Interaction between the Btk PH Domain and Phosphatidylinositol-3,4,5-trisphosphate Directly Regulates Btk*

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Bruton's tyrosine kinase (Btk) binds to phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P3) through the Btk pleckstrin homology (PH) domain, an interaction thought to be required for Btk membrane translocation during B cell receptor signaling. Here, we report that interaction of PtdIns-3,4,5-P3 with the PH domain of Btk directly induces Btk enzymatic activity in an in vitro kinase assay. A point mutation that reduces interaction of PtdIns-3,4,5-P3 with the Btk PH domain blocks in vitro PtdIns-3,4,5-P3-dependent Btk activation, whereas the PH domain deletion enhances Btk basal activity but eliminates the PtdIns-3,4,5-P3-dependent stimulation. Btk kinase activity and the Btk activation loop phosphorylation site are both required for the PtdIns-3,4,5-P3-mediated stimulation of Btk kinase activity. Together, these results suggest that the Btk PH domain is positioned such that it normally suppresses both Btk kinase activity and access to substrates; when interacting with PtdIns-3,4,5-P3, this suppression is relieved, producing apparent Btk activation. In addition, using Src family kinase inhibitors and Btk catalytically inactive mutants, we demonstrate that in vivo, the activation of Btk is due to both Lyn phosphorylation and PtdIns-3,4,5-P3-mediated direct activation. Thus, the Btk-PtdIns-3,4,5-P3 interaction serves to translocate Btk to the membrane and directly regulate its signaling function.

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The abbreviations used are: BCR, B cell antigen receptor; IP3, inositol-1,4,5-trisphosphate (IP3); PI5-P, phosphoinositide-dependent 3-kinase or phospholipase Cγ2, to produce highly similar B cell immunodeficiencies in the mouse (7–11) and by the association of Btk mutations that abrogates phospholipase Cγ2 activation with X-linked agammaglobulinemia in humans (12, 13). Furthermore, Btk/PtdIns-3,4,5-P3-dependent IP3 signaling is blocked by the FcγRIIb1 inhibitory receptor, whose engagement with the BCR has been shown to inhibit BCR-mediated IP3 production, BCR-mediated calcium signaling, BCR-dependent antibody production, and B cell proliferation (14).

The Btk recruitment into activated BCR signaling complexes is thought to be a direct result of its interaction with PtdIns-3,4,5-P3 in the plasma membrane following local accumulation after BCR engagement (4, 5). This model of Btk activation is based on evidence from direct binding of the Btk PH domain and PtdIns-3,4,5-P3 in vitro (15, 16) and by phosphatidylinositol 3-kinase-dependent membrane translocation of the Btk PH domain fused to green fluorescent protein (15–17). After binding to PtdIns-3,4,5-P3, Btk then becomes activated via transphosphorylation through Src family kinase-dependent mechanisms and autophosphorylation of its kinase domain activation loop tyrosine (18). However, whether Btk activation also might be directly regulated by the Btk PH domain/PtdIns-3,4,5-P3 interaction has not been addressed. Here, we have investigated this question using an in vitro Btk kinase assay. Our data show that PtdIns-3,4,5-P3 is able to directly induce Btk enzymatic activity in vitro and, therefore, support a dual role for PtdIns-3,4,5-P3 in the activation of Btk signaling function, namely membrane translocation in proximity to activated B cell receptor complexes and direct regulation of Btk kinase domain signaling function.

EXPERIMENTAL PROCEDURES

Cell Culture, Recombinant Virus Production and cDNAs—A20 mouse B cells and Ramos human B cells were grown in RPMI 1640 with 10% fetal bovine serum and 10% fetal bovine serum and 10% calf serum. NIH3T3 cells were grown in RPMI 1640 with 10% calf serum.

A20 infections were performed by adding 5 plaque-forming unit/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 plaque-forming unit/cell. A recombinant virus containing a cDNA-encoding human Gβl inserted in an antisense orientation was used as the control virus because the transcript generated was similar in length to that of Btk.

The pSC-FLAG4 vector was constructed by ligating the FLAG epitope coding sequence and the following multienzyme restriction site sequence (GTGATTGGGCGCCGACCATGAGGCGGCGGCTAAGGAGCCAGAGGTGAACAAGGCGGGCGGTACCACTAGTGAATGGCGGCGGCGG) into the pSC-66 vaccinia recombination plasmid using 5 SalI and 3 KpnI restriction sites.

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ase selection, amplified, and titered using standard techniques (19). Other recombinant Btk and vaccinia viruses have been described previously (4, 6, 18).

Pharmacologic Reagents, Antibodies, Cell Lysis, Immunoprecipitations, and Western Blotting—Phosphatidylinerine (PS) was purchased from Avanti Polar. Lipids, phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2), and PtdIns-3,4,5-P_3 were purchased from Biomol. PS2 was purchased from Calbiochem. The enolase substrate was purchased from Sigma. Anti-FLAG M2 antibody was obtained from Sigma. Anti-stimulatory protein-tyrosine antibody 4G10 was obtained from Upstate Biotechnology.

Cells were lysed in Tris-HCl-buffered saline (pH 7.4) containing 1% Brij 97 or 0.5% Triton X-100, 2 mM sodium vanadate, 5 mM EDTA, 5 mM sodium fluoride, and 5 μM of leupeptin and pepstatin. Immunoprecipitations, Western transfer, and immunoblotting were described previously (20). The precipitates were subjected to Western blot analysis using enhanced chemiluminescent detection system (Amersham Pharmacia Biotech). The blots were analyzed using a Molecular Dynamics STORM imager and ImageQuant software.

Stimulation by Lipids and in Vitro Kinase Assays—In vitro kinase assay was carried out utilizing previously described standard protein kinase C vesicle assay (21). Briefly, mixed vesicles containing PS, PtdIns-4,5-P_2, or PtdIns-3,4,5-P_3, were solubilized on ice for 10 min in 10 mM Heps, pH 7.5. Reaction mixtures (50 μl) contained 20 mM Heps, pH 7.5, 5 mM MgCl_2, 5 mM MnCl_2, 10 μM ATP, 10 μCi of (γ-32P)ATP, and mixed phospholipid/phosphoinositide vesicles. For transphosphorylation experiments, 5 μM of acid-denatured enolase were added to the reaction mixture. Reactions were started by addition of Btk and Btk mutant-precipitated beads and incubated at room temperature for 3 min. Reactions were stopped by addition of 2× SDS-polyacrylamide gel electrophoresis sample buffer and heating to 100 °C for 5 min. Proteins were separated by 8% PAGE and transferred to nitrocellulose membranes. The blots were analyzed using a Molecular Dynamics STORM imager and ImageQuant software. For some experiments, the bands of Btk and enolase were excised, and the volume of the bands was measured using ImageQuant software.

Results

PtdIns-3,4,5-P_3 Induces Btk Phosphorylation—To analyze whether interactions between PtdIns-3,4,5-P_3 and the Btk PH domain might regulate Btk function, we first examined whether the presence of PtdIns-3,4,5-P_3 significantly in-
creased Btk autophosphorylation under these assay conditions and was highly specific. The effect observed with PtdIns-3,4,5-P₃ present was not detected with the substitution of the related polar lipid, PtdIns-4,5-P₂ (compare middle and right lanes of top panel). When added to whole cell lysates, PtdIns-3,4,5-P₃ also dramatically increased the phosphorylation of a 75–80-kDa protein in the whole cell lysates; the size of this protein corresponded precisely with the size of expressed Btk by Western blot against the anti-FLAG tag antibody (bottom panel).

Next, to eliminate the potential contribution of other B cell-specific signaling pathways, such as through the endogenous Lyn as well as through other hematopoietic phosphatases, we examined Btk produced in a non-hematopoietic cell line (Fig. 1B). As little as 1 μM PtdIns-3,4,5-P₃ significantly activated Btk produced in NIH3T3 cells compared with PtdIns-3,4-P₂ or

**Fig. 2. Activation of Btk as a function of PtdIns-3,4,5-P₃ concentration.** NIH3T3 cells were infected for 3 h with recombinant vaccinia viruses expressing FLAG-tagged Btk. Precipitates of Btk were prepared and processed in the same fashion as in Fig. 1B. The bands of Btk and enolase were excised and the incorporated radioactivity was measured by liquid scintillation counter. A dose response curve for activation of Btk by PtdIns-3,4,5-P₃ was obtained by varying the concentration of PtdIns-3,4,5-P₃ and maintaining a fixed concentration of total phospholipids (200 μM) by adjusting the concentration of PS. The dose response curve of Btk was representative of three separate experiments.

**Fig. 3. PtdIns-3,4,5-P₃-induced Btk phosphorylation is due to autophosphorylation: evidence for allosteric regulation of Btk activity.** A, the Src family kinase-specific inhibitor does not affect the PtdIns-3,4,5-P₃-enhanced *in vitro* kinase activity of Btk. Precipitates of Btk were prepared in the same fashion as in the top panel of Fig. 1A. Precipitates were treated with the indicated concentration of PP2 at 37 °C for 1 h and then assayed in the same fashion as in the top panel of Fig. 1A. B–D, analysis of various Btk mutants. NIH3T3 fibroblasts were infected for 3 h with the indicated vaccinia viruses, harvested, and lysed, and postnuclear supernatants were immunoprecipitated with the indicated antibody or analyzed directly. Top panels, precipitates were divided in two aliquots, treated or untreated with PtdIns-3,4,5-P₃, and analyzed for *in vitro* kinase activity as in Fig. 1. Bottom panels, whole cell lysates were analyzed by Western blotting using the indicated antibody. *In vitro* kinase activities of Btk mutants were representative from three sets of different experiments. Stimulation indexes (SI) were calculated as described under "Experimental Procedures." From the calculation of ³²P incorporation, ΔPH mutant showed 4.0–4.3-fold compared to the basal level of *in vitro* kinase activity of wild type Btk. The basal level of *in vitro* kinase activity of R28C mutant was reduced by 50–90% of the wild type. No ³²P incorporation was detected in the precipitates of the Δkinase or the K430R mutant. IP, immunoprecipitated; IB, immunoblotted; IVK, *in vitro* kinase.
endogenous Lyn or other Src family kinases, we first compared the effect of the Src family-specific inhibitor, PP2, to the enhanced phosphorylation of Btk by PtdIns-3,4,5-P3. Even at a concentration of 10 μM, PP2 did not affect the enhanced phosphorylation (Fig. 3A). (Note that we will show in Fig. 4, A and B that 10 μM PP2 inhibits Lyn activity efficiently but not Btk."

We then analyzed two different inactive versions of Btk (a kinase domain deletion mutant and the K430R ATP binding site mutant) (see Fig. 3, B and C). Neither of these mutants demonstrated any apparent kinase activity (either basal or PtdIns-3,4,5-P3-dependent) in the in vitro assay conditions. These data demonstrate unequivocally that the kinase activity observed in these assays is entirely due to the autophosphorylation kinase activity of Btk.

FIG. 4. Effect of the Src family kinase inhibitor on the tyrosine phosphorylation of Btk. A and B, incubation of A20 B cells with PP2 specifically affects the kinase activity of Lyn but does not affect the kinase activity of Btk. A20 B cells were infected with recombinant vaccinia viruses expressing FLAG-tagged Btk as shown in Fig. 1A. Cells were then incubated with the indicated concentration of PP2 at 37 °C for 30 min. Precipitates of Btk were prepared, treated with PS/PIP3, and tested with in vitro kinase assay in the same fashion as in the top panel of Fig. 1A. C, at least half of Btk can be autophosphorylated by PtdIns-3,4,5-P3 without transphosphorylation by Lyn. A20 B cells were incubated with indicated viruses as in Fig. 1A. Cells were incubated or not with 10 μM PP2 at 37 °C for 30 min, harvested, washed, and lysed, and postnuclear supernatants were immunoprecipitated with anti-Btk antibody. Precipitates were then subjected to SDS-polyacrylamide gel electrophoresis and analyzed by Western blot using antiphosphotyrosine antibody (4G10) and anti-Btk antibody. The volume of the tyrosine phosphorylation and the content of protein bands were measured. Tyrosine phosphorylation indexes were calculated as described under “Experimental Procedures.” The results are represented from two sets of different experiments. IP, immunoprecipitated; IVK, in vitro kinase.

Furthermore, we observed the enhanced kinase activity of Btk by PtdIns-3,4,5-P3 toward acid-denatured enolase used here as a substrate (Fig. 1D).

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We then examined the dose/response relationship of PtdIns-3,4,5-P3-dependent activation of Btk in vitro by measuring the phosphorylation of Btk and acid-denatured enolase (Fig. 2). The activation of Btk by various amounts of PtdIns-3,4,5-P3 was studied in the presence of PS to maintain a constant total lipid concentration (200 μM). The activation of Btk was observed at concentrations of 10 nM to 10 μM, thus yielding a 50% maximal activation (EC50) of ~100 nm. This result closely corresponds to the value reported by others for the affinity of the Btk PH domain for PtdIns-3,4,5-P3 (100–700 nM) and is consistent with a direct interaction between PtdIns-3,4,5-P3 and the Btk PH domain being involved in the kinase activation effect (16, 22).

PtdIns-3,4,5-P3 Dependent Phosphorylation of Btk Is Due to Autophosphorylation—To discriminate between autophosphorylation of Btk versus the presence of contaminating kinases such as PS alone. We also observed the same activation of endogenous Btk in the human B cell line (Fig. 1C).

At Least Half of Btk Is Autophosphorylated by the Interaction of PtdIns-3,4,5-P3 in Vivo—The above results showed direct interaction of PtdIns-3,4,5-P3 with the Btk PH domain with an enhancement of the kinase activity of Btk. On the other hand, we and others have described that Btk is also activated by the transphosphorylation of Src family kinase, Lyn, in B cells. Indeed, Btk is activated by both interaction of PtdIns-3,4,5-P3 and by transphosphorylation with Lyn. How significant is the autophosphorylation of Btk by the interaction of PtdIns-3,4,5-P3 while transphosphorylated by Lyn? To answer this question, we examined the tyrosine phosphorylation of Btk in A20 B cells with the overexpression of the constitutively active form of phosphatidylinositol 3-kinase, P110α, using PP2 and the K430R Btk to eliminate each one of the phosphorylation pathways. As seen in Fig. 4, incubation of A20 cells with 10 μM PP2 did not affect the kinase activity of Btk (Fig. 4A), whereas it significantly inhibited the kinase activity of Lyn (Fig. 4B).
The tyrosine phosphorylation of wild type Btk in PP2-treated cells was half that found in PP2-untreated cells; whereas in the K430R mutants, tyrosine phosphorylation of Btk was equivalent to that of wild type Btk in PP2-treated cells. These data suggest that in vivo, at least half of the Btk is autophosphorylated by PtdIns-3,4,5-P3 (Fig. 4C).

**DISCUSSION**

We have presented biochemical evidence that the interaction between PtdIns-3,4,5-P_3 and the Btk PH domain directly regulates the catalytic activity of Btk. This conclusion is based on the following results: the stimulation of the in vitro activity of wild type Btk by PtdIns-3,4,5-P_3, the disruption of this effect either by a PH domain mutation (R28C) known to eliminate its PtdIns-3,4,5-P3 binding activity or by deletion of the Btk PH domain, and the observation of enhanced basal activity in our Btk PH domain deletion mutant. Together, these findings suggest that the Btk PH domain normally acts to suppress Btk kinase activity and that this domain may in addition hinder access of the kinase domain to substrates. In either case, interaction with PtdIns-3,4,5-P_3 or PH domain deletion relieves the inhibition, producing a form of Btk that is now able to more effectively autophosphorylate and transphosphorylate substrates.

We and others have previously noted significant similarity between the role of PtdIns-3,4,5-P_3 in Btk activation and that proposed for d-3-phosphoinositides in regulation of the serine-threonine kinase, Akt/protein kinase B (Fig. 5) (5, 23). Full activation of wild type Akt requires phosphorylation on its activation loop (24). This phosphorylation occurs only in the presence of d-3-phosphoinositides. d-3-Phosphoinositides fail to promote the 3-phosphoinositide-dependent kinase 1 (PDK1)-mediated phosphorylation of a phosphoinositide-binding PH-deficient Akt mutant (R25C) and deletion of the Akt PH domain allows the Akt ΔPH molecule to be phosphorylated by PDK1 in the absence of phosphoinositides in vitro. These findings have led to the proposal that the Akt PH domain acts as an inhibitor of PDK1-dependent phosphorylation of Akt activation loop residues required for full activation and that d-3-phosphoinositide binding to the PH domain relieves these inhibitory effects (Fig. 5A). In contrast, the Btk activation mechanism has been proposed to consist of membrane targeting by the binding of PtdIns-3,4,5-P_3 to the Btk PH domain. Then, tyrosine phosphorylation of its activation loop occurs through Src family kinase, Lyn-dependent transphosphorylation and Btk auto-phosphorylation (Fig. 5B). Our data demonstrate that enhanced PtdIns-3,4,5-P_3-dependent Btk autophosphorylation is blocked in the Btk-Y551F activation loop mutant. This latter result demonstrates that this PtdIns-3,4,5-P_3-dependent Btk autophosphorylation primarily occurs at the activation loop tyrosine. This result is consistent with previous data (18) and, in conjunction with the Btk PH domain mutant data, suggests that in this "opened" form of Btk the activation tyrosine Y551 is more accessible to both Lyn and Btk (Fig. 5B). Modification of our previous Btk activation model, in light of these data, suggests that PtdIns-3,4,5-P_3-mediated regulation of Btk resembles the model proposed for d-3-phosphoinositide-mediated regulation of Akt.

**FIG. 5. Proposed mechanism for Btk activation by comparison with Akt activation.** The PH domains of PDK1, Akt, and Btk are depicted in red, and their kinase domains are depicted in dark blue. The yellow circles represent phosphorylation sites whereas the orange hexagons show d-3-phosphoinositides. A, the PH domains of PDK1 and Akt bind to membrane-bound d-3-phosphoinositides. This binding opens the enzymes and relieves the inhibitory effect of PH domains, allowing PDK1 to phosphorylate the activation loop threonine 308 (T308) of Akt. The R25C Akt, a phosphoinositide-binding, PH-deficient mutant, fails to phosphorylate on T308; deletion of the Akt PH domain results in the phosphorylation of T308 by PDK1 in vitro, in the absence of phosphoinositides. B, the PH domains of Btk binds to membrane-bound d-3-phosphoinositides. This binding relieves the inhibitory effect of the PH domain and provides access to membrane-attached Src family kinases such as Lyn. This allows Btk to autophosphorylate and permits Lyn-dependent transphosphorylation of Btk on tyrosine 551 (Y551). The R28C Btk, phosphoinositide-binding, PH-deficient mutant fails to phosphorylate on Y551; deletion of the Btk PH domain results in autophosphorylation of Y551 in vitro.
In conclusion, together with previous data, our results suggest that PtdIns-3,4,5-P$_3$ plays dual and probably functionally inseparable roles in the activation of PH-containing Tec family kinases; it provides proximity to target effector molecules (by targeting the kinase to the plasma membrane) as well as an activated and more accessible kinase domain (by opening up the kinase as suggested in this paper). These data suggest a modified model of Btk activation, which is similar to one previously proposed for the D-3-phosphoinositide-regulated serine-threonine kinase, Akt, in which D-3-phosphoinositides also played a dual localization and allosteric regulatory role. Such a mechanism is theoretically attractive for the control of signaling molecules like Tec kinases and Akt whose functions are closely linked to cell fate decisions as it provides a high level of assurance that their signaling function is directed toward the proper targets in the proper context.

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REFERENCES