, to determine whether SF-stimulated draining of intracellular calcium stores was, as in extracellular calcium entry, dependent on PIP₃, intracellular calcium measurements carried out in the presence of EGTA were performed in the presence and absence of LY294002. As shown in Figure 6D, SF-induced calcium release from intracellular stores, in both SHIP^{+/+} and SHIP^{-/-} BMMCs, was decreased and delayed with increasing concentrations of LY294002. Complete inhibition of intracellular calcium release was achieved with 100 μ M LY294002. These results demonstrate that SF-induced activation of PI-3-kinase is not only upstream of extracellular calcium entry but also of intracellular calcium release.

Discussion

Taken together, our results suggest a model (Figure 7) in which binding of SF to normal primary mast cells (i.e. SHIP^{+/+} BMMCs) very rapidly activates PI-3-kinase. Previous studies have established that this activation is through the direct binding of the p85 subunit of PI 3-kinase, via its SH2 domains, to pY⁷¹⁹ within the activated SF receptor, c-kit (Lev *et al.*, 1992; Serve *et al.*, 1994).

Previous studies have also established that phosphorylation of Y⁷¹⁹ within c-kit is essential for SF-induced enhancement of Fc ϵ R1-mediated degranulation of normal mast cells (Vosseller *et al.*, 1997), consistent with an important role for PI-3-kinase in enhancing degranulation. Since the SF-induced release of intracellular calcium (which occurs within 20–40 s of exposure to SF) is inhibitable in both SHIP^{+/+} and SHIP^{-/-} BMMCs by PI-3-kinase inhibitors, it follows that PI-3-kinase is also activated within 20–40 s of exposure to SF. This activation of PI-3-kinase leads to the generation of PIP₃. In SHIP^{+/+} BMMCs, SHIP is then attracted to the plasma membrane, perhaps via its SH2 domain (Liu *et al.*, 1997b; Huber *et al.*, 1998) and degrades PIP₃ to PI-3,4-P₂ (Damen *et al.*, 1996). The dramatic difference in SF-induced PIP₃ levels achieved in SHIP^{+/+} and SHIP^{-/-} BMMCs reveals the critical *in vivo* role that SHIP plays in hydrolyzing this phosphoinositide in these cells. Interestingly, however, since we find that SF stimulates a similar draining of intracellular calcium stores within SHIP^{+/+} and SHIP^{-/-} BMMCs, we hypothesize that the level of PIP₃ achieved in SHIP^{+/+} BMMCs is sufficient (i.e. not limiting) to maximally trigger this signalling step. The lack of a detectable difference in the SF-induced intracellular calcium release in SHIP^{-/-} and SHIP^{+/+} BMMCs is consistent with our finding that the IP₃ levels are elevated to the same degree in SHIP^{+/+} and SHIP^{-/-} BMMCs and that there is no effect of the sphingosine kinase inhibitor, DHS, on SF-induced intracellular calcium release or degranulation in these cells. Our finding that SF increased the intracellular IP₃ levels to the same degree in SHIP^{+/+} and SHIP^{-/-} BMMCs

Fig. 6. (A) SF-induced release of calcium from intracellular stores was measured in BMMCs from four SHIP^{+/+} and SHIP^{-/-} mice. EGTA (5 mM) was added 100 s before the addition of 400 ng/ml SF (arrow). (B) SF-induced intracellular calcium levels in selected SHIP^{+/+} and SHIP^{-/-} BMMCs, in the presence and absence of EGTA. (C) SF-induced degranulation of SHIP^{+/+} and SHIP^{-/-} BMMC clones displaying low and high intracellular calcium release. (D) LY294002, at the concentrations indicated, or vehicle (DMSO) was added 25 min prior to addition of 5 mM EGTA to SHIP^{+/+} (left panel) or SHIP^{-/-} (right panel) BMMCs. SF was added 100 s later (arrow). Identical results were obtained in two separate experiments.

is also consistent with a recent study showing that overexpression of the p110 subunit of PI 3-kinase had no significant effect on IP₃ levels induced by BCR activation in A20 B cells (Scharenberg *et al.*, 1998) since PI-3-kinase overexpression might be predicted to have the same effect on PIP₃ levels as deleting SHIP.

Our results also demonstrate that, following this draining of intracellular calcium stores, there is another PIP₃-regulated step leading to or amplifying extracellular calcium entry that requires a higher and/or more prolonged

elevation of PIP₃ than occurs in SF-stimulated normal BMMCs. Deletion of SHIP results in PIP₃ increasing to levels that are sufficient for this step to occur (Figure 7). The possibility that PIP₃ might be playing a role downstream of intracellular calcium release has also been suggested recently by Bolland *et al.* (1998) from studies of SHIP's role in mediating FcγRIIB inhibition of B-cell receptor activation in the chicken DT40 B-cell line.

Exactly how PIP₃ mediates both the SF-stimulated intracellular release and extracellular entry of calcium in

Fig. 7. A model of SF-induced degranulation in SHIP^{+/+} and SHIP^{-/-} BMMCs.

BMMCs remains to be determined. However, pertinent to this issue, two recent reports involving overexpression of Btk have elegantly demonstrated a central role for Btk in the intracellular calcium release from B-cell lines (Fluckiger *et al.*, 1998; Scharenberg *et al.*, 1998). From their studies they hypothesize that Btk, via its PH domain, is attracted to PIP₃ generated in response to BCR activation. Binding of this tyrosine kinase to PIP₃ not only protects PIP₃ from hydrolysis by 5'-phosphatases like SHIP but also leads to the transphosphorylation [most likely by a Src family member (Rawlings *et al.*, 1996)] and autophosphorylation of Btk. This activates Btk and it phosphorylates/activates PLCγ1 and 2, which in turn generate IP₃ and intracellular calcium release. A similar scenario may be taking place in our SF-stimulated SHIP^{+/+} and SHIP^{-/-} BMMCs since Btk has been shown to be activated in response to FcεR1 crosslinking in BMMCs (Kawakami *et al.*, 1994; Hata *et al.*, 1998). Moreover, our results suggest that Btk rather than PIP₃ levels limit the release of intracellular calcium stores from SF-stimulated BMMCs since higher PIP₃ levels do not increase calcium release. This is consistent with recent studies showing that overexpression of Btk in A20 B cells dramatically increases the generation of IP₃ (Fluckiger *et al.*, 1998; Scharenberg *et al.*, 1998) while introduction of the p110 of PI-3-kinase does not (Scharenberg *et al.*, 1998).

As to the PIP₃ mediator(s) that might be playing a role downstream of intracellular calcium release, Bolland *et al.*

(1998) have suggested from studies of SHIP's role in mediating FcγRIIB inhibition of B-cell receptor activation in the chicken DT40 B-cell line, that SHIP inhibits extracellular calcium entry at a stage following intracellular calcium release, by limiting Btk association with the plasma membrane. However, it is difficult to reconcile, at least in our system, how Btk, if it is the only mediator involved, is limiting for intracellular calcium release but not for extracellular calcium entry (i.e. how does more PIP₃ at this latter stage attract more Btk when the latter was limiting for the first stage). It is also worth mentioning at this point that while B cells lacking Btk fail to proliferate in response to BCR activation (Rigley *et al.*, 1989), Btk-deficient mast cells develop normally and there is only a mild impairment in FcεR1-induced degranulation (Hata *et al.*, 1998). It is therefore possible that another Tec kinase family member and/or another PH-containing (e.g. PLCγ1; Barker *et al.*, 1998; Falasca *et al.*, 1998) or SH2-containing (Rameh *et al.*, 1995) protein acts, in addition to or instead of Btk in SF-stimulated BMMCs to mediate PIP₃-induced calcium fluxes.

In summary, we have clearly demonstrated that SHIP modulates growth factor-induced signalling events. Specifically, we have shown that in normal primary mast cells, SHIP is the major enzyme responsible for hydrolyzing SF-induced increases in PIP₃. We have also shown for the first time that PI-3-kinase is upstream of SF-stimulated intracellular calcium release and that

substantially less PIP_3 is required for this release than for extracellular calcium entry. Our results suggest that mutations rendering SHIP less active could lead to unregulated mast cell degranulation and it will be interesting to determine whether a subset of human atopic disorders are the result of mutations in this 5'-phosphatase. It will also be interesting to determine if the very recently cloned SHIP-2 (Pesesse *et al.*, 1997; Habib *et al.*, 1998), which is probably the non-hemopoietic homolog of SHIP, acts in a similar fashion.

Materials and methods

Mast cell isolation, Steel factor treatment and degranulation assay

Bone marrow cells from 4- to 8-week-old SHIP^{+/+}, SHIP^{+/-} and SHIP^{-/-} littermates were plated in methylcellulose (StemCell Technologies, Methocult M3234) supplemented with 30 ng/ml murine IL-3, 50 ng/ml murine SF and 10 ng/ml human IL-6 for 10–14 days and then harvested and grown in suspension in Iscove's modified Dulbecco's medium (IMDM) containing 15% FCS (StemCell Technologies), 150 μM monothioglycerol (MTG) (Sigma) and 30 ng/ml IL-3 (with or without 40 ng/ml SF). For degranulation studies, cells were washed with IMDM, starved for 2–4 h in IMDM, 10% fetal calf serum (FCS) and 150 μM MTG, resuspended in Tyrode's buffer (Ortega *et al.*, 1989) and then treated for 15 min with or without 400 ng/ml SF or 400 ng/ml IL-3. One minute prior to the addition of cytokine 5 mM EGTA was added when degranulation in the absence of extracellular calcium was determined. The degree of degranulation was determined by measuring the release of β -hexosaminidase (Nishizumi and Yamamoto, 1997).

Calcium measurements

SHIP^{+/+} and SHIP^{-/-} BMMCs (5×10^6 cells/ml) were incubated with 2 μM fura-2/AM (Molecular Probes) in Tyrode's buffer at 23°C for 45 min. Then the cells were washed, resuspended in 1 ml of the same buffer at 5×10^5 cell/ml in a stirring cuvette. Following stimulation by addition of 400 ng/ml SF or 300 ng/ml IL-3 $\pm$ 5 mM EGTA (added 50 s before stimulation), cytosolic calcium was measured by monitoring fluorescence intensity at 510 nm, after excitation of the sample with two different wavelengths (340 and 380 nm) using an MC200 monochromator from SLM AMINCO with a 8100 V3.0 software program.

Immunoprecipitation and immunoblotting

SHIP^{+/+} and SHIP^{-/-} BMMCs were starved as above and incubated for various times at 37°C with 400 ng/ml SF. Then cells were washed with phosphate-buffered saline (PBS), solubilized at 1.5×10^7 cells/ml with 0.5% NP-40 in 4°C phosphorylation solubilization buffer (Liu *et al.*, 1994) and subjected to immunoprecipitation as indicated. Western blotting was carried out as described previously (Liu *et al.*, 1994).

IP₃ measurements

Determinations of IP_3 levels were performed essentially as described by Scheid and Duronio (1996). Briefly, SHIP^{-/-} and SHIP^{+/+} BMMCs were incubated for several hours in cytokine-free RPMI 1640 plus 10% FCS, washed and resuspended at 10^7 /ml in phosphate-free RPMI 1640 containing 20 mM HEPES pH 7.4 and 1 mCi/ml ^{32}P -orthophosphate (carrier free, ICN) for 90 min at 37°C. Then SF (200 ng/ml) was added for various times and reactions were stopped by the addition of 3.75 ml of methanol:chloroform (2:1, v:v). Next, 2.4 N HCl (1.25 ml) and chloroform (1.25 ml) were added and the lower organic phase isolated. The upper aqueous phase was re-extracted with 1 ml of chloroform: methanol (2:1, v:v). The combined lower phases were then washed twice with 1 ml methanol:0.1 M EDTA in water (1:1, v:v) and dried under nitrogen. The dried lipids were then deacylated by adding 1.8 ml of methylamine reagent (methanol:25% methylamine:1-butanol, 45:7:42.8:11.4, v:v:v) and incubated for 50 min at 53°C. The deacylated lipids were dried *in vacuo*, resuspended with 1 ml of H₂O and dried again to remove the methylamine. The dried lipids were then resuspended in 2 ml of H₂O and extracted twice with 2 ml of 1-butanol:ethyl ether:ethyl formate (20:4:1, v:v:v) and the aqueous phase dried again and resuspended in 240 ml of H₂O. Two hundred microliters of each sample were applied to a Partisil 10 SAX ion exchange column. Following a 10 min wash with water, lipids were separated by a 60 min

linear gradient from 0 to 0.25 M ammonium phosphate pH 3.8, followed by a 50 min linear gradient from 0.25 to 1 M ammonium phosphate pH 3.8. One milliliter fractions were collected and analyzed by liquid scintillation counting. ATP, ^3H -I-1,4,5- P_3 (ICN) and ^3H -I-1,3,4,5- P_4 (ICN) were used to calibrate the column and eluted at 78, 90 and 110 min, respectively.

IP₃ measurements

IP_3 measurements were carried out using a commercial IP_3 radioreceptor assay (NEN Dupont) according to the manufacturer's instructions.

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