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Phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3)/Tec kinase-dependent calcium signaling pathway: a target for SHIP-mediated inhibitory signals

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Tec family non-receptor tyrosine kinases have been implicated in signal transduction events initiated by cell surface receptors from a broad range of cell types, including an essential role in B-cell development. A unique feature of several Tec members among known tyrosine kinases is the presence of an N-terminal pleckstrin homology (PH) domain. We directly demonstrate that phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3) interacting with the PH domain acts as an upstream activation signal for Tec kinases, resulting in Tec kinase-dependent phospholipase Cγ (PLCγ) tyrosine phosphorylation and inositol trisphosphate production. In addition, we show that this pathway is blocked when an SH2-containing inositol phosphatase (SHIP)-dependent inhibitory receptor is engaged. Together, our results suggest a general mechanism whereby PtdIns-3,4,5-P_3 regulates receptor-dependent calcium signals through the function of Tec kinases.

Keywords: B-cells/inositol trisphosphate/phospholipase C/receptor/tyrosine kinases

Introduction

Receptors of virtually every type have been shown to stimulate the production of the phosphoinositides phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3) and PtdIns-3,4-P_2 (Auger *et al.*, 1989; Serunian *et al.*, 1991; Gold and Aebersold, 1994; Gold *et al.*, 1994; Okada *et al.*, 1996). These lipids are produced by the action of a family of enzymes which specifically phosphorylate the D3 position of the inositol ring in phosphoinositides (reviewed in Carpenter and Cantley, 1996; Vanhaesebroeck *et al.*, 1997), and are thought to act as inducible membrane targeting signals for proteins which carry domains capable of interacting with them. Several examples of domains with the ability to interact with PtdIns-3,4,5-P_3 or PtdIns-3,4-P_2 *in vitro* have now been described (Rameh *et al.*, 1995, 1997; Klarlund *et al.*, 1997). Recently, the task of determining whether and/or how each of these *in vitro* interactions translate into cellular functions was begun by three different groups who provided evidence that PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 act as upstream activation signals for the AKT serine/threonine kinase via an interaction with its pleckstrin homology (PH) domain (Franke *et al.*, 1995, 1997; Alessi *et al.*, 1997; Klippel *et al.*, 1997; Stokoe *et al.*, 1997). The cellular functions attributable to other *in vitro* protein–PtdIns-3,4,5-P_3/PtdIns-3,4-P_2 interactions remain uncharacterized, but the diversity of proteins which carry these domains suggests that PtdIns-3,4,5-P_3/PtdIns-3,4-P_2 are likely to act in an analogously diverse range of processes depending on the cellular context in which they are produced.

The PH domain of Bruton's tyrosine kinase (Btk), a member of the Tec non-receptor tyrosine kinase family, is among the protein domains capable of interacting with PtdIns-3,4,5-P_3 *in vitro* (Salim *et al.*, 1996; Rameh *et al.*, 1997). Tec kinases are an emerging family of proteins which are expressed in both hematopoietic and non-hematopoietic tissues. Four Tec members (Tec, Btk, Itk and Bmx) have closely homologous structures which include an N-terminal PH domain, followed by SH3, SH2 and tyrosine kinase domains (reviewed in Desiderio and Siliciano, 1994; Rawlings and Witte, 1995). These Tec kinases have been implicated in the signaling pathways of a variety of hematopoietic receptors, including several types of cytokine and antigen receptors (reviewed in Rawlings and Witte, 1995). The mechanism of their activation by these receptors is thought to involve a two-step process in which they receive a currently undefined signal which targets them to the vicinity of activated Src kinases, after which their activation occurs through a transphosphorylation/autophosphorylation mechanism (Mahajan *et al.*, 1995; Rawlings *et al.*, 1996). In terms of downstream targets, Btk is thought to play an important role in apoptotic signaling as well as the activation of Jnk kinases and phospholipase Cγ2 (PLCγ2) (Rigley *et al.*, 1989; Takata and Kurosaki, 1996; Uckun *et al.*, 1996; Kawakami *et al.*, 1997). Furthermore, B-cell overexpression experiments show that participation in PLCγ2 activation is a general property of Tec kinases, that their role in PLCγ2 activation is distinct from that of Src or Syk family kinases, and that they are particularly important for producing the sustained production of inositol trisphosphate (IP_3) required for store-operated calcium influx (Fluckiger *et al.*, 1998).

While progress has been made in identifying signaling pathways lying upstream and downstream from Tec kinases, little is known about Tec kinase structure–function relationships. Many investigators have attempted to under-

stand Tec signaling mechanisms by identifying ligands for the subdomains of these kinases. Four potential cellular ligands for Tec family PH domains have emerged from *in vitro* studies including: (i) G-protein β/γ subunits (Tsukada *et al.*, 1994); (ii) protein kinase C (most isoforms) (Yao *et al.*, 1994; Kawakami *et al.*, 1995); (iii) BAP-135, a 135 kDa protein of unknown function (Yang and Desiderio, 1997); and (iv) inositol-1,3,4,5-tetrakisphosphate (IP_4, a soluble inositol phosphate) and PtdIns-3,4,5-P_3 (Fukuda *et al.*, 1996; Salim *et al.*, 1996; Rameh *et al.*, 1997). Several proteins, including CBL, WASP and SAM-68 among others, have been proposed to function in Tec family signaling via their ability to interact with Tec family SH3 domains *in vitro* (Cory *et al.*, 1995; Bunnell *et al.*, 1996; Andreotti *et al.*, 1997). Finally, Tec has been proposed to function in linking cytokine receptors to Vav (through a direct association) and phosphatidylinositol 3-kinase (PI3K)-dependent signaling pathways (through an association with the p85 subunit of PI3K) based on co-precipitation and *in vitro* binding data (Machide *et al.*, 1995; Takahashi-Tezuka *et al.*, 1997). It is currently unclear whether and/or how each of the binding interactions described for various Tec family subdomains are integrated into the signaling function of these kinases. However, compelling evidence for the relevance of PH domain function to Tec kinase function exists in the form of spontaneously arising mutations in the Btk PH domain which cause X-linked agammaglobulinemia in humans and mice (reviewed in Conley and Rohrer, 1995; Rawlings and Witte, 1995). Furthermore, one such mutation, an arginine to cysteine substitution at position 28, has been shown to reduce the *in vitro* inositol polyphosphate- and phosphoinositide-binding activity of the isolated Btk PH domain (Fukuda *et al.*, 1996; Salim *et al.*, 1996; Rameh *et al.*, 1997).

Given the confusing picture of Tec kinase function which has emerged from studying the interactions of its various domains *in vitro*, we undertook a functional approach to studying Tec kinase signaling by examining how co-expressing them with other signaling proteins affects Tec kinase-regulated signaling events *in vivo*. This approach identified an activated form of the p110 subunit of PI3K (p110) as a prominent synergizing molecule when expressed with the Tec kinase prototype Btk in a fibroblast transformation assay. Subsequent expression of Btk, Itk and Tec with p110 in B cells showed a similar functional synergy at the level of the B-cell receptor (BCR)-initiated calcium signal, and this system was used for a detailed molecular analysis of the synergy mechanism. The results of this analysis suggest that PtdIns-3,4,5-P_3 initiates PLCγ2-dependent IP_3 production at least in part through its ability to interact with and activate Tec kinases. In addition, we show that FcγRIIb1, an inhibitory receptor which recruits the SHIP inositol 5′-phosphatase (Ono *et al.*, 1996), eliminates BCR-induced PtdIns-3,4,5-P_3 accumulation and results in blocked Tec kinase-dependent calcium signaling.

Results

p110-PI3K synergizes with Btk in a transformation assay

In order to identify signaling proteins which functionally interact with Tec kinases, proteins of interest were screened for their ability to transform rat fibroblasts synergistically with Btk. This screen involves assaying the transforming activity of Btk alone or in combination with other molecules using retroviral expression of the proteins of interest in a soft agar growth transformation assay, and has previously permitted receptor-independent analysis of Btk activation and signaling (Afar *et al.*, 1996). Using this assay, Btk or an activated form of p110, known as p110* (Hu *et al.*, 1995), each produce <10 colonies per plate, while their co-expression results in an ~6-fold enhancement in number of colonies. In contrast, co-expression of Btk with Cbl or Vav, both of which have also been shown to interact with Btk *in vitro*, does not produce a similar enhancement (Z.Li, D.J.Rawlings, H.Park and O.N.Witte, unpublished data).

p110–Tec kinase co-expression synergistically enhances BCR-mediated IP_3 production and calcium signals

Increased expression of Tec kinases in B cells produces an enhanced calcium signal characterized in particular by sustained calcium influx (Fluckiger *et al.*, 1998). Based on the results of the transformation screen, we investigated whether the p110 subunit of PI3K could functionally interact with Tec kinases during calcium signaling (Figure 1A). In a typical experiment, p110 expression produces only a minor change in the overall calcium flux relative to control infections, while Btk, Itk and Tec expression produce an enhanced calcium signal most evident at later time points. However, when expressed together with p110, the effects of Btk, Itk and Tec are substantially larger and more sustained—reproducibly greater than the additive effects of p110 and the kinase alone. Blots of the lysate (data not shown) demonstrate that similar amounts of each kinase are expressed when Btk, Itk and Tec are expressed alone or with p110.

In order to test whether the PI3K activity of p110 and not some other factor related to its increased expression level was responsible for the observed synergistic effects on the calcium signal, we examined the effect of wortmannin on the calcium flux generated by expression of Tec kinases alone or in combination with p110 (Figure 1B, note that the traces in this experiment were obtained from the same experiment as the Btk traces without wortmannin pre-treatment in Figure 1A). As expected and as previously reported, wortmannin inhibits the calcium fluxes of uninfected A20 B cells and of A20 B cells infected with a control recombinant vaccinia virus (Kiener *et al.*, 1997; A.M.Scharenberg and J.-P.Kinet, unpublished data; Figure 1B). Wortmannin is also able to reverse the minor enhancement of calcium flux seen with infection with the p110 virus alone, indicating that the effect of p110 on calcium mobilization is mediated through its phosphoinositide metabolites. In addition, wortmannin inhibits essentially all of the calcium flux enhancement generated by Btk expression alone, and substantially but incompletely inhibits the effect of p110–Btk co-expression. The complete inhibition of the effect of Btk expressed alone has the important implication that the action of endogenous PI3K is required for Btk to function in calcium signaling. The less complete inhibition of calcium mobilization in Btk–p110-expressing cells is in part expected, as the amount of wortmannin used would produce less complete

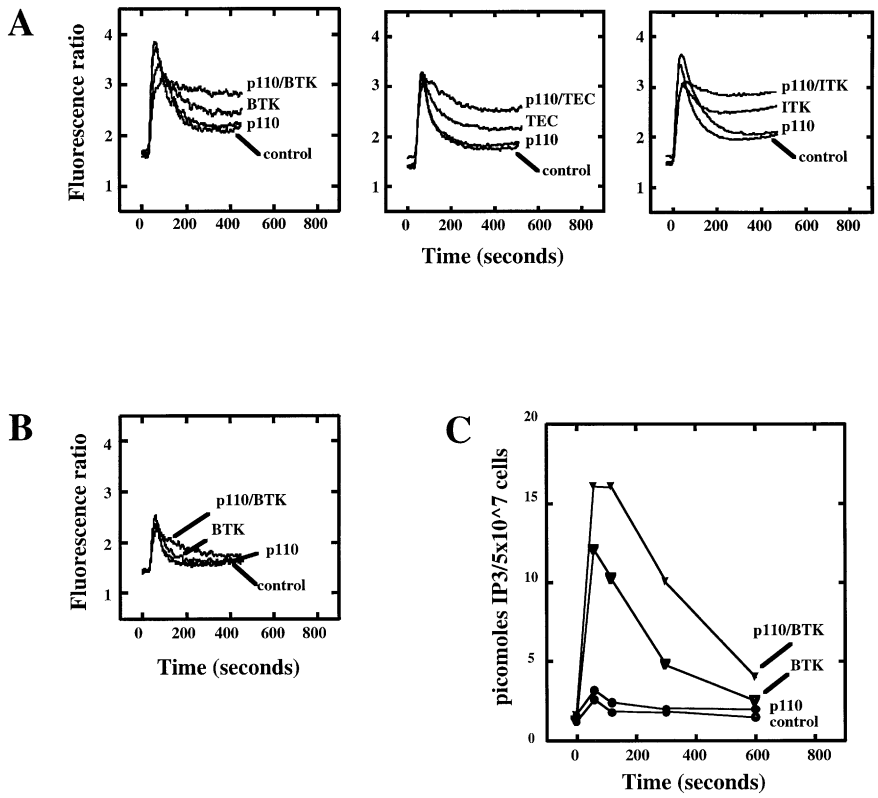

Fig. 1. p110–Tec kinase co-expression synergistically enhances BCR-mediated IP_3 production and calcium signals. (**A**) p110–Tec kinase co-expression produces synergistically increased calcium signals. Upper panels: A20 B cells were plated to 20% confluency and subsequently infected for 13 h with the indicated combinations of recombinant vaccinia viruses. The following morning, the cells were harvested, loaded with fura-2, and calcium mobilization in response to 15 μg/ml Fab′$_2$ rabbit anti-mouse IgG was measured in a bulk spectrofluorimeter at 30°C. (**B**) Btk-dependent enhancement of calcium signaling is inhibitable by wortmannin. The same experiment as (A), except that cells were pre-treated with 100 nM wortmannin for 10 min prior to the assay. (**C**) p110–Btk co-expression produces a synergistically increased IP_3 signal. Cells were infected and harvested identically to those in (A). After harvest, they were suspended at 5×10^7 cells/ml in calcium buffer and stimulated with 30 μg/ml of Fab′$_2$ rabbit anti-mouse IgG. After the indicated times of stimulation, cells were spun down rapidly and lysed in 20% trichloroacetic acid (w/v in H_2O), and prepared for IP_3 radioreceptor assay as specified in the manufacturer's protocol.

inactivation of PI3K activity in these cells because its pharmacologic target, the p110 PI3K subunit, is present in greater abundance. Similar results are obtained with other Tec kinases in the A20 cells (data not shown).

Tec kinase-dependent enhancement of calcium signaling occurs at least in part via enhanced production of IP_3 (Fluckiger *et al.*, 1998). Using Btk as a prototype, we evaluated whether increased Tec kinase expression in combination with p110 was affecting IP_3 production (Figure 1C). Control infected A20 cells produce an ~2-fold increase in IP_3 at 1 min after BCR engagement, which then decays back to just above baseline by 10 min. While expression of p110 alone increases IP_3 production only slightly relative to control, Btk expression alone produces a substantial enhancement, and p110–Btk co-expression results in synergistically higher and more sustained IP_3 production than Btk alone. Therefore, the synergy between p110 and Tec kinases observed at the level of the calcium signal correlates well with the production of IP_3, suggesting that p110 and Tec kinases are both somehow involved in the regulation of PLCγ2, the principal PLC isozyme utilized during BCR signaling.

p110–Tec synergy requires intact PH, SH2 and kinase domains

In order to begin to analyze the mechanism through which p110 and Tec kinases were acting on IP_3 production, we examined whether p110 would be able to complement the signaling capabilities of mutant versions of Tec kinases. Because of the availability of a variety of Btk mutants in our laboratory, Btk was again used as the prototype Tec kinase for these experiments. Btk constructs carrying inactivating mutations within each major domain [kinase (K430R), PH-R28C, ΔSH3 (full deletion) and SH2-R307K (Figure 2A–D)] were expressed in A20 B cells, and calcium flux (left panel) and IP_3 generation (right panel) were analyzed for expression of each mutant kinase alone or with p110 (designated black traces). Traces representing control and p110 alone infections for each experiment are shown in gray for purposes of comparison. As shown by the designated black traces in Figure 2A, the catalytically inactive mutant expressed alone is not able to enhance the calcium (left panel) or IP_3 signals (right panel) significantly, and co-expression with p110 is not able to complement its loss of signaling function. Of the Btk interaction domain mutants, the Btk-ΔSH3 produces both enhancement of signaling when expressed alone and synergistic signaling when co-expressed with p110 (Figure 2C, direct comparison of the ΔSH3 and wild-type Btk in other experiments showed that the ΔSH3 mutant reproducibly generated similar but somewhat larger effects than wild-type Btk, data not shown). The R28C (Figure 2B) and R307K (Figure 2D) mutants were unable to enhance calcium signaling nor did they show significant comple-

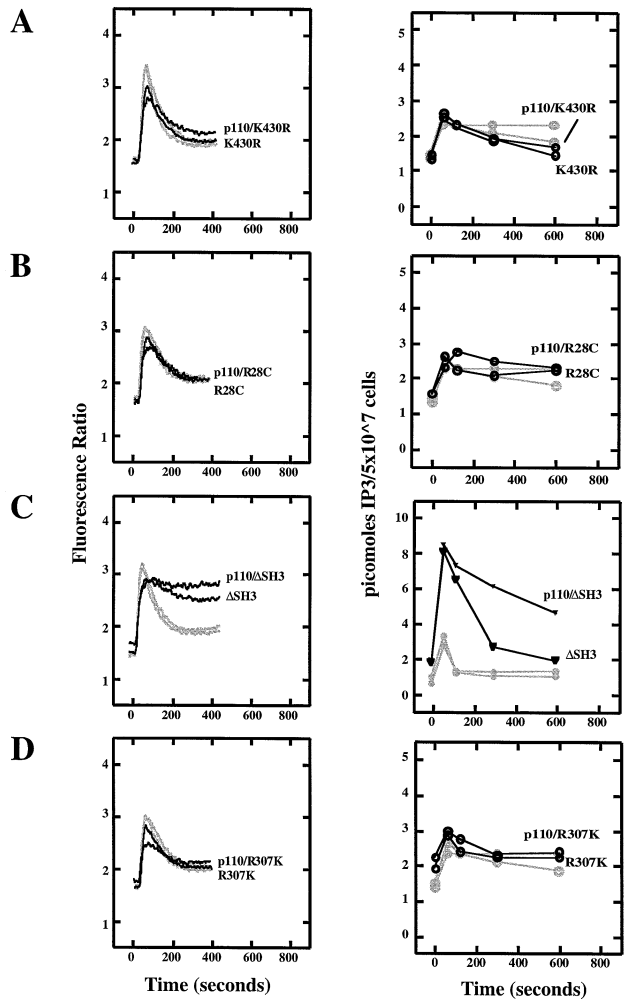

Fig. 2. p110–Tec synergy requires intact Btk PH, SH2 and kinase domains. (**A–D**) For calcium assays, A20 B cells were infected with the indicated vaccinia viruses, harvested, loaded with fura-2, stimulated and assayed in a manner identical to those in Figure 1. For IP_3 assays, A20 B cells were infected with the indicated vaccinia viruses, harvested, stimulated, lysed in 20% TCA, and processed for IP_3 assay in a manner identical to those in Figure 1. Note that for both the calcium and IP_3 assays, the K430R, R28C and R307K mutants were assayed in the same experiment, so that their control and p110 alone curves (shown in gray) are identical. The ΔSH3 was assayed in a separate experiment, so the control curves from that particular experiment are shown for comparison.

mentation of their signaling defects when expressed with p110.

p110–Tec co-expression synergistically enhances detectable PtdIns-3,4,5-P_3

Based on published reports of ligands of the various domains of Tec kinases (see Introduction) and the recent suggestion that p110 might be a cofactor for PLCγ activation (Rhee and Bae, 1997), the p110–Tec kinase synergy could reasonably be explained by four possible mechanisms: (i) p110-dependent enhancement of Tec kinase signaling; (ii) Tec kinase-dependent enhancement of p110 signaling; (iii) a combination of both of these mechanisms; or (iv) a cooperative mechanism in which Tec kinases and p110 act independently on PLCγ2 function. While identifying direct targets of protein kinases can be difficult in a cellular context due to the confounding effects of activation of other kinases, the direct products of p110-PI3K action are known—PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2. Therefore we decided to begin determining which of the above scenarios best explained the p110–Tec synergy by testing the hypothesis that Tec kinases were enhancing p110 signaling.

One implication of the hypothesis that Tec kinases act upstream from p110 would be that production of the direct p110 product would be increased. Available evidence suggests that PtdIns-3,4,5-P_3 is the primary direct product generated by p110 (via 3′ phosphorylation of PtdIns-4,5-P_2) while PtdIns-3,4-P_2 is generated indirectly via 5′ dephosphorylation of PtdIns-3,4,5-P_3 (Stephens *et al.*, 1991). Therefore, we first examined the effect of co-expression of p110 and Btk on the basal and stimulated levels of PtdIns-3,4,5-P_3 in A20 B cells (Figure 3A). In control cells, PtdIns-3,4,5-P_3 is just detectable in our TLC assay after 2 min of stimulation, consistent with what has been previously published on PtdIns-3,4,5-P_3 production in tumor B-cell lines (Gold and Aebersold, 1994). This production is enhanced significantly by expression of p110 alone, and increased marginally by expression of Btk alone at these expression levels (at higher expression levels achieved by longer infections, significant elevations in PtdIns-3,4,5-P_3 are detectable with Btk expression alone; A.M.Scharenberg, O.El-Hillal and J.-P.Kinet, unpublished observations; see also Figure 3C below). However, co-expression of Btk and p110 results in much higher levels of PtdIns-3,4,5-P_3 than p110 alone or Btk alone in both basal and stimulated conditions, indicating the presence of a p110–Tec kinase-dependent synergy on the level of cellular PI3K lipid products. As substitution of other Tec kinases in these experiments produced identical synergy effects (data not shown), we focused on Btk for subsequent experiments because of the ready availability of Btk mutants and immunological reagents in our laboratory.

A second implication of the hypothesis that Tec kinases act upstream from p110 is that loss-of-function Tec mutants would be expected to show a loss of the synergy at the level of PtdIns-3,4,5-P_3. Therefore, we compared the PtdIns-3,4,5-P_3 signal generated by co-expression of p110 and wild-type Btk with that generated by co-expression of p110 and inactive Btk (Btk-K430R, which is unable to calcium signal and is not complemented by p110, see Figure 2) (Figure 3B, left panel). Similar experiments were performed in NIH 3T3 fibroblasts in the absence of receptor stimulation (right panel). These experiments show that the inactive Btk mutant has equivalent or greater ability than wild-type Btk to enhance the PtdIns-3,4,5-P_3 signal. They also demonstrate that no hematopoietic-specific component is required for the synergistic PtdIns-3,4,5-P_3 signal.

Again assuming that Tec kinases act upstream from p110, the ability of inactive Btk to produce synergistic accumulation of PtdIns-3,4,5-P_3 when co-expressed with p110 could only be accounted for if p110 activation was being enhanced by Btk in a manner which was independent of Btk activity. In order to address this possibility, we used HPLC to analyze the effect of increased Btk expression on both PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2 (Figure 3C, left and right panels). Increased Btk expression is associated with a large (~3-fold) increase in PtdIns-3,4,5-P_3 levels with essentially no effect on PtdIns-3,4-P_2 levels. Since

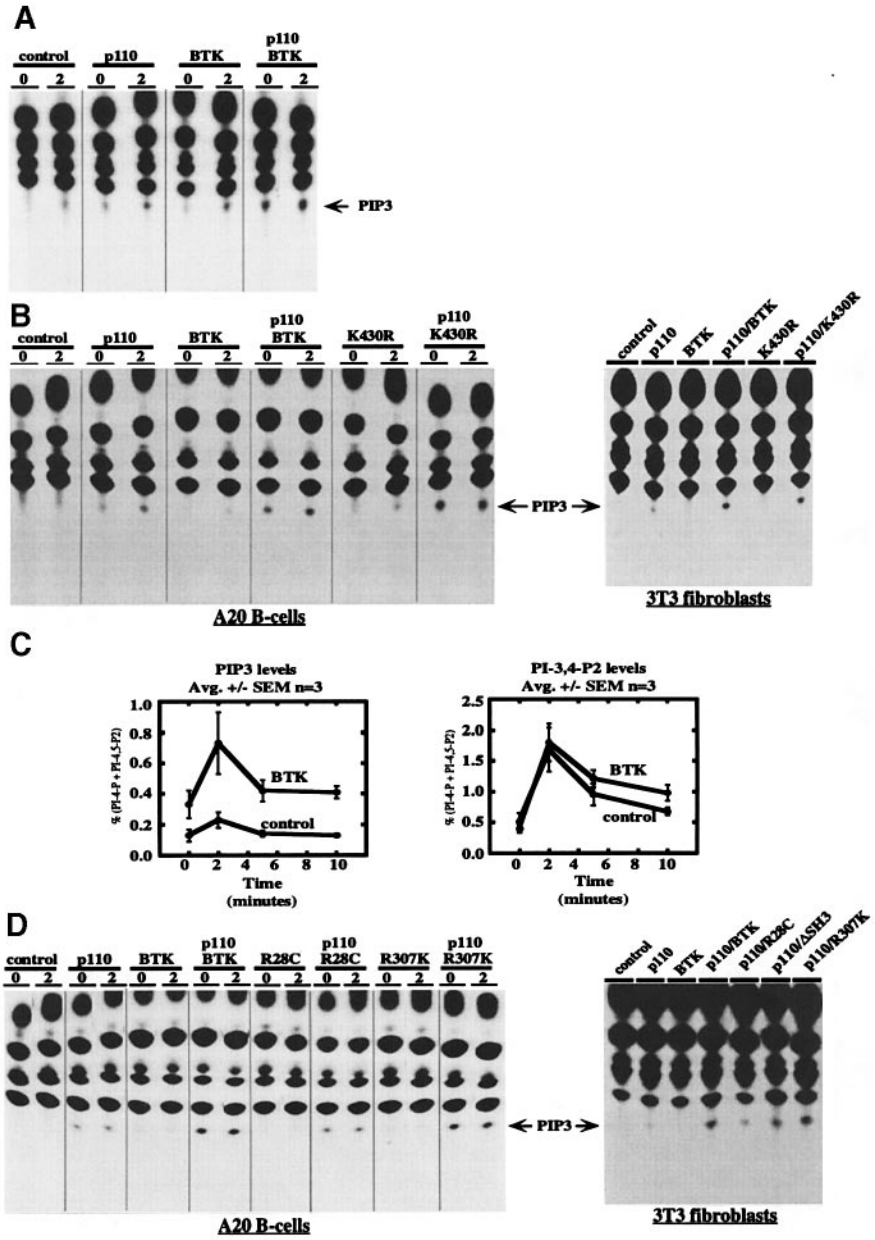

Fig. 3. p110–Tec co-expression synergistically enhances detectable PtdIns-3,4,5-P_3. (**A**) p110–Btk co-expression produces a synergistic enhancement of the amount of detectable PtdIns-3,4,5-P_3 (PIP3). Both panels: A20 B cells were infected for 13 h with the indicated vaccinia viruses, harvested, labeled with ^{32}P and stimulated or not for 2 min with 30 μg/ml Fab′$_2$ rabbit anti-mouse IgG. Lipids were then extracted and analyzed by TLC. (**B**) Btk kinase activity is not required to produce an enhanced PtdIns-3,4,5-P_3 signal. Left panel: A20 B cells were infected with the indicated vaccinia viruses, and then analyzed for PtdIns-3,4,5-P_3 levels as in (A). Right panel: NIH 3T3 fibroblasts were infected for 6 h with the indicated vaccinia viruses, harvested and labeled with ^{32}P. Lipids were then extracted and analyzed by TLC. (**C**) Increased Btk expression does not significantly affect production of PtdIns-3,4-P_2. A20 B cells were infected for 16 h with Btk alone or control vaccinia viruses. Cells were then harvested, labeled with ^{32}P, stimulated or not for 2 min with 30 μglml Fab′$_2$ rabbit anti-mouse IgG and lipids were extracted and analyzed by HPLC. Note that expression when using a single virus is typically greater than when co-infections are performed, so that Btk expression in these experiments was in the range of 2-fold greater than in all other experiments. (**D**) An intact PH domain but not SH2 domain is required to produce synergistic enhancement of PtdIns-3,4,5-P_3. Left panel: A20 B cells were infected for 13 h with the indicated vaccinia viruses, and then analyzed for PtdIns-3,4,5-P_3 levels as in (A). Right panel: NIH 3T3 fibroblasts were infected for 6 h with the indicated vaccinia viruses, and then otherwise processed as in (B).

p110 activation typically produces proportional accumulation of both PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2 (Hu *et al.*, 1995; Klippel *et al.*, 1996), the selective enhancement in PtdIns-3,4,5-P_3 levels suggests that Btk is not significantly affecting cellular PI3K activity.

Based on the above two experiments, we felt that it was unlikely that Tec kinases were acting upstream to activate p110 and enhance D3 phosphoinositide synthesis. The remaining explanation for the ability of increased Tec kinase expression to increase the accumulation of PtdIns-3,4,5-P_3 was that it was somehow reducing the rate of PtdIns-3,4,5-P_3 degradation. Since Btk is known to be capable of binding PtdIns-3,4,5-P_3 via its PH domain, the simplest explanation for how increased Tec kinase expression could decrease PtdIns-3,4,5-P_3 degradation was by binding to PtdIns-3,4,5-P_3 and thereby protecting it

from endogenous inositol phosphatases. We therefore tested which interaction domains of Btk were participating in the enhancement of PtdIns-3,4,5-P_3 accumulation by expressing the R28C and R307K Btk mutants alone or with p110-PI3K in A20 B cells (Figure 3D, left panel). The R28C PH domain mutation (which greatly reduces the binding of the isolated Btk PH domain to PtdIns-3,4,5-P_3 *in vitro*) eliminates the enhancement of the PtdIns-3,4,5-P_3 signal seen after co-expression of p110. In contrast, an enhanced PtdIns-3,4,5-P_3 signal identical to that of wild-type Btk is seen for the R307K SH2 FLVR mutant. A comparable result is obtained if the co-expression is performed in fibroblasts (right panel), again confirming that additional hematopoietic-specific components are not required. This experiment also shows that the ability of the ΔSH3 mutant to enhance PtdIns-3,4,5-P_3 detection is comparable with that of other forms of Btk (right panel), consistent with its containing an intact PH domain and its ability to produce enhanced calcium signaling. Finally, together with the lack of significant enhancement of PtdIns-3,4-P_2 accumulation in the HPLC experiment in Figure 3B above, this experiment provides *in vivo* data to support the previously described binding specificity of the Btk PH domain for PtdIns-3,4,5-P_3.

Btk-dependent PLCγ2 tyrosine phosphorylation is PI3K-dependent

The above data demonstrate that Tec kinase PH domains are interacting with PtdIns-3,4,5-P_3 in the context of our co-expression system, and that Tec kinases do not act as major upstream activation signals for p110. They therefore constrain the potential mechanisms for the functional synergy between Tec kinases and p110 to those in which PtdIns-3,4,5-P_3 acts either upstream from or together with the Tec kinase to promote IP_3 production and calcium mobilization through the activation of PLCγ2.

Since Btk is known to participate in the tyrosine phosphorylation of PLCγ2, we first evaluated whether the synergistic effect on PLCγ2 activation (as assessed by IP_3 production) could be accounted for via synergistic effects on PLCγ2 tyrosine phosphorylation. Using conditions identical to those used in the calcium synergy and IP_3 experiments, we expressed various combinations of p110 and Btk in the A20 B-cell line, and then analyzed the level of BCR-induced PLCγ2 tyrosine phosphorylation (Figure 4A). Relative to control infection, expression of p110 alone produces a slight enhancement of PLCγ2 tyrosine phosphorylation, consistent with the idea that p110 is able to functionally interact with endogenous Btk. In contrast, Btk expression alone produces an easily detectable enhancement, which is then enhanced further by co-expression with p110.

If enhanced PLCγ2 tyrosine phosphorylation is responsible for the functional synergy between p110 and Btk, then PLCγ2 tyrosine phosphorylation should be eliminated by Btk mutants which eliminate the synergy seen at the levels of IP_3 and calcium. We tested this by expressing p110 alone or with wild-type or loss-of-function Btk mutants and analyzing BCR-induced PLCγ2 tyrosine phosphorylation as above (Figure 4B). All loss-of-function Btk mutations greatly reduce the enhancement of PLCγ2 tyrosine phosphorylation produced by co-expression of p110 and wild-type Btk, consistent with the inability

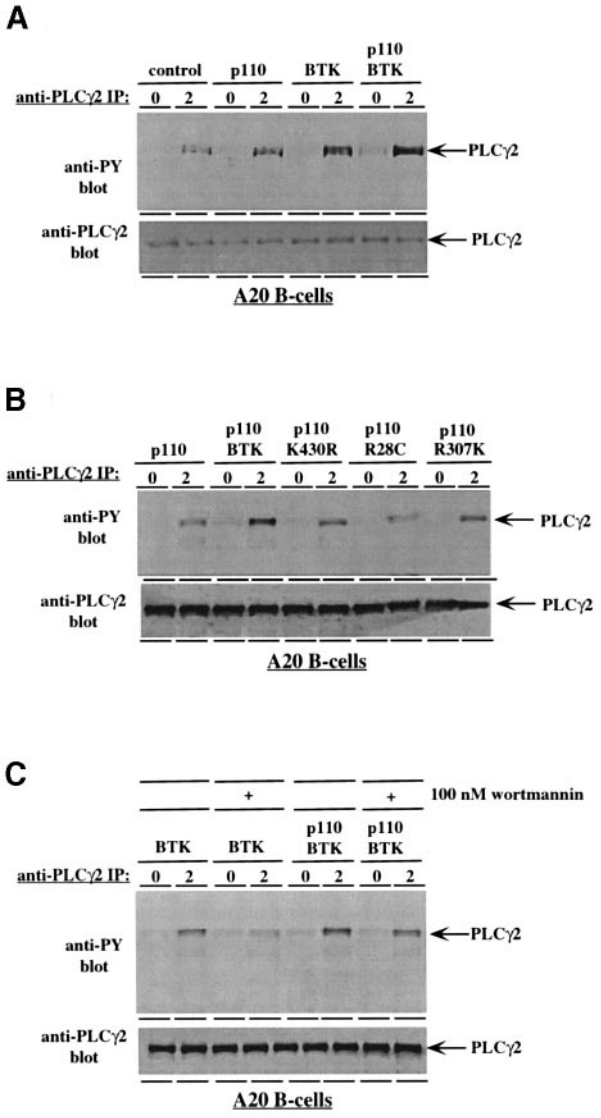

Fig. 4. Btk-dependent PLCγ2 tyrosine phosphorylation is PI3K-dependent. (**A**) BCR-mediated PLCγ2 tyrosine phosphorylation is enhanced by p110–Btk co-expression. A20 B cells were infected for 13 h with the indicated vaccinia viruses, harvested, washed once with calcium buffer, resuspended at 10^7 cells/ml in calcium buffer and stimulated or not for 2 min with 30 μg/ml Fab′$_2$ rabbit anti-mouse IgG. Cells were then quickly spun down and lysed, and post-nuclear supernatants were immunoprecipitated with the indicated antibodies or directly analyzed. (**B**) p110–Btk-dependent PLCγ2 tyrosine phosphorylation requires kinase activity and intact PH and SH2 domains. A20 B cells were infected for 13 h with the indicated vaccinia viruses, and otherwise processed identically to those in (A). (**C**) Wortmannin treatment of A20 B cells overexpressing Btk or p110–Btk inhibits PLCγ2 tyrosine phosphorylation. A20 B cells were infected for 13 h with the indicated vaccinia viruses, and otherwise processed identically to those in (A), except that the indicated samples were pre-treated for 10 min with 100 nM wortmannin.

of p110 to complement them in calcium signaling and IP_3 assays.

As a further test of the hypothesis that modulation of PLCγ2 tyrosine phosphorylation by p110–Btk accounted for the synergistic enhancement of calcium signaling, we treated cells overexpressing Btk or p110–Btk with 100 nM wortmannin for 10 min and analyzed PLCγ2 tyrosine phosphorylation (Figure 4C). Consistent with the effect of wortmannin on the calcium signal (see Figure 1

above), PLCγ2 tyrosine phosphorylation is inhibited by wortmannin pre-treatment in both Btk- and p110–Btk-overexpressing cells. The greater residual level of PLCγ2 tyrosine phosphorylation in p110–Btk-overexpressing cells than in cells expressing Btk alone may partially explain why wortmannin does not completely revert the effect of p110–Btk co-expression on the calcium signal (see Figure 1B above). Finally, the ability of wortmannin to attenuate the enhancement of PLCγ2 tyrosine phosphorylation produced by Btk expression alone has the important implication that Btk-dependent tyrosine phosphorylation of PLCγ2 is normally a PI3K-dependent process.

PtdIns-3,4,5-P$_3$ interaction with the Btk PH domain induces Btk autophosphorylation

The synergistic effect of p110–Btk co-expression on PLCγ2 tyrosine phosphorylation suggests that co-expression of Btk and p110 is either directly affecting Btk activation or is somehow co-localizing Btk and PLCγ2, with either scenario resulting in enhanced Btk-dependent tyrosine phosphorylation of PLCγ2. We and others have shown previously that Btk activation, which is identical to that seen after BCR stimulation, can be induced by co-expression of Btk with Src kinases, and that this occurs by enhanced autophosphorylation of Btk (Mahajan *et al.*, 1995; Rawlings *et al.*, 1996). Since enhanced Btk activation would explain the enhanced PLCγ2 tyrosine phosphorylation observed with p110–Btk co-expression, our initial investigations focused on whether p110 could be involved in promoting Src kinase-induced Btk autophosphorylation.

We first examined whether p110 alone was able to induce Btk autophosphorylation using the fibroblast co-expression system in which our previous studies were performed (Figure 5A, top left panel). p110 induces an easily detectable enhancement of wild-type Btk tyrosine phosphorylation which is entirely dependent on Btk activity. We then investigated whether Src kinases could synergize with p110 in inducing Btk phosphorylation (Figure 5A, top right panel) and whether Src kinase-induced Btk phosphorylation was wortmannin inhibitable (bottom left panel). Co-expression of Lyn and Btk or of p110 and Btk both induce Btk tyrosine phosphorylation, and expression of all three produces a further enhancement of this. In addition, treatment of cells expressing Lyn and Btk with 100 nM wortmannin substantially inhibits the Lyn-dependent Btk tyrosine phosphorylation. Alterations in Lyn activity (as assessed by its ability to induce tyrosine phosphorylation of the Syk tyrosine kinase under identical conditions) by wortmannin or p110 expression do not occur (data not shown). In further support of the idea that p110 acts in concert with Src kinases, p110γ produces a similar enhancement of Btk tyrosine phosphorylation only when it is co-expressed with Btk in a rat fibroblast line which constitutively expresses an activated Src mutant (srcE387G) (Li *et al.*, 1997). Identical p110-dependent enhancement of Btk tyrosine phosphorylation also occurs in the A20 B-cell environment when Btk and p110 are co-expressed (Figure 5A, bottom right panel), and is wortmannin inhibitable (data not shown). These results closely parallel the observed Btk-dependent PLCγ2 tyrosine phosphorylation and IP$_3$ production, consistent with our previous findings that Btk autophosphorylation is required for its activation (Rawlings *et al.*, 1996).

In order to analyze the mechanism of p110-induced Btk autophosphorylation, we examined the effect of the various Btk interaction domain mutations on the ability of p110 to induce Btk autophosphorylation (Figure 5B) in A20 B-cell and fibroblast environments. The R28C PH domain mutant eliminates p110-dependent Btk tyrosine phosphorylation in both environments. In comparison, the level of tyrosine phosphorylation of the ΔSH3 mutant is similar to that of wild-type Btk, and that of the R307K mutant is markedly enhanced. Together with the results from Figures 1–4, these results suggest that PtdIns-3,4,5-P$_3$ acts as an upstream activation signal for Btk through an interaction with the Btk PH domain, and that the SH3 and SH2 domains are dispensable for this process. It is important to note that the ΔSH3 mutant is missing one phosphorylation site at residue 223, so its phosphorylation level would normally be expected to be ~50% of wild-type Btk (Park *et al.*, 1996). The enhanced autophosphorylation observed for the R307K mutant was unexpected, but we speculate that since the Btk SH2 domain is required for Btk-dependent phosphorylation of PLCγ2, this mutation might somehow inhibit access of Btk to substrates, thereby enhancing its opportunities for autophosphorylation.

The FcγRIIB1–SHIP inhibitory receptor complex blocks PtdIns-3,4,5-P$_3$/Tec kinase-dependent calcium signaling

The inhibitory receptor FcγRIIb1 is expressed on B cells, where its co-engagement with the BCR results in an inhibitory signal which is thought to provide an important negative feedback mechanism for antibody production. The FcγRIIb1 inhibitory signal is known to cause a block in sustained calcium signaling due to its apparent ability to specifically block calcium influx (Bijsterbosch and Klaus, 1985; Amigorena *et al.*, 1992; Muta *et al.*, 1994), and recently has been shown to require the SHIP inositol phosphatase, an enzyme with the *in vitro* capability to degrade PtdIns-3,4,5-P$_3$ (Gupta *et al.*, 1997; Ono *et al.*, 1997). Since PtdIns-3,4,5-P$_3$/Tec kinase-dependent calcium signals are sustained as the result of enhanced calcium influx (Fluckiger *et al.*, 1998), we examined how co-engagement of the BCR and FcγRIIb1 affected PtdIns-3,4,5-P$_3$ levels and Tec kinase-dependent signaling (Figure 6). We first compared the levels of PtdIns-3,4,5-P$_3$ observed after either BCR engagement alone or co-engagement with FcγRIIb1 (Figure 6A). While BCR engagement resulted in a sustained PtdIns-3,4,5-P$_3$ signal, no PtdIns-3,4,5-P$_3$ was detectable in this experiment at any time point after BCR–FcγRIIb1 co-engagement, although in other experiments small amounts of PtdIns-3,4,5-P$_3$ could be detected at early time points only (data not shown). We then examined the effect of BCR–FcγRIIb1 co-engagement on Tec kinase-dependent calcium signaling and PLCγ2 tyrosine phosphorylation. In both control and Btk-overexpressing B cells, FcγRIIb1 was able to inhibit sustained calcium signals (Figure 6B) and Btk-dependent PLCγ2 tyrosine phosphorylation (Figure 6C). Similar results for the calcium signal were obtained when other Tec kinases were expressed (data not shown). Strikingly, analysis of Btk tyrosine phosphorylation in both control and Btk-overexpressing B cells showed no differences in Btk tyrosine phosphorylation after either BCR

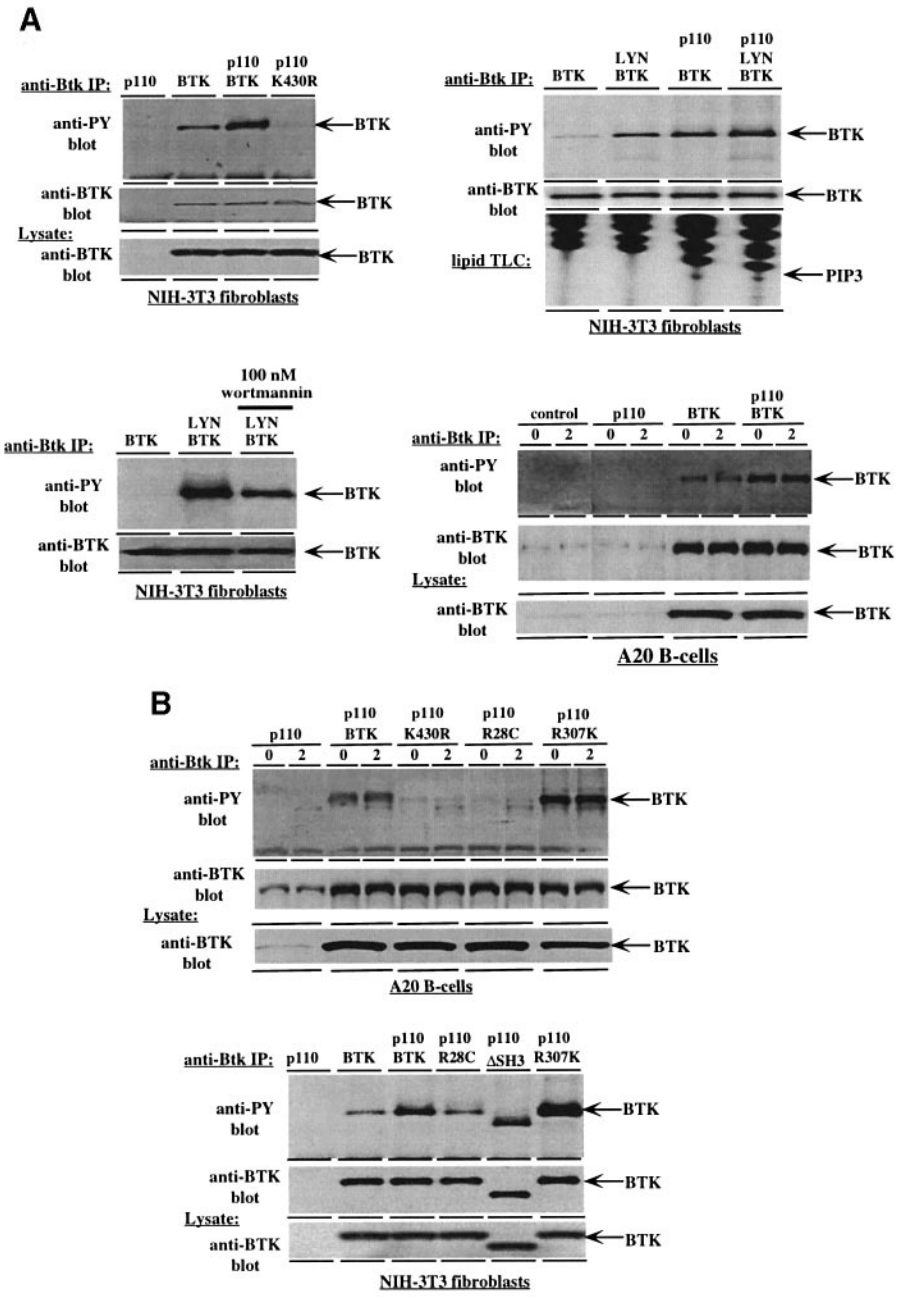

Fig. 5. PtdIns-3,4,5-P_3 interaction with the Btk PH domain induces Btk autophosphorylation. (**A**) Active p110 is able to induce Btk autophosphorylation. Top left and top right panels: NIH 3T3 fibroblasts were infected for 6 h with the indicated vaccinia viruses, harvested and resuspended at 10^7 cells/ml in calcium buffer. Cells were then quickly spun down and lysed, and post-nuclear supernatants were immunoprecipitated with the indicated antibodies or analyzed directly. Bottom left panel: NIH 3T3 cells were infected as indicated, and 100 nM wortmannin was added as indicated to the infection media for the last 1 h of infection. Cells were then processed in the same fashion as in the top panels. Bottom right panel: A20 B cells were infected for 14 h with the indicated vaccinia viruses and then harvested, stimulated and lysed as in Figure 4. Post-nuclear supernatants subsequently were immunoprecipitated with the indicated antibodies or analyzed directly. (**B**) p110-dependent Btk phosphorylation requires kinase activity and an intact PH domain but not an SH2 domain. Top panel: A20 B cells were infected for 14 h with the indicated vaccinia viruses and then otherwise analyzed as in the bottom right panel of (A). Bottom panel: NIH 3T3 cells were infected for 6 h with the indicated viruses, and then otherwise analyzed as in the top left panel of (A).

engagement alone or BCR–FcγRIIb1 co-engagement (data not shown). We therefore reasoned that perhaps only a small fraction of the total Tec kinases in the cells was participating in the receptor-induced signaling events. In order to produce an environment in which the high local concentration of signaling proteins likely to be present in BCR–FcγRIIb1 complexes would be able to affect the majority of the expressed Btk, we examined the effect of SHIP expression on both Lyn-induced and Lyn–p110-induced tyrosine phosphorylation of Btk in NIH 3T3 cells. Co-expression of SHIP, but not a catalytically inactive SHIP mutant, was able to block Lyn-induced Btk tyrosine phosphorylation (Figure 6D, left), and Lyn–p110-induced Btk phosphorylation (Figure 6D, right top). The expression of SHIP was also associated with a complete loss of the p110-

dependent PtdIns-3,4,5-P_3 signal, confirming the primary role of PtdIns-3,4,5-P_3 among other D3 phosphoinositides in Tec kinase activation (Figure 6D, right bottom).

Discussion

We have presented genetic, pharmacological and biochemical evidence that PtdIns-3,4,5-P_3 initiates Tec kinase activation and subsequent PLCγ2 tyrosine phosphorylation and IP_3 production. Our results provide a mechanistic link between D3 phosphoinositides and regulation of intracellular calcium levels, and represent only the second example of a cellular process which has been shown to be controlled directly through an interaction with a D3 phosphoinositide, the other being the activation of the Akt serine/threonine kinase during its role in anti-apoptotic signaling (reviewed in Toker and Cantley, 1997). The mechanisms through which Akt and Tec kinases are activated by interaction with D3 phosphoinositides appear to be strikingly analogous: in each case, phosphoinositide binding to the PH domain mediates their ability to be activated by an upstream kinase. In the case of Akt, PtdIns-3,4-P_2 interacts with the Akt PH domain and induces its partial activation. This mechanism is enhanced further by a second serine/threonine kinase, PDK-1, which is able to phosphorylate Akt and lock it into a fully active state. In the case of Btk, the prototype Tec kinase, interaction of its PH domain with PtdIns-3,4,5-P_3 is sufficient to produce Btk autophosphorylation in a cellular context. This effect is enhanced further by Src family kinases, at least in part through their ability to phosphorylate Btk within its activation loop (Rawlings *et al.*, 1996). Therefore, while interacting with distinct effector proteins in the context of quite different cellular processes, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 appear to utilize functionally equivalent mechanisms in which they promote the membrane association of their respective target kinase for the purpose of promoting its activation by a second kinase.

Taking into account what has been shown previously regarding the mechanism of Btk activation, a complete molecular mechanism for a Tec kinase calcium signaling pathway emerges based on our data: PtdIns-3,4,5-P_3 initiates Tec kinase activation in concert with Src kinases, as well as probably targeting the kinase to the plasma membrane. Once the Tec kinase is bound to PtdIns-3,4,5-P_3 and activated, an interaction between its SH2 domain and a tyrosine-phosphorylated ligand induced by BCR engagement is required to co-localize the activated Tec kinase and PLCγ2 for the purpose of tyrosine phosphorylating PLCγ2. The importance of the latter interaction is supported by (i) the normal to enhanced PtdIns-3,4,5-P_3-induced activation of the Btk-R307K SH2 mutant coupled with its inability to participate in BCR-mediated PLCγ2 tyrosine phosphorylation; and (ii) our previous results indicating that the Btk SH2 domain is required for Btk (activated by co-expression with Lyn) to phosphorylate

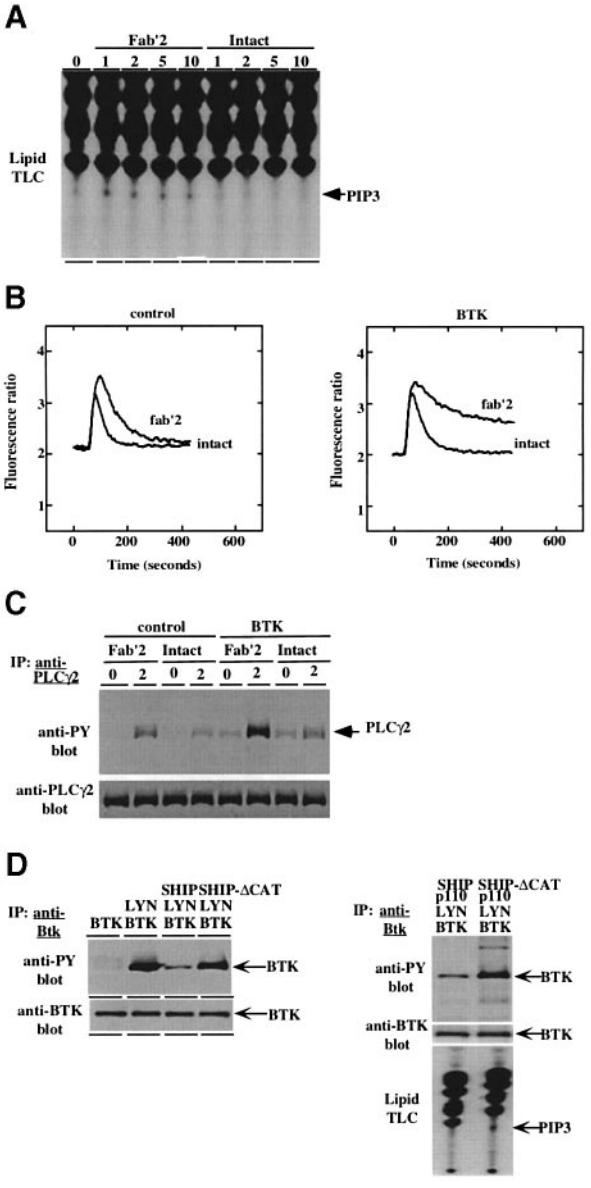

Fig. 6. SHIP-dependent PtdIns-3,4,5-P_3 degradation blocks PtdIns-3,4,5-P_3/Tec kinase-dependent signaling. (**A**) Co-engagement of the BCR and FcγRIIb1 blocks BCR-induced PtdIns-3,4,5-P_3 accumulation. Uninfected A20 B cells were labeled with ^{32}P and prepared for stimulation as above, then stimulated with either 15 μg/ml of Fab′$_2$ rabbit anti-mouse IgG or 30 μg/ml of intact rabbit anti-mouse IgG, followed by extraction and analysis of lipids as in Figure 3 above. Similar results were obtained with infected cells, although the magnitude of the Fab′$_2$-stimulated PtdIns-3,4,5-P_3 production was reduced. (**B**) Co-engagement of the BCR and FcγRIIb1 blocks Tec kinase-dependent calcium signaling. A20 B cells were infected either with control virus or Btk, loaded with fura-2, and prepared for calcium assays as described above. Cells were then stimulated with either 15 μg/ml Fab′$_2$ or 30 μg/ml rabbit anti-mouse IgG, while fura-2 fluorescence was monitored by bulk spectrofluorimetry. (**C**) Co-engagement of the BCR and FcγRIIb1 blocks Tec kinase-dependent phosphorylation of PLCγ2. A20 B cells were infected either with control virus or Btk, stimulated with either 15 μg/ml Fab′$_2$ or 30 μg/ml intact rabbit anti-mouse IgG, and PLCγ2 tyrosine phosphorylation was analyzed as described above. Identical results were obtained when stimulations were performed with intact rat anti-mouse IgG with or without pre-treatment with 2.4G2 (to block binding of the Fc fragment of intact rat IgG with the inhibitory Fc receptors), demonstrating that the differences in observed PLCγ2 tyrosine phosphorylation are not due to differences in the activation of the BCR. (**D**) SHIP blocks Lyn- and Lyn–p110-dependent activation of Btk. Left panel: Btk was expressed alone or with the indicated viruses, followed by analysis of phosphotyrosine content as in Figure 5. Right panel: Lyn, Btk and p110 were expressed with SHIP or with a catalytically inactive form of SHIP in NIH 3T3 cells. Each sample was divided into two parts. One part was used for analysis of Btk phosphotyrosine content as in Figure 5 (top), while the second part was used for lipid extraction and analysis of the level of PtdIns-3,4,5-P_3 as in (A) (bottom).

PLCγ2 when they are co-expressed in fibroblasts in the absence of receptor stimulation (Fluckiger *et al.*, 1998). In the case of BCR-mediated signals, candidate Tec SH2 ligands would be the Syk tyrosine kinase, which is also required for BCR-mediated calcium mobilization, and which is capable of binding both PLCγ2 and Btk *in vitro* (Takata *et al.*, 1994; Law *et al.*, 1996; Wan *et al.*, 1997), or PLCγ2 itself. In other systems, other receptor-associated kinases or a structural feature of the receptor itself might provide the co-localizing signal. This mechanism provides a full molecular framework with which to predict how mutations in Btk which cause X-linked agammaglobulinemia are able to interrupt signaling: PH mutations block Btk membrane targeting and all subsequent events including its activation, SH2 mutations block the ability of membrane targeted/activated Btk to co-localize with PLCγ2 or other substrates, and kinase domain mutations block the ability of membrane targeted/co-localized Btk to tyrosine phosphorylate PLCγ2 or other substrates.

The ability of PtdIns-3,4,5-P_3 to initiate Tec-dependent PLCγ2 tyrosine phosphorylation provides at least a partial molecular explanation for previous observations that wortmannin is able to inhibit BCR-mediated IP_3 production and calcium mobilization (Hippen *et al.*, 1997; Kiener *et al.*, 1997). In addition, our data further suggest that D3 phosphoinositides may play a second role in the regulation of PLCγ2, either alone or with Tec kinases. Comparison of the effect of wortmannin on PLCγ2 tyrosine phosphorylation (Figure 5C, compare lanes 2 and 8) and calcium fluxes (Figure 2A and B) in cells expressing Btk alone but not treated with wortmannin with those in cells co-expressing p110–Btk but treated with wortmannin, shows similar levels of PLCγ2 tyrosine phosphorylation but markedly different sustained levels of intracellular calcium. This implies that in addition to tyrosine phosphorylation, PLCγ2 requires another factor(s) to be present for it to hydrolyze its PtdIns-4,5-P_2 substrate to IP_3 effectively, a finding consistent with the normal levels of BCR-induced PLCγ2 tyrosine phosphorylation found in XLA-derived B-cell lines (Fluckiger *et al.*, 1998). Assuming that wortmannin is only blocking D3 phosphoinositide production, this second factor may involve direct regulation of PLCγ1/2 by PtdIns-3,4,5-P_3, as has been suggested for PLCγ1 (Rhee and Bae, 1997; Falasca *et al.*, 1998). However, we favor the possibility that the second factor involves PtdIns-3,4,5-P_3 acting together with Tec kinases independently of their activity based on the following: (i) the relatively small effect of overexpression of p110 alone on IP_3 and calcium (see Figure 1); (ii) the lack of dominant-negative effect on IP_3 and calcium when inactive Btk is overexpressed (see Figure 2); and (iii) a Btk-deficient form of the DT-40 cell line which produces no calcium signal, but regains a partial signal when it is re-transfected with inactive Btk (Takata and Kurosaki, 1996). A potential unifying explanation for these data is that PtdIns-3,4,5-P_3 functions as an inducible 'tag' for caveolae islands of PtdIns-4,5-P_2 (Pike and Casey, 1996); and that PtdIns-3,4,5-P_3/Tec kinase complexes function as adaptors to bring PtdIns-4,5-P_2 into proximity with activated PLCγ2. Identifying which of the above scenarios best explains PLCγ1/2 function is an important goal for future investigations.

The mechanism by which engagement of FcγRIIb1 with the BCR or other antigen receptor is able to inhibit sustained calcium signals has been the subject of much interest since the effect of FcγRIIb1 was first described. While FcγRIIb1 is known to recruit SHIP, it has been unclear whether SHIP-mediated breakdown of IP_4 (which has been linked to calcium influx in some cell types) or PtdIns-3,4,5-P_3 is responsible for the block in sustained calcium signaling. The results presented here demonstrate that engagement of FcγRIIb1 is associated with a lack of accumulation of PtdIns-3,4,5-P_3, inhibition of Tec kinase-dependent PLCγ2 tyrosine phosphorylation and inhibition of Tec kinase-dependent calcium signaling, and that SHIP is able to degrade PtdIns-3,4,5-P_3 and block Tec kinase activation in a heterologous system. Together, these data suggest that SHIP-dependent degradation of PtdIns-3,4,5-P_3 in the local neighborhood of BCR signaling complexes blocks calcium signaling by producing local deactivation of Tec kinases and consequent decreased PLCγ2 activation, as well as loss of the cofactor/substrate access functions of PtdIns-3,4,5-P_3 (as discussed above). As demonstrated in Fluckiger *et al.* (1998), the resulting inhibition of IP_3 production allows an initial endoplasmic reticulum (ER) calcium release followed by ER calcium store refilling and so a loss of the store-operated calcium influx required for sustained calcium signals, accounting for the observed apparent selectivity of FcγRIIb1 for blocking calcium influx.

In summary, our data implicate PtdIns-3,4,5-P_3 as a critical regulator of calcium signaling at least in part through its ability to initiate Tec kinase activation and resulting tyrosine phosphorylation of PLCγ2 and IP_3 production. A role for PtdIns-3,4,5-P_3–PH domain interactions in Tec kinase-dependent PLCγ activation provides a molecular basis for why mutations which eliminate this interaction in the B cell-specific Tec member Btk cause aberrant B-cell development in humans and mice, and a molecular explanation for the inhibition of calcium signaling mediated by the association of the SHIP inositol 5′-phosphatase with the inhibitory receptor FcγRIIb1. As exemplified by BCR–FcγRIIb1 co-engagement, the PtdIns-3,4,5-P_3/Tec kinase calcium signaling pathway provides a mechanism for cells to control the magnitude of calcium signals initiated by receptors which utilize PLCγ isozymes independently of the strength of the external stimulus to those receptors, and may explain the apparent involvement of PI3K/Tec signaling in signaling through co-stimulatory receptors such as CD19 or CD28. The tissue distribution of Tec kinases, the diversity of receptors capable of activating them, and the recent appreciation of the significance of receptor-mediated inhibitory signals involving inositol 5′-phosphatases (Scharenberg and Kinet, 1996) suggest that the PtdIns-3,4,5-P_3/Tec signaling mechanism is likely to be of broad importance.

Materials and methods

Cell culture, transformation assays, recombinant virus production and cDNAs

A20 B cells were grown in RPMI-1640 with 10% fetal bovine serum and 10^{-5} M 2-mercaptoethanol. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum.

A20 infections were performed by adding 5 p.f.u./cell of recombinant virus to ~20% confluent A20 cells and allowing infections to proceed for 12–15 h. Where appropriate, control recombinant virus was added so that all samples were exposed to an equal number of p.f.u./cell. A recombinant virus containing a cDNA encoding human Gβ1 inserted in an antisense orientation was used as the control virus because the transcript generated was similar in length to that of Btk.

The cDNAs for p110α, Tec, wild-type Btk and all mutant forms of Btk have been described previously (Hiles *et al.*, 1992; Rawlings *et al.*, 1996; Fluckiger *et al.*, 1998). The cDNA for the p110* construct has also been described previously (Hu *et al.*, 1995).

The retrovirus expressing p110* was constructed by subcloning the p110* cDNA into the pSRαMSVtk-neo retroviral recombination vector and selecting for recombinant retroviruses. Procedures used for the production of retroviruses for transformation assays have been described, as has the recombinant Btk retrovirus and the procedures used for the transformation assays (Afar *et al.*, 1996).

The p110-expressing recombinant vaccinia virus was constructed by subcloning a p110α cDNA into the pSC-66 vaccinia recombination plasmid. Recombinant viruses were then selected and amplified using standard techniques (Earl *et al.*, 1987). All recombinant Btk vaccinia viruses have been described previously (Rawlings *et al.*, 1996).

Pharmacologic reagents, antibodies, cell lysis, immunoprecipitations and Western blotting

Anti-phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology. Polyclonal anti-Btk has been described previously (Rawlings *et al.*, 1996). Polyclonal anti-PLCγ2 was obtained from Santa Cruz Biotechnologies. Stimulations of A20 B cells were performed with Fab'_2 rabbit anti-mouse IgG (15 μg/ml) or intact rabbit anti-mouse IgG (30 μg/ml) purchased from Jackson Immunoresearch. Wortmannin was obtained from Sigma.

Cells were lysed in borate-buffered saline (pH 8.0) containing 0.5% Triton X-100, 2 mM Na vanadate, 5 mM EDTA, 5 mM NaF and 5 μg/ml of leupeptin, aprotinin and pepstatin. Immunoprecipitations, Western transfer and Western immunoblotting were performed using standard techniques.

Phosphoinositide analyses

TLC analyses of phosphoinositides were performed by ^{32}P labeling of cells, extraction of lipids and TLC analysis as below. HPLC analysis of phosphoinositides was performed by ^{32}P labeling of cells, extraction of lipids, deacylation of lipids and HPLC analysis of glycero-phosphoinositides as previously described (Serunian *et al.*, 1991).

^{32}P-labeling of both B cells and fibroblasts was performed by placing cells in calcium buffer [135 mM NaCl, 10 mM KCl, 10 mM HEPES (pH 7.5), 5.6 mM glucose, 0.1% bovine serum albumin (BSA), 1 mM $MgCl_2$, 1 mM $CaCl_2$) for ~15 min, followed by addition of 1 mCi of [^{32}P]orthophosphate per 10^7 cells in 5–7 ml total volume and labeling for 1 h at 37°C with gentle agitation. Cells were then washed once in calcium buffer, resuspended in calcium buffer and stimulated, followed by lipid extraction and drying of lipids under N_2. Acidic chloroform/methanol lipid extraction was performed as described in Gold and Aebersold (1994), except that the method was adapated for use with microfuge tubes. TLC analyses of labeled lipids was performed by spotting 20% of lipid extract on silica gel 60 plates (Fischer) and chromatography for 5–6 h in 2:1 1-propanol/2 M acetic acid. Autoradiograms of TLC plates were obtained with an overnight exposure to a storage phosphor screen and measurement of incorporated radioactivity using a Molecular Dynamics STORM imager.

IP_3 and calcium assays

IP_3 levels were assayed using a commercial IP_3 assay kit (NEN Dupont) according to the manufacturer's protocols. Calcium mobilization was measured using fura-2 in a bulk spectrofluorimeter as previously described (Scharenberg *et al.*, 1995).

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