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Two Distinct Waves of Membrane-Proximal B Cell Antigen Receptor Signaling Differentially Regulated by Src Homology 2-Containing Inositol Polyphosphate 5-Phosphatase

Allyson K. Krahn,* Kewei Ma,† Sen Hou,* Vincent Duronio,† and Aaron J. Marshall2*

The phosphatidylinositol 3-kinase (PI3K) pathway plays a critical role in B cell activation and differentiation. Recruitment of pleckstrin homology (PH) domain-containing signal transduction proteins to the plasma membrane through binding to 3-phosphoinositide second messengers represents a major effector mechanism for PI3Ks. We have found that the PH domains of Bam32 and tandem PH domain-containing protein 2 (TAPP2) specify a temporally distinct wave of membrane recruitment compared with that of Bruton’s tyrosine kinase (Btk), with recruitment of these two adaptors representing a later stage of the response. In this study we provide direct evidence that PH domain-dependent recruitment of Btk to the membrane is blocked by coligation of the inhibitory receptor FcγRII in human B lymphoma cells. In contrast, recruitment specified by the Bam32 or TAPP2 PH domains is completely insensitive to FcγRII inhibition. This differential regulation can be accounted for by Src homology 2-containing inositol polyphosphate 5-phosphatase (SHIP) activity alone, as expression of membrane-targeted SHIP completely abrogated Btk recruitment, but had no inhibitory effect on Bam32 or TAPP2 recruitment. Strikingly, kinetic analysis revealed that membrane recruitment of Bam32 and TAPP2 is actually more rapid under “inhibitory” signaling conditions. Analysis of 3-phosphoinositide generation under activating and inhibitory signaling conditions indicated that recruitment of Bam32 and TAPP2 is inversely correlated with the SHIP substrate/product ratio (phosphatidylinositol 3,4,5-trisphosphate/phosphatidylinositol 3,4-bisphosphate). Overexpression of TAPP2 in B cells led to an increase in the sustained phase of the calcium response and increased NF-AT-dependent transcriptional activation after B cell Ag receptor ligation. Together, these results suggest that Bam32 and TAPP2 adaptors define a novel group of SHIP-activated targets of PI3K that regulate B cell Ag receptor signaling. The Journal of Immunology, 2004, 172: 331–339.

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he phosphatidylinositol 3-kinase (PI3K) pathway represents a broadly important regulator of cell growth and differentiation (1, 2). This signaling pathway is activated by several large classes of cell surface receptors, including receptor tyrosine kinases, kinase-linked receptors, and heterotrimeric G protein-coupled receptors. PI3Ks are phospholipid kinases that specifically bind the PI3K-generated second messenger phosphatidylinositol 3-phosphate (PtdIns(3)P), leading to their recruitment to the plasma membrane and/or conformational changes that activate signaling events that are dependent on PI3K in B cells is initiated by Bruton’s tyrosine kinase (Btk) (19). The PH domain of Btk specifically binds the PI3K-generated second messenger phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) in vitro (8, 9) and mediates membrane recruitment of Btk in vivo (20). Btk has been implicated in phosphorylation and activation of phospholipase Cγ (PLCγ), leading to influx of Ca2+ across the plasma membrane (4, 21). As Btk-deficient and PLCγ-deficient mice have defects in B cell development and activation similar to those of the p85 and p110α subunits (16) or the p110δ catalytic subunit (17, 18) have demonstrated that PI3K activity is involved in both early B cell differentiation and B cell activation. One well-established set of signaling events that are dependent on PI3K in B cells is initiated by Bruton’s tyrosine kinase (Btk) (19). The PH domain of Btk specifies the PI3K-generated second messenger phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) in vitro (8, 9) and mediates membrane recruitment of Btk in vivo (20). Btk has been implicated in phosphorylation and activation of phospholipase Cγ (PLCγ), leading to influx of Ca2+ across the plasma membrane (4, 21). As Btk-deficient and PLCγ-deficient mice have defects in B cell development and activation similar to those of the p85 and p110α subunits (16) or the p110δ catalytic subunit (17, 18) have

Abbreviations used in this paper: PI3K, phosphatidylinositol 3-kinase; Bam32, B cell adaptor molecule of 32 kDa; BCR, B cell Ag receptor; Btk, Bruton’s tyrosine kinase; EGFP, enhanced green fluorescent protein; PH, pleckstrin homology; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,4,5)P3, phosphatidylinositol 3,4,5-bisphosphate; PI(3)P, phosphatidylinositol 3-phosphate; PLCγ, phospholipase Cγ; PTEN, phosphatase and tensin homologue on chromosome 10; SHIP, Src homology 2-containing inositol polyphosphate 5-phosphatase; TAPP, tandem PH domain-containing protein.

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PI3K subunit knockouts, it has been postulated that these molecules form an important signalsome regulating B cell function (22).

Consistent with the functional importance of the PI3K pathway in B cell activation, the activation of this pathway is modulated by B cell coreceptors such as CD19/CD21, which enhances PI3K activation when coligated with the BCR (23, 24), and FcγRII, which antagonizes PI3K signaling when coligated with the BCR (25–27).

One important mechanism used by FcγRII to inhibit PI3K signaling is recruitment of the inositol phosphate Src homology 2-containing inositol polyphosphate 5-phosphatase (SHIP) and the resulting enhanced breakdown of PIP3 (25, 26, 28). The inhibitory effects of SHIP on BCR-induced PLCγ activation, Ca2+ flux, and inositol triphosphate production have been largely attributed to blockade of Btk activation, which is highly dependent on PIP3 (4, 21, 25). Thus, current models for PI3K signaling in B cells have largely focused on this Btk/PLCγ signalsome (22), with SHIP as a physiological negative regulator of this pathway.

In contrast to PTEN, SHIP phosphatase activity does not reverse the D3-phosphorylation of PIP3, but, rather, removes the D5-phosphate to produce a distinct product called phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2) (29). After BCR ligation, both PI3K and SHIP are activated, and PIP3 is rapidly and transiently produced, whereas PI(3,4)P2 appears to remain elevated after PIP3 has returned to baseline levels (30, 31), potentially due to the continued activity of SHIP. As PI(3,4)P2 is produced upon cellular activation in a PI3K-dependent manner (30, 32), it is assumed that this lipid has second messenger activities of its own that remain to be discovered (7).

Interestingly, different transformed lymphocyte cell lines show marked differences in levels of PIP3 and PI(3,4)P2 produced at baseline and upon activation (30, 31, 33). One study found that PIP3/PI(3,4)P2 levels positively correlate with SHIP expression, but negatively correlate with PTEN expression in T cell lines (33), suggesting that this lipid may be under the regulatory control of both SHIP and PTEN. In the absence of SHIP, mouse B cells and mast cells were shown to produce lower levels of PIP3 and PI(3,4)P2, but not PI(3)P, in response to stimulation (34, 35). Thus, SHIP expression and/or activation may be a critical fulcrum regulating the balance of PIP3 vs PI(3,4)P2 signaling.

Some recent studies have found positive signaling roles for SHIP or PI(3,4)P2 in IL-4 (36) and c-Kit (34) signaling, suggesting that there may be specific signaling mechanisms activated by PI(3,4)P2; however, the cellular mediators have not been clearly defined. We and others have recently described Bam32/DAPPP1, tandem PH domain-containing protein 1 (TAPP1), and TAPP2, a group of related PH domain adaptor proteins that are recruited to the plasma membrane in response to PI3K activation (31, 37–39). Intriguingly, the PH domains from these proteins were shown to bind strongly to PI(3,4)P2 in vitro, with this lipid representing the sole 3-phosphoinositide binding partner in the cases of TAPP1 and TAPP2 (38). Consistent with this in vitro binding specificity, TAPP1 was recently found to translocate to the membrane in response to H2O2, which selectively activates PI(3,4)P2 production in fibroblasts (40), and in response to CD28 stimulation, which activates PI(3,4)P2 production in SHIP-positive T cells (33). In this study we have tested the hypothesis that Bam32 and TAPP2 are positively, rather than negatively, regulated by FcγRII and SHIP and therefore specify a distinct wave of PI3K signaling in B cells.

Materials and Methods

Cells and reagents

BJAB and A20 B lymphoma cells were cultured in RPMI 1640 medium supplemented with 10% FCS, antibiotics, and 50 μM 2-ME (A20 cells only). BJAB cells were stimulated with 10 μg/ml goat anti-human IgM F(ab)2, or intact goat anti-human IgM (Jackson ImmunoResearch Laboratories, West Grove, PA). A20 cells were stimulated with 10 μg/ml rabbit anti-mouse IgG F(ab)2, or intact rabbit anti-mouse IgG (Zymed, San Francisco, CA). A plasmid construct encoding Myc-tagged TAPP2 was generated by cloning the full-length TAPP2 cDNA into pcdNA3.1 Myc/His vector (Invitrogen, Carlsbad, CA), in-frame with the C-terminal Myc tag. Constructs expressing enhanced green fluorescent protein (EGFP)-Bam32-Ph, EGFP-Btk-Ph, and EGFP-TAPP2-Ph fusions were described previously (31, 37). A plasmid encoding murine SHIP lacking its SH2 domain fused to the extracellular and transmembrane portions of murine FcγRII (25) was a gift from Dr. I. Ravetch (Rockefeller University, New York, NY). All constructs were verified by DNA sequencing before use. After G418 selection and cloning, stably transfected clones were screened for protein expression either by FACS (2.4G2 anti-murine FcγRII mAb for the FcγRII/SHIP fusion) or Western blot (9E10 anti-Myc mAb for TAPP2-Myc). The expression of endogenous human FcγRII and surface BCR was assessed using FITC-labeled anti-CD52 mAb (BD PharMingen, San Diego, CA) or FITC-labeled goat anti-human IgM (Fab')2 (Jackson ImmunoResearch Laboratories), respectively. Tyrosine phosphorylation was assessed by immunoblotting with 4G10 anti-phosphotyrosine (Upstate Biotechnology, Lake Placid, NY), according to the manufacturer’s specifications.

Live cell imaging

BJAB cells were transiently transfected with fluorescent protein fusions and imaged essentially as previously described (31). Briefly, cells were electroporated with the indicated construct, resuspended in medium containing 1% FCS, and plated in eight-well LabTek chambered coverglass slides (Nunc, Naperville, IL). After overnight incubation, cells were directly imaged in the chambered coverglass on an inverted laser-scanning confocal microscope (Fluoview; Olympus, New Hyde Park, NY) equipped with a stage-mounted microincubator (Harvard Instruments, Boston, MA) using settings calibrated to keep the four corner wells at 37 ± 1°C (only the corner wells were used for imaging). Stimulating Abs were added to the wells, and images were acquired at 30-s intervals. A similar procedure was followed for A20 mouse B lymphoma cells, but using the following electroporation conditions: 20 μg of plasmid DNA and 1 × 106 cells in 0.5 ml of complete medium were electroporated using a GenePulse instrument (Bio-Rad, Hercules, CA) set at 340 V and 950 μF.

Image analysis

The ratio of membrane vs cytoplasmic fluorescence was determined using ImageQuant software (Molecular Dynamics, Sunnyvale, CA) essentially as previously described (37). Briefly, regions of interest were established encompassing plasma membrane or cytoplasmic areas, and the average pixel intensities within those areas were calculated for cells pre- and poststimulation (see Fig. 4). The membrane intensities are divided by the cytoplasmic intensities for each cell, and the data are presented as the mean ratios obtained from 5–10 cells/condition. For the kinetic studies, a large image dataset was scored postacquisition by viewing high quality laser printouts of each image series. The first time point showing visible accumulation of fluorescence at the plasma membrane was identified for each individual cell. More than 50 cells were scored for each experimental condition. To control for observer bias in scoring, the same image dataset was scored by a second observer with no knowledge of the experimental variables, and no significant difference in the scoring results was observed.

Calcium mobilization assay

The relative intracellular Ca2+ concentration was determined by a ratio-metric method using Fluo-4 and Fura-Red dyes (31, 41). BJAB cells were resuspended at 1 × 106 cells/ml in prewarmed serum-free RPMI 1640 medium and incubated with 5 μg/ml Fluo-4/AM and 10 μg/ml Fura-Red/AM (Molecular Probes, Eugene, OR) for 30 min at 37°C. Cells were washed with room temperature HBSS (Life Technologies, Gaithersburg, MD) and resuspended at 2 × 106/ml in HBSS containing 1 mM CaCl2. Cells were analyzed for green (FL1) and red (FL3) fluorescence using a FACScan Calibur instrument (BD Immunocytometry Systems, Mountain View, CA), set to time-resolved acquisition mode. The indicated stimuli were added after 30 s, and acquisition was continued for a total of 7 min. Flow data were analyzed using FCS Assistant software (http://fcspress.com) to obtain the green/red fluorescence ratio over time. For some experiments EGTA was added to a 1.2-mM final concentration immediately before running the samples.
NF-AT activation assay

NF-AT activation was assayed using a NF-AT-luciferase reporter construct essentially as previously described (37). Eighteen to 20 h after transfection with the reporter construct, cells were harvested and plated in round-bottom, 96-well plates at 2 × 10^4/well. Triplicate cultures were incubated in medium alone, with the indicated concentrations of F(ab')_2 or intact goat anti-human IgM, or with 50 nM PdBu and 2.5 μM ionomycin (Sigma-Aldrich, St. Louis, MO). After 6 h the cells were lysed in 100 μl of reporter lysis buffer (Promega, Madison, WI), 10 μl of lyase was mixed with 50 μl of luciferase substrate (Promega), and luminescence was immediately measured on a 96-well plate format luminometer (MicroLumat Plus, Berthold, Bad Wildbad, Germany).

Measurement of 3-phosphoinositide levels

Cells were washed three times with phosphate-free medium before resuspension at 10^5/ml in 99% phosphate-free medium, in which the cells were starved for 1 h. Cells were then labeled with 0.5 mCi of orthophosphate (ICN, Costa Mesa, CA)/ml for 1.5 h at 37°C and stimulated with 10 μg/ml F(ab')_2, or intact goat anti-human IgM for the indicated times. Extraction of inositol phospholipids and HPLC analysis of deacylated lipids were performed as previously described (31). The amount of radioactivity contained in the elution peak for each lipid (two to five fractions) was summed to give the total counts for each lipid, and data were normalized to the phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) peak to adjust for fluctuations in total lipid labeling and recovery between samples.

Results

Effect of FcγRII coligation on PH domain-directed membrane recruitment events

Our previous work using cells expressing different GFP fusions indicated a marked difference in the membrane recruitment kinetics directed by the PH domains of Btk vs Bam32 and TAPP2 (31). To test whether these recruitment events are differently regulated at the level of inhibitory signaling through FcγRII, we compared membrane recruitment responses after stimulation with either F(ab')_2, anti-BCR Abs or intact anti-BCR Abs, which can coligate FcγRII with the BCR (42). As FcγRII-mediated inhibitory signaling has been primarily studied in murine cells, we first confirmed that this pathway is functional in our human B lymphoma model (Fig. 1). We confirmed that BJAB cells express human FcγRII using FACS staining (Fig. 1A) and found that FcγRII coligation inhibited PIP3 accumulation (Fig. 1B) and subsequently calcium mobilization and NF-AT transcriptional activation (Fig. 1, C and D). However, global levels of tyrosine phosphorylation were not detectably affected by FcγRII coligation (Fig. 1E), confirming that the upstream events in BCR signaling are intact.

The current model for FcγRII inhibitory signaling postulates that FcγRII coligation inhibits the PH domain-directed membrane recruitment of Btk (4, 21, 25); however, to our knowledge, this has not yet been demonstrated directly. We therefore compared the membrane recruitment of Btk-EGFP (20) after BCR stimulation with or without coligation of FcγRII (Fig. 2A). It was found that FcγRII coligation led to a nearly complete block in membrane recruitment, confirming the inhibitory effect at the level of Btk recruitment to the membrane. Over 50 cells per condition were scored for membrane recruitment, and membrane recruitment could be observed in 68% of F(ab')_2-stimulated cells, but in only 12% of intact Ab-stimulated cells. In contrast, membrane recruitment of Bam32 or TAPP2 PH domains was not detectably inhibited by FcγRII coligation (Fig. 2, B and C), with strong membrane recruitment observable in >85% of cells within 5 min after stimulation with either Ab. To confirm this lack of inhibition, we examined PH domain-mediated recruitment events in the murine B lymphoma A20, a well-characterized model for FcγRII inhibitory signaling (4, 27, 43). We also found no evidence for FcγRII inhibition of Bam32 recruitment in these cells (Fig. 2B). As shown previously (31, 37), BCR-induced recruitment of Bam32, TAPP2, and Btk is abrogated by pretreatment with the PI3K inhibitors wortmannin and LY294002 (Fig. 2 and data not shown), confirming the dependence of these events on PI3K activity. These results demonstrate that different PH domains can specify not only temporally distinct PI3K-dependent responses, but also distinct sensitivity to inhibitory signaling through FcγRII.

Differential regulation of PH domain-directed membrane recruitment events by SHIP

To determine whether SHIP activity can, in isolation, account for this differential regulation, BJAB cells stably expressing a chimeric protein consisting of the extracellular and transmembrane portions of FcγRII directly fused to SHIP were generated (Fig. 3A). These cells exhibited strong inhibition of BCR-induced PIP3 accumulation, calcium mobilization, and NF-AT transcriptional activation, even using F(ab')_2 stimulating Abs (Fig. 3, B–D), confirming the inhibitory function of this chimeric protein (25). It should be noted that this construct lacks both the cytoplasmic tail...
of FcγRII and the SH2 domain of SHIP, so the effects of this chimeric protein can be attributed to SHIP activity at the membrane. We then assessed membrane recruitment of Btk, Bam32, and TAPP2 fluorescent proteins in these membrane SHIP-expressing cells, and it was found that membrane recruitment of Btk was blocked, whereas there was no detectable inhibition of Bam32 or TAPP2 membrane recruitment (Fig. 3E). These results confirm that FcγRII inhibition of Btk recruitment is mediated through SHIP and demonstrate that membrane recruitment of Bam32 and TAPP2 is resistant to even very strong SHIP-mediated inhibitory signaling.

**Bam32 and TAPP2 as SHIP-activated targets of PI 3-kinase: more rapid kinetics of recruitment under inhibitory signaling conditions**

We applied image analysis and visual scoring to our confocal microscopy data to determine whether inhibitory signaling through FcγRII or SHIP affected the quantity or kinetics of membrane
recruitment. Inhibitory signaling did not appear to affect the overall percentage of cells showing visible membrane recruitment of Bam32 and TAPP2 within the 10-min imaging period after stimulation, with 80–90% of cells showing recruitment under all stimulation conditions. Quantitative analysis of the fluorescence in membrane vs cytoplasmic regions indicated that there was no decrease in the quantity of Bam32 or TAPP2 membrane recruitment under inhibitory signaling conditions; in fact, marginal increases were observed at 5 min poststimulation (Fig. 4). To address whether the kinetics of recruitment may be affected, a large image dataset was visually scored for membrane recruitment, and it was found that that membrane recruitment directed by the Bam32 and TAPP2 PH domains occurs more rapidly on the average under inhibitory signaling conditions than with BCR ligation alone (Fig. 5). This difference is evident within the first 3 min after stimulation, when a significantly higher proportion of cells treated under inhibitory signaling conditions shows membrane recruitment. However, within 5–10 min the cells stimulated with BCR ligation alone reached similar levels of membrane recruitment. These results indicate that not only are Bam32 and TAPP2 resistant to FcγRII/SHIP inhibitory signaling, but they may be preferentially recruited at early stages of the response in the presence of these signals.

Bam32 and TAPP2 recruitment correlates with the PI(3,4)P2/PIP3 ratio

To determine whether membrane recruitment correlates with specific 3-phosphoinositide levels under the different signaling conditions, we determined the relative cellular PIP3 and PI(3,4)P2 levels.
levels after BCR ligation in the presence or the absence of FcγRII coligation or membrane SHIP expression. As expected, PIP3 accumulation was very rapid, was markedly decreased by FcγRII coligation, and was severely inhibited in membrane SHIP-expressing cells (Fig. 6A), correlating well with the pattern of Btk PH domain-mediated recruitment. In contrast, PI(3,4)P2 levels peaked later, were only slightly decreased by FcγRII coligation, and were increased in membrane SHIP-expressing cells (Fig. 6B). The different effects of FcγRII coligation and membrane SHIP expression may be attributable to other SHIP-independent inhibitory effects of FcγRII that may generally depress PI3K activation and therefore limit the generation of PI(3,4)P2. Thus, although depletion of PIP3 can at least partially account for inhibition of Btk recruitment under inhibitory signaling conditions, it does not appear that differences in the absolute levels of PI(3,4)P2 can solely account for the more rapid recruitment of Bam32 and TAPP2 under these conditions. However, it is clear that under inhibitory signaling conditions, the PIP3/PI(3,4)P2 ratio is substantially decreased at the early time points encompassing the peak of the PIP3 response (Fig. 6C), and this shift in the SHIP substrate to product ratio correlates very well with Bam32 and TAPP2 recruitment. Together, these results provide support for the model that, whereas Btk recruitment is solely dependent on PIP3 levels, Bam32 and TAPP2 recruitment is largely dependent on PI(3,4)P2.

TAPP2 expression enhances calcium flux and NF-AT-dependent transcriptional activation

To test the hypothesis that TAPP2 may function as an effector of SHIP during BCR signaling, we generated BJAB cells stably expressing epitope-tagged TAPP2. As it is known that SHIP regulates BCR-induced calcium mobilization and consequently NF-AT activation, we assessed the effect of TAPP2 expression on these events. Surprisingly, TAPP2 expression had the opposite effect to that seen in cells expressing membrane SHIP: increased calcium flux and NF-AT activation were observed (Fig. 7). The effect on the calcium response is primarily on the sustained phase and appears to result from increased influx across the plasma membrane, as it is blocked by chelating extracellular calcium with EGTA. However, expression of the TAPP2 PH-EGFP fusion protein had no effect on BCR-induced NF-AT activation (data not shown), suggesting that this protein does not significantly affect calcium signaling. This result suggests that TAPP2 can regulate BCR signaling and may functionally interact with other SHIP-regulated pathways.

Discussion

After ~10 years of work on PH domain structure and function, an attractive model for the function of these domains as phospholipid-binding modules or, in some cases, dual lipid- and protein-binding modules has become the prevailing paradigm (7). In terms of B cell activation, the PH domain of Btk has become a prototype for this paradigm. Our data provide additional support for this model in a human B lymphoma model by directly demonstrating BCR-induced membrane recruitment of Btk and its inhibition by FcγRII or SHIP. Furthermore, we show a strong correlation between Btk PH domain-directed membrane recruitment and PIP3 levels in both kinetics and regulation by inhibitory signals.

This lipid-binding module paradigm is made more powerful by the data from in vitro binding studies of large panels of PH domains that provide support for the idea that individual PH domains have very distinctive patterns of specific lipid-binding activity (8, 38, 44), perhaps allowing them to sense distinctive activation states or subregions of cellular membranes. In terms of the PI3K signaling pathway, there are multiple PH domain proteins reported to bind to the PI3K substrates (PI(4)P and PI(4,5)P2) and each of the PI3K products (PI(3)P, PI(3,4)P2, and PI(3,4,5)P3), suggesting a strong potential for PI3Ks to regulate the localization and function of many proteins in distinctive ways. However, these possibilities largely remain to be proven in a cellular context. In the present study we have examined the possibility that PI3K-dependant, PH domain-directed membrane recruitment of Bam32 and TAPP2 is regulated in a fundamentally different way than that of Btk due to their apparent different lipid-binding patterns. Our results provide strong evidence that this is indeed the case, with the distinction occurring in their responses to inhibitory signaling through SHIP.

**FIGURE 6.** TAPP2 PH domain-directed recruitment inversely correlates with the PIP3/PI(3,4)P2 ratio. 32P-labeled phospholipids were extracted at the indicated times after stimulation, and PIP3 and PI(3,4)P2 were quantified by HPLC assay. A, PIP3 production. B, PI(3,4)P2 production. C, The peak PIP3/PI(3,4)P2 ratios under the different activation conditions were averaged over four experiments.
Both Bam32 and TAPP1/2 were reported to bind specifically to the SHIP product PI(3,4)P2 in vitro (38, 39); thus, their differential responses to SHIP inhibitory signaling in the context of B cells fit with their in vitro lipid binding specificities. However, it does not appear to be universally possible to predict in vivo responses from in vitro binding results. For example, Bam32 was also found to bind PIP3 with similar affinity to PI(3,4)P2, whereas the TAPPs showed no PIP3-binding ability; however, in our cellular model, both proteins behave similarly, and we have not observed recruitment of Bam32 at early time points corresponding to peak PIP3 accumulation and Btk recruitment. This suggests that PIP3 is not an important determinant of Bam32 recruitment in this cellular environment; however, other functions for PIP3 binding cannot be ruled out. Another well-studied example is the serine-threonine kinase Akt/PKB, whose PH domain binds both PIP3 and PI(3,4)P2 in vitro (45), similar to that of Bam32. However, unlike Bam32, Akt membrane recruitment and activation have been reported by several groups to be inhibited by FcγRII and/or SHIP inhibitory signaling (43, 46, 47). Thus, it is essential that the functional significance of in vitro binding activities is verified in a relevant cellular context.

One surprising finding from our study was the observation of more rapid recruitment of Bam32 and TAPP2 under inhibitory signaling conditions. This rapid recruitment does not appear to correlate with an increase in the overall level of PI(3,4)P2. One report found that overexpression of full-length Btk lead to increased cellular levels of PIP3, suggesting that in some cases PH domain proteins may be able to regulate levels of their lipid ligands, perhaps through protecting them from degradation by lipid phosphatases (4). Although its not clear whether this is a generalized phenomenon for PH domain proteins, we cannot rule out the possibility that PI(3,4)P2 levels may differ between cells transiently expressing Bam32 or TAPP2 PH domains and the untransfected cells used for lipid measurements. It is also possible that inhibitory signaling results in higher localized concentrations of PI(3,4)P2 in plasma membrane niches supporting rapid recruitment of Bam32 and TAPP2, or that the observed rapid recruitment reflects the alteration in the PIP3 to PI(3,4)P2 balance, as a reduction in the PIP3/PI(3,4)P2 ratio consistently correlates with rapid recruitment in our study. Our working hypothesis is that the altered PIP3/PI(3,4)P2 balance during “inhibitory” signaling leads to fundamental alterations in the assembly of membrane-proximal signaling complexes, perhaps favoring the stable membrane docking of PI(3,4)P2 effector molecules such as Bam32 and TAPP2.

The regulation of PI(3,4)P2 production as a whole is still poorly understood. PI(3,4)P2 production is reduced, but not completely abrogated, in SHIP-deficient cells (34, 35), suggesting that PI(3,4)P2 can also be produced by PI3K-dependent, SHIP-independent mechanisms, perhaps including phosphorylation of PI(4)P. It is intriguing that transformed lymphocyte cell lines vary markedly in the amounts of PI(3,4)P2 produced (30, 31, 33). High levels of PI(3,4)P2 appear to correlate with a SHIP-expressing, PTEN-nonexpressing phenotype among a group of four T cell lines (33), suggesting that PI(3,4)P2 levels may also be influenced by the presence of extracellular calcium. Similar results were observed with an independent, stably transfected clone (not shown). B, Wild-type or TAPP2-expressing BJAB cells were transiently co-transfected with an NF-AT-regulated luciferase reporter plasmid and stimulated with 1.25, 5, or 20 μg/ml F(ab’2)2 anti-IgM. Alternatively, wild-type BJAB cells were transiently cotransfected with luciferase reporter and either empty vector (Vector Tx) or TAPP2 vector (TAPP2 Tx). Note the markedly increased NF-AT activation in cells expressing TAPP2 either transiently or stably.
by PTEN. As BJAB cells produce exceptionally high levels of PI(3,4)P2, it will be important to determine the lipid phosphatase activity profile of these cells. It will also be important to determine what other factors may regulate PI(3,4)P2 levels; for example, both BCR and TCR signaling may activate the production of reactive oxygen species (48), which may lead to selective elevation of PI(3,4)P2 (49).

Our results clearly demonstrate that SHIP is not a blanket inhibitor of all PI3K-dependent signaling events and can have positive effects on a subset of PI3K effector molecules. We hypothesize that the PI3K activation leads to the assembly of at least two distinct types of signalosomes tethered to the inner leaflet of the plasma membrane: one dependent on PIP3, which in B cells involves Btk, B cell linker protein, and other molecules, and a second dependent on PI(3,4)P2, which involves Bam32, TAPP2, and other molecules (Fig. 8). SHIP could therefore change the “flavor” of the PI3K signal by tipping the balance between PIP3 and PI(3,4)P2 signalosomes. As PI(3,4)P2 is produced during positive signaling through Ag receptors and cytokine receptors, it is reasonable to assume that the function of this lipid and its targets will extend beyond inhibitory signaling. Its tempting to speculate that PTEN, the lipid phosphatase that directly reverses the enzymatic addition of the 3-phosphate added by PI3K, may serve the role of a global inhibitor to keep the PI3K pathway in check, whereas SHIP may instead function to modify specific cellular responses triggered upon PI3K signaling. Consistent with this idea, heterozygous deficiency of PTEN in mice leads to a high incidence of cancer, including lymphomas (50), whereas SHIP-deficient mice are not reported to have a predisposition to tumors (51, 52). This raises the possibility that deregulation of PIP3 (i.e., SHIP knockout mice) is not sufficient for cellular transformation, as deregulation of both the PIP3 and PI(3,4)P2 arms of the PI3K response (i.e., PTEN knockout mice) may be required.

The functions of Bam32 and TAPP2 as mediators of PI3K signaling have not been completely characterized, but it is clear that these molecules can play an important role in B cell activation. Bam32-deficient DT40 B cells have a variety of defects in BCR signaling, including defects in calcium signaling (53). Preliminary results from human cells overexpressing mutant Bam32 (A. Allam and A. J. Marshall, unpublished observations) and from Bam32-deficient mice (54) have confirmed that this molecule is required for normal BCR signaling. In the present study we demonstrated that TAPP2 can regulate BCR-induced calcium flux and activation of NF-AT, which is the first demonstration of a functional role for this molecule in signaling. It is intriguing that TAPP2 overexpression has positive effects, as this suggests that at least some PI(3,4)P2-dependent signaling events may generate positive signaling outcomes. Like other adaptors, TAPP2 probably functions by regulating the assembly of signaling complexes, thus determining the protein interaction partners of TAPP2 that will be critical for delineating its function at the molecular level. It is intriguing that several studies have identified positive signaling functions dependent on SHIP and/or PI(3,4)P2, such as protection from FcgRII-induced apoptosis in B cells (55), proliferation of myeloid cells in response to IL-4 (36), and phosphorylation of Akt on Ser473 in c-Kit ligand-stimulated mast cells (34). Our results suggest that Bam32 and TAPP1/2 are prime candidates for linking SHIP to such positive signaling functions.

Note added in proof. A study just published by Alessi and colleagues (56) found that both TAPP1 and TAPP2 can associate with the protein-tyrosine-phosphatase-like protein-1 (also known as FAP-1) and regulate its association with the plasma membrane, suggesting a potential mechanism through which these molecules can regulate signaling.

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References

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