A Role for Bruton's Tyrosine Kinase in B Cell Antigen Receptor-mediated Activation of Phospholipase C-γ2

By Minoru Takata and Tomohiro Kurosaki

From the Department of Oncology and Immunology, Wyeth-Ayerst Research, Pearl River, New York 10965, and Section of Immunobiology, Yale University School of Medicine, New Haven, Connecticut 06510-8023

Summary

Defects in the gene encoding Bruton's tyrosine kinase (Btk) result in a disease called X-linked agammaglobulinemia, in which there is a profound decrease of mature B cells due to a block in B cell development. Recent studies have shown that Btk is tyrosine phosphorylated and activated upon B cell antigen receptor (BCR) stimulation. To elucidate the functions of this kinase, we examined BCR signaling of DT40 B cells deficient in Btk. Tyrosine phosphorylation of phospholipase C (PLC)–γ2 upon receptor stimulation was significantly reduced in the mutant cells, leading to the loss of both BCR-coupled phosphatidylinositol hydrolysis and calcium mobilization. Pleckstrin homology and Src-homology 2 domains of Btk were required for PLC-γ2 activation. Since Syk is also required for the BCR-induced PLC-γ2 activation, our findings indicate that PLC-γ2 activation is regulated by Btk and Syk through their concerted actions.

M utations in the gene encoding the Bruton's tyrosine kinase (Btk)[1] are responsible for X-linked agammaglobulinemia (XLA), a severe primary immunodeficiency of boys characterized by the virtually complete absence of circulating B lymphocytes. An early developmental block is evidenced by an increase in pro-B cells and inefficient expansion and proliferation of pre-B cells in the bone marrow (for reviews see references 1, 2). A point mutation in the NH$_2$-terminal pleckstrin homology (PH) domain of the murine Btk causes a less severe X-linked immunodeficiency (Xid) of CBA/N mice (3, 4). Comparison of the phenotypes seen in XLA and Xid suggested the possibility that murine Xid mutation may not be a complete loss of function. Analyses of recently established btk$^{-/-}$ mice (5, 6), however, prove that lack of Btk function results in Xid phenotypes and suggest a differential requirement for Btk between murine and human B cell development.

Btk, along with Tec and Itk, comprises the Tec/Btk subfamily of Src-related tyrosine kinases (7–13). Btk has a unique NH$_2$-terminal region containing a PH domain and a proline-rich stretch, followed by Src-homology (SH) 3, SH2, and kinase domains (14, 15). SH3 domains have been shown to interact with proteins containing a short proline-rich motif, whereas SH2 domains interact with motifs containing phosphorylated tyrosine residues (for a review see reference 16). The PH domains have been found in many proteins involved in intracellular signaling pathways and, although of unknown function, have been speculated to be involved in protein–protein interactions. XLA patients display heterogeneity in mutations, which include deletions, insertions, and substitutions. Mutations in the kinase domain, as well as genetic alterations affecting PH, SH3, or SH2 domains can lead to the XLA phenotype (1, 2, 17). These observations indicate that multiple protein–protein interactions are essential for Btk function.

Xid mice are unable to respond to thymus-independent type II antigens (18, 19). In vitro studies also have shown that Xid B cells do not proliferate when triggered through their surface B cell receptor for antigen (BCR) (20, 21) and show hyporeactivity to LPS stimulation (19, 22). Inactivation by homologous recombination of membrane Igμ or of surrogate light chain encoding the λ5 gene leads to a block in progression from the pro-B cell to pre-B cell (23, 24), generating a phenotype in mice that resembles that seen in XLA patients. Thus, it is speculated that Btk is involved in the signaling activity through pre–B cell receptor as well as BCR.

The BCR complex contains, in addition to mIg, a heterodimer of Igα/Igβ, which is essential for signal transduction (for a review see reference 25). The BCR is endowed with a tyrosine kinase function by associating Src-family kinases,

Lyn, Fyn, and Blk, as well as Syk (for reviews see references 26–29). Src-family kinases are rapidly activated after BCR engagement (30, 31), and their activation correlates with the initial tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) on the BCR Igα and Igβ subunits. Syk is recruited to the phosphorylated Igα/Igβ ITAM (32) and is subsequently phosphorylated by Src kinase-mediated transphosphorylation and autophosphorylation at the 518 and/or 519 tyrosine sites, leading to the activation of Syk (33). In addition to Src kinase and Syk, it has been shown that Btk is also activated after ligation of the BCR in mature and immature B cells (34–37) and may be constitutively activated in pre-B cells (34). However, none of its in vivo substrates have thus far been identified.

Here we report that BCR-induced phosphatidylinositol (PI) hydrolysis and calcium mobilization are abolished in Btk-deficient DT40 B cells. Induction of tyrosine phosphorylation of phospholipase C (PLC)–γ2 was significantly reduced in Btk-deficient cells. These observations provide the evidence that PLC-γ2 could be a potential target of Btk and support a model for the action of at least three types of protein tyrosine kinases in BCR signaling.

Materials and Methods

Cells, Expression Constructs, and Abs. Wild-type DT40 and its mutant cells were cultured in RPMI 1640 medium supplemented with 10% FCS, antibiotics, and glutamine. Human Btk cDNA was obtained from UK DNA probe bank (Human Genome Mapping Project Resource Centre, Harrow, Middlesex, UK). Mutations in PH, SH2, or kinase domains were introduced by PCR using PFU polymerase (Stratagene Inc., La Jolla, CA), confirmed by sequencing, and subcloned into expression vector pApuro (38). Transfection into Btk-deficient cells was done by electroporation. Clones were selected in the presence of puromycin (0.5 μg/ml), and expression of Btk cDNA was verified by Western blotting. Anti–chicken IgM mAb M4, anti-PLC-γ2 serum, and anti-Syk serum were previously described (39). Anti-Btk mAb, anti-Cbl Ab, anti–mitogen-activated protein kinase (MAPK) Ab, anti-Shc Ab, and antiphosphotyrosine mAb 4G10 were purchased from PharMingen (San Diego, CA), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), New England Biolabs, Inc. (Beverly, MA), Upstate Biotechnology, Inc. (Lake Placid, NY), and Upstate Biotechnology, Inc., respectively.

Generation of Btk- and Lyn/Syk–deficient DT40 Cells. Chicken *btk* cDNA clones were isolated from a chicken spleen cDNA library (Clontech Inc., Palo Alto, CA) using human *btk* cDNA fragment as a probe under a low stringent condition. Chicken *btk* 7-kb EcoRI genomic clone was obtained by screening a genomic library that was constructed by ligating EcoRI-digested DT40 genomic DNA (∼6 to ∼9 kb) with vector arms of the Lambda ZAP II (Stratagene, Inc.). The targeting vectors, pcBTK-neo and pcBTK-hisD, were constructed by replacing the 0.7-kb BglII-BamHI genomic fragment containing exons that correspond to human Btk amino acid residues 91-124 with neo or hisD cassette. The targeting vector pcBTK-neo was linearized and introduced into wild-type DT40 cells by electroporation. Selection was done in the presence of 2 mg/ml G418. Clones were screened by

Southern blot analysis using 3' flanking probe (0.5-kb BglII-BglII fragment). The *neo*-targeted clone was again transfected with pcBTK-hisD and selected with both G418 (2 mg/ml) and histidinol (1 mg/ml). For generation of Lyn/Syk double-deficient cells, the targeting vector pSyk–blasticidin S resistance (bsr) was made by replacing the *neo* gene of pSyk-neo (38) with the *bsr* gene (Funakoshi, Tokyo, Japan) (40) and transfected into Lyn-deficient cells (38). Clones were selected in the presence of 50 μg/ml blasticidin S. Correct targeting was confirmed by reprobing the blots with internal neo, hisD, or bsr probe.

Immunoprecipitation and Western Blot Analysis. Cells were solubilized in lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris, pH 7.5, and 1 mM EDTA) containing 50 mM NaF, 10 μM molybdate, and 0.2 mM sodium vanadate supplemented with protease inhibitors (1 mM PMSF, 2 mg/ml aprotinin, 0.5 mM benzamidine hydrochloride, 10 mg/ml chymostatin, 0.1 mM Nα-p-tosyl-L-lysine chlorometyl ketone (TLCK), 0.1 mM N-1-tosyl-amide-2-phenylethylethylchlorometyl ketone (TPCK), 10 mg/ml leupeptin, 10 mg/ml antipain, 10 mg/ml calpain inhibitor I, and 10 mg/ml pepstatin) (41). For immunoprecipitation, precleared lysates were sequentially incubated with Abs and protein A–agarose. Lysates or immunoprecipitates were separated by SDS-PAGE gel, transferred to nitrocellulose membrane, and detected by appropriate Abs and the enhanced chemiluminescence system (ECL); (Amersham Corp., Arlington Heights, IL). Deprobing and reprobing were done according to the manufacturer's recommendations. To examine in vitro kinase activity of human Btk and its mutants, immunoprecipitated Btk was suspended in kinase buffer (20 mM Tris, pH 7.5, 10 mM MgCl$_2$, and 0.1% Triton X–100) containing γ-[^{32}P]ATP (>3,000 Ci/mmol; Amersham Corp.), and incubated at room temperature for 10 min. For in vitro kinase assay of Lyn and Syk (see Fig. 4), cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (1% NP–40, 0.25% sodium deoxycholate, 150 mM NaCl, 20 mM Tris, pH 7.5, and 1 mM EDTA) supplemented with the phosphatase and protease inhibitors described above. Immunoprecipitates were suspended in kinase buffer (20 mM Tris, pH 7.4, 10 mM MnCl$_2$ for Lyn, or 20 mM Hepes, pH 8, 150 mM NaCl, and 10 mM magnesium acetate for Syk) containing γ-[^{32}P]ATP, and incubated at room temperature for 10 min.

Northern Blot Analysis. RNA was prepared from wild-type and mutant DT40 cells using the guanidium thiocyanate method. Total RNA (20 μg) was separated by 1.5% agarose, transferred to Hybond-N membrane, and probed with a chicken Btk cDNA fragment.

Calcium Measurements. Cells (5 × 10^6) were suspended in PBS containing 20 mM Hepes, pH 7.2, 5 mM glucose, 0.025% BSA, and 1 mM CaCl$_2$, and loaded with 3 μM Fura-2/AM at 37°C for 45 min. Cells were washed twice and adjusted to 10^6 cells/ml. Continuous monitoring of fluorescence from the cell suspension was performed using a fluorescence spectrophotometer (model F-2000; Hitachi, San Jose, CA) at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. Calibration and calculation of calcium levels were done as described (42).

Phosphoinositide Analysis. Cells (10^6/ml) were labeled with myo-[^{3}H]inositol (10 μCi/ml, 105 Ci/mmol; Amersham Corp.) for 5–6 h in inositol-free RPMI-1640 supplemented with 10% dialyzed FCS, then stimulated in the presence of 10 mM LiCl with M4 mAb. The soluble inositol phosphates were extracted with TCA at indicated time points, and applied to AG1-X8 (formate form) ion exchange columns (Bio-Rad Laboratories, Richmond, CA) preequilibrated with 0.1 M formic acid. The columns were washed with 10 ml water and 10 ml of 60 mM ammonium
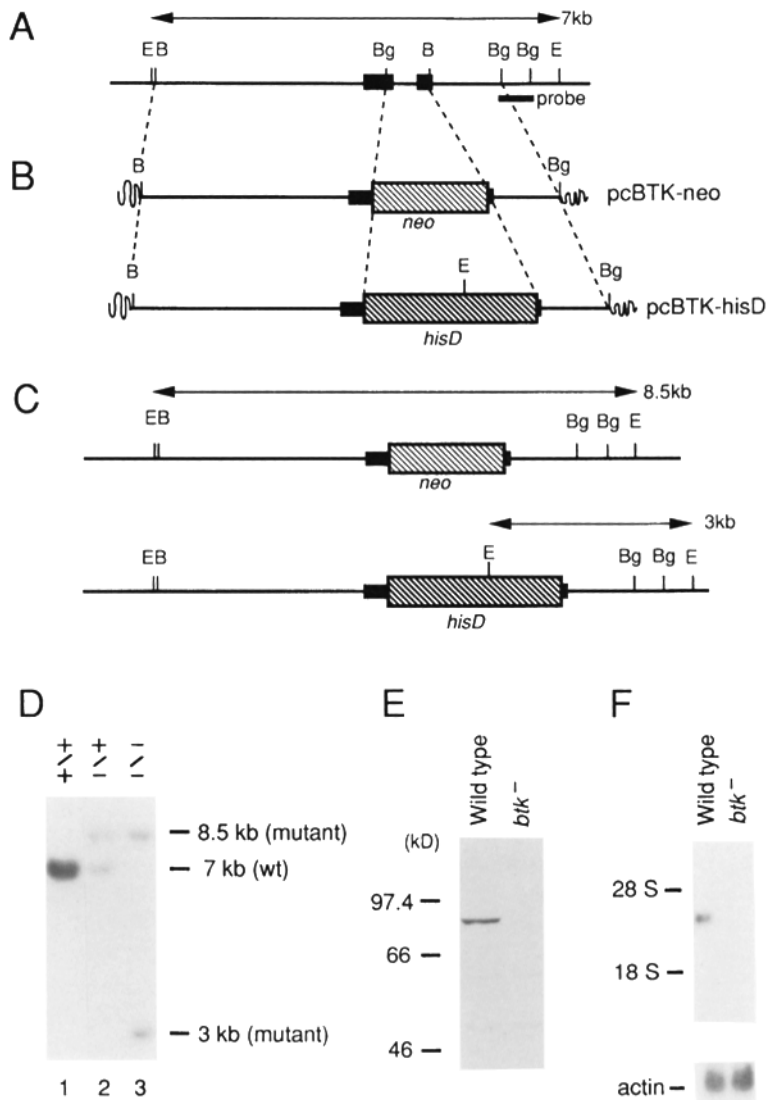
Figure 1. Disruption of the *btk* gene in chicken B cell line DT40. (*A*) Partial restriction map of chicken *btk* locus. (*B*) Targeting constructs. (*C*) Predicted structure of the targeted *btk* alleles. Only disrupted exons are shown as black boxes. The restriction endonuclease cleavage sites are abbreviated: *B*, BamHI; *Bg*, BglII; *E*, EcoRI. (*D*) Southern blot analysis of genomic DNA from wild-type DT40 cells (+/+, lane *1*), *neo*-targeted cells (+/−, lane *2*), and *neo*- and *hisD*-targeted cells (−/−, lane *3*). Genomic DNA was digested by EcoRI. (*E*)Western blot analysis of Btk protein expression in wild-type and targeted DT40 cells. Cells were lysed by NP-40 buffer, separated by 8% SDS-PAGE gel. The blotted membrane was incubated with anti-Btk Ab. (*F*) RNA expression was analyzed by Northern blot analysis using chicken cDNA probe for Btk (*top*) or β-actin (*bottom*). Positions of 28S and 18S RNA are shown.

formate/5 mM sodium tetraborate. Elution was performed with increasing concentrations of ammonium formate (0.1–0.7 M) (43).

Flow Cytometric Analysis for Surface Expression of BCR. Cells were washed, stained with FITC-conjugated anti–chicken IgM (Bethyl Laboratories, Inc., Montgomery, TX) , and analyzed by FACSort (Becton Dickinson & Co., Mountain View, CA).

Results

To disrupt the *Btk* gene, targeting constructs containing neomycin or histidinol resistance gene cassette were sequentially transfected into chicken B cell line DT40 (Fig. 1 *B*). Screening by Southern blot analysis identified two independent clones that underwent homologous recombination at both *btk* loci (Fig. 1 *D*). Hybridization with a neo and hisD probe indicated that the targeted clone had incorporated a single copy of each construct (data not shown). Lack of Btk expression was confirmed by Northern and Western blot analyses in these clones (Fig. 1, *E* and *F*). Another mu-

tant cell line that was deficient in both Lyn and Syk was also established by disrupting *syk* gene in Lyn-deficient cells (38). Flow cytometric analysis showed that BCR expression on all of these mutant cell lines was essentially the same level as that of parental DT40 cells (Fig. 2).

Engagement of BCR rapidly activates protein tyrosine kinases, leading to the induction of tyrosine phosphorylation on a number of cellular proteins. To characterize early signaling events through BCR stimulation, we evaluated the induction of tyrosine phosphorylation in whole cell lysates of Btk-deficient cells in response to stimulation by anti-IgM mAb M4. There were no significant differences between wild-type and Btk-deficient cells by antiphosphotyrosine Western blot (Fig. 3). Cells deficient in both Lyn and Syk (Lyn/Syk double-deficient cells), which express Btk, showed an almost complete defect in induction of tyrosine phosphorylation. This tyrosine phosphorylation pattern was essentially the same as that of BCR-deficient DT40 cells (data not shown). These data, together with our previous results
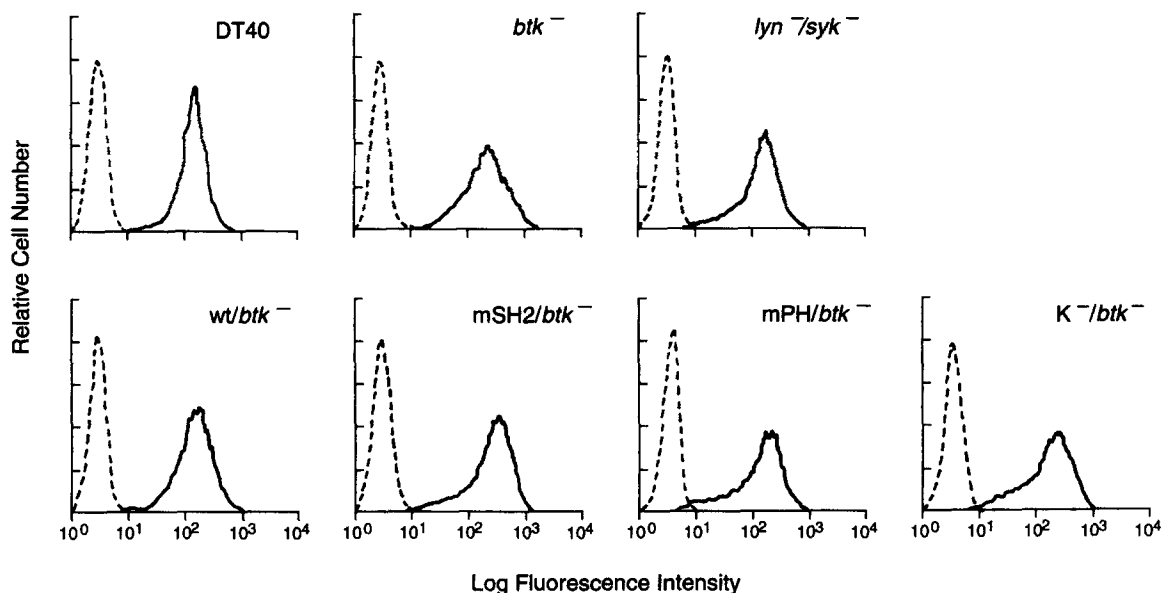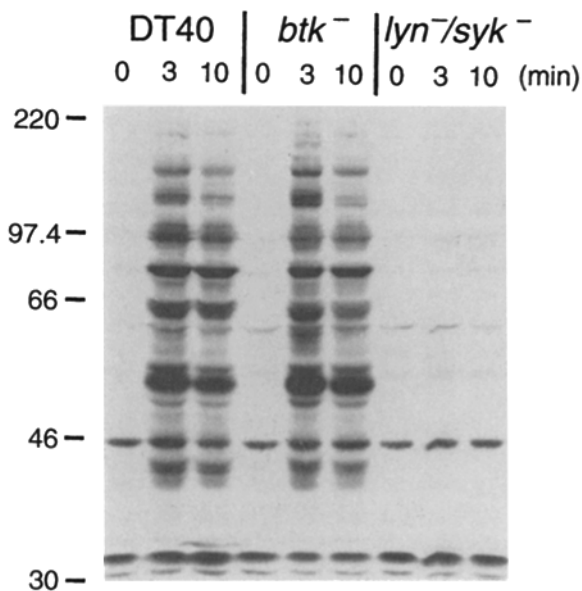
Figure 2. Cell surface expression of BCR on various DT40 mutants. Unstained cells were used as negative controls. Btk-deficient cells expressing wild type, SH2, PH, and kinase mutants of Btk are indicated as wt/*btk⁻*, mSH2/*btk⁻*, mPH/*btk⁻*, and K⁻/*btk⁻*, respectively.

(38), demonstrate that Lyn and Syk, not Btk, are primarily responsible for overall induction of tyrosine phosphorylation on multiple cellular substrates upon BCR stimulation.
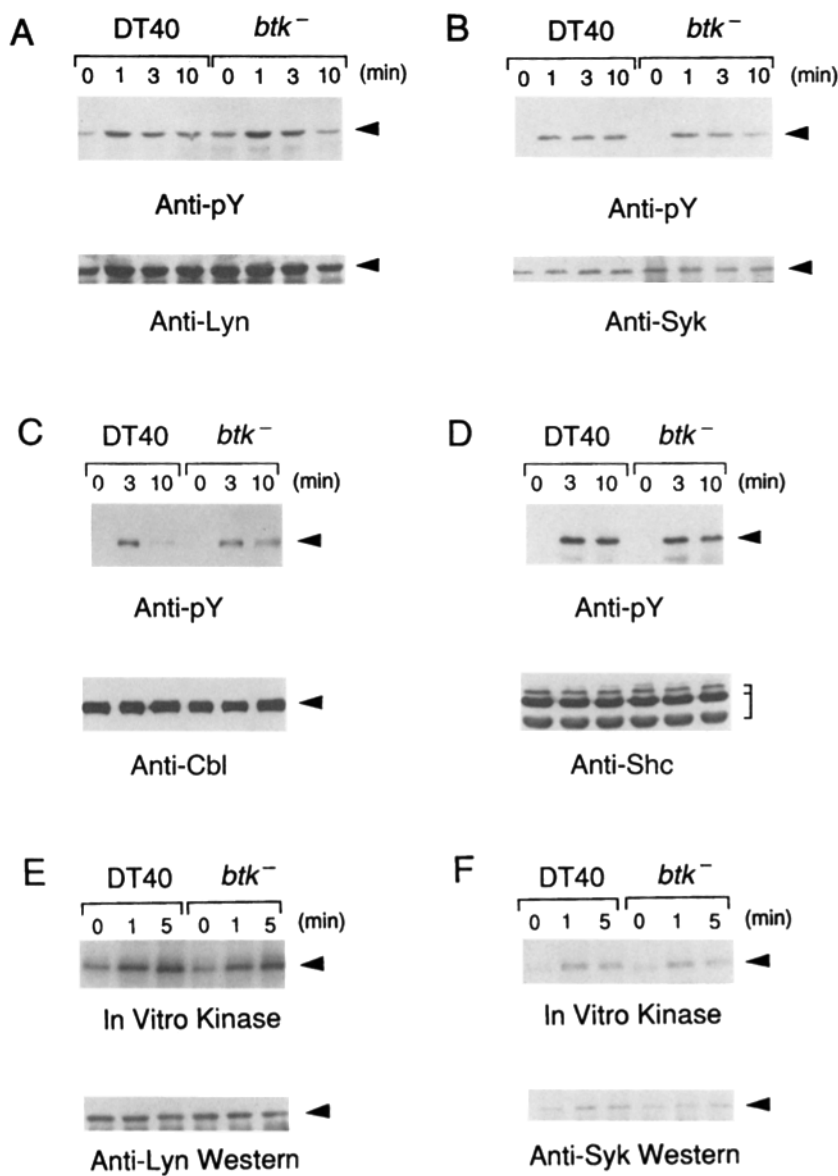
Btk-deficient cells allowed us to examine the effects of Btk on BCR-induced Lyn and Syk activation. We previously showed that receptor-induced tyrosine phosphorylation of Cbl and Syk is mediated by Lyn (44, 45). Thus, to determine the effects of Btk on Lyn activation, induction of

Figure 3. Tyrosine phosphorylation of whole cell proteins in Btk- and Lyn/Syk-deficient DT40 cells. At the indicated time points after stimulation with M4 (4 μg/ml), whole cell lysates (2.5 × 10⁶ cells) were prepared using SDS sample buffer, and loaded onto 8% SDS-PAGE gel. The blotted membrane was incubated with antiphosphotyrosine mAb.

phosphorylation of these molecules was examined in wild-type and Btk-deficient cells. As shown in Fig. 4, B and C, the BCR-induced tyrosine phosphorylation of both Cbl and Syk was not affected by the loss of Btk, although tyrosine phosphorylation of Cbl at 10 min was a little increased compared with wild-type cells. Furthermore, tyrosine phosphorylation of Lyn upon receptor ligation in Btk-deficient cells was similar to that of wild-type cells (Fig. 4 A). These observations indicate that BCR-induced activation of Lyn is independent of Btk. To determine the effects of Btk on Syk activation, we monitored tyrosine phosphorylation of Shc, since receptor-induced phosphorylation of Shc is dependent on Syk (46). Loss of Btk did not affect the tyrosine phosphorylation of Shc (Fig. 4 D), demonstrating that Syk activation is also independent of Btk. To strengthen these conclusions, in vitro kinase assay of Lyn and Syk after BCR stimulation was carried out in wild-type and Btk-deficient cells. As shown in Fig. 4, E and F, no differences were observed between wild-type and mutant cells.

Wild-type DT40 cells show a brisk elevation of intracellular calcium levels after BCR stimulation, whereas Btk-deficient cells completely abrogated this calcium response (Fig. 5 A). As shown in Fig. 5 B, the BCR-induced inositol 1,4,5-trisphosphate (IP₃) generation was also completely abolished in the mutant cells, indicating that Btk is essential for PLC-γ2 activation. Since these early events are known to be dependent on tyrosine phosphorylation of PLC-γ2 (39), induction of tyrosine phosphorylation of PLC-γ2 was examined. Although Btk-deficient cells still induced tyrosine phosphorylation of PLC-γ2 upon BCR ligation, its extent was clearly lower (about threefold) and more transient than that of wild-type cells (Fig. 5 C). These results suggest that Btk mediates phosphorylation of tyrosine residue(s) of PLC-γ2 which is critical for PLC-γ2 activation.

A DT40   *btk⁻*

0 1 3 10 | 0 1 3 10 (min)

◄

Anti-pY

◄

Anti-Lyn

B DT40   *btk⁻*

0 1 3 10 | 0 1 3 10 (min)

◄

Anti-pY

◄

Anti-Syk

C DT40   *btk⁻*

0 3 10 | 0 3 10 (min)

◄

Anti-pY

◄

Anti-Cbl

D DT40   *btk⁻*

0 3 10 | 0 3 10 (min)

◄

Anti-pY

]

Anti-Shc

E DT40   *btk⁻*

0 1 5 | 0 1 5 (min)

◄

In Vitro Kinase

◄

Anti-Lyn Western

F DT40   *btk⁻*

0 1 5 | 0 1 5 (min)

◄

In Vitro Kinase

◄

Anti-Syk Western

Figure 4. Activation of Lyn and Syk in wild-type and Btk-deficient DT40 cells. Tyrosine phosphorylation of Lyn, Syk, Cbl, and Shc (*A–D*) and in vitro kinase activity of Lyn and Syk (*E, F*) upon BCR engagement. At the indicated time points after M4 stimulation (4 μg/ ml), cells were lysed in 1% NP-40 lysis buffer, and immunoprecipitated with anti-Lyn (*A*), anti-Syk (*B*), anti-Cbl (*C*), or anti-Shc Abs (*D*). Immunoprecipitates were loaded onto 8% SDS-PAGE gel, and the blotted membrane was incubated with antiphosphotyrosine mAb. After the filter was stripped, the same blot was reprobed with Abs that were used for immunoprecipitation (*bottom*). For in vitro kinase assay, cells were lysed by modified RIPA buffer, and immunoprecipitated with anti-Lyn (*E*) and anti-Syk (*F*) Abs. Immunoprecipitated were divided, and half of them were used for in vitro kinase assay. The remaining half were used for Western blotting. Samples were electrophoresed on 8% SDS-PAGE gels.

BCR engagement results in the activation of at least two divergent signaling pathways (for a review see reference 47). The first is activation of PLC-γ2 and a second pathway involves Ras with subsequent phosphorylation and activation of MAPK. The contribution of Btk to the Ras pathway was measured by the ability of the BCR to mediate activation of MAPK. Activation of MAPKs is mediated by phosphorylation of Thr and Tyr residues within their catalytic domains (48). Cross-linking of the BCR in both wild-type and mutant cells resulted in activation of MAPK as measured by immunoblotting with an Ab that recognizes only the activated forms of MAPK (Fig. 6), suggesting that Btk is not essential for coupling the BCR to the Ras pathway.

Btk has a unique NH₂-terminal region containing a PH domain and a proline-rich stretch, followed by SH3, SH2, and kinase domains (14, 15). To examine whether mutated Btk exhibits a functional defect through BCR signaling, human Btk cDNA harboring mutations either in PH (Arg[28]

to Cys), SH2 (Arg[307] to Ala), or kinase (Arg[525] to Gln) domains were transfected into Btk-deficient cells (Fig. 7 *A*). In addition, wild-type Btk was introduced into the Btk-deficient cells. BCR-induced calcium mobilization and PI hydrolysis were examined in the transfectants expressing similar levels of Btk among wild-type and these mutants (Fig. 7 *B*). As expected, only the kinase domain mutant abrogated its in vitro kinase activity (data not shown). Expression levels of BCR on these transfectants were similar to those of wild-type cells (Fig. 2).

Upon BCR stimulation, Btk-deficient cells expressing wild-type Btk (wt/*btk⁻*) evoked even more vigorous calcium mobilization and IP₃ generation than wild-type cells, confirming the role of Btk in PLC-γ2 activation. In contrast, transfectants expressing Btk mutants in the PH (mPH/*btk⁻*), SH2 (mSH2/*btk⁻*), or kinase (K⁻/*btk⁻*) domain showed a much smaller calcium response and IP₃ generation after BCR ligation (Fig. 8, *A* and *B*). Consistent with these data,

A

B

C

Anti-pY

Anti-PLC-γ2
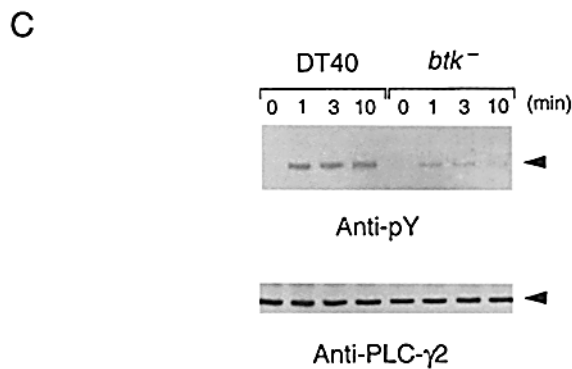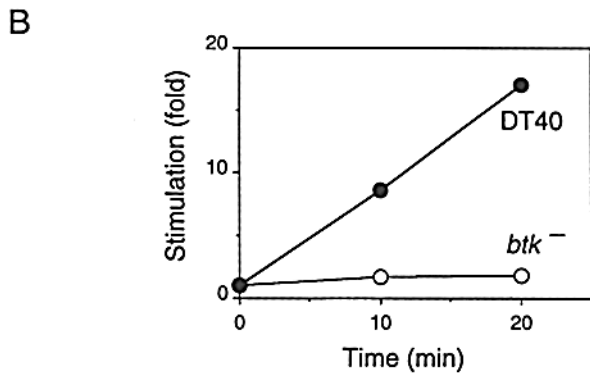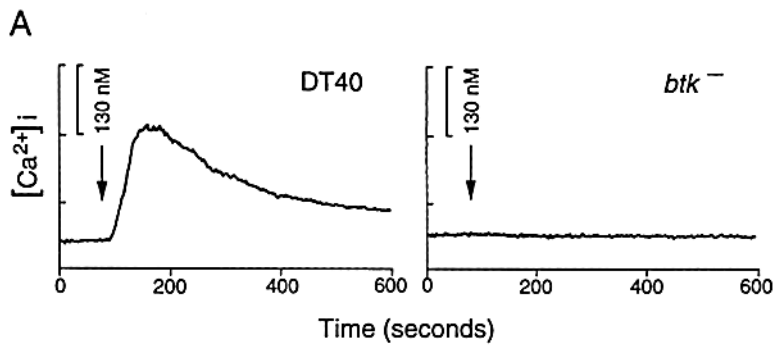
Figure 5. Calcium mobilization, IP₃ generation, and tyrosine phosphorylation of PLC-γ2 in wild-type and Btk-deficient DT40 cells. (*A*) Intracellular free calcium levels in Fura-2–loaded cells were monitored by a spectrophotometer after stimulation with M4 (2 μg/ml). (*B*) For IP₃ detection, soluble inositol was extracted from cells stimulated with M4 (2 μg/ml) and subjected to AG1-X8 anion exchange columns. (*C*) Tyrosine phosphorylation of PLC-γ2 was carried out as described in the legend to Fig. 4.

Anti-phospho MAPK
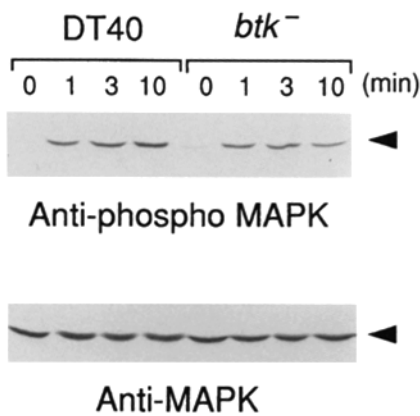
Anti-MAPK

Figure 6. MAPK activation in wild-type and Btk-deficient cells. Cells were stimulated with M4 (4 μg/ml). Cell lysates were then analyzed by immunoblotting with anti-phospho MAPK (*top*) or anti-MAPK (*bottom*) Abs.

only wild-type Btk restored the BCR-induced tyrosine phosphorylation of PLC-γ2 that was seen in wild-type DT40 cells (Fig. 8 *C*). These results establish the importance of PH, SH2, and kinase domains of Btk in PLC-γ2 activation through BCR stimulation, and correlate well with the fact that mutations of these Btk residues (Arg[28] to Cys, Arg[307] to Gly, and Arg[525] to Gln) cause a typical XLA phenotype (15, 49, 50). The residual calcium mobilization and IP₃ generation might be due to overexpression of these mutants, because cells expressing lower amounts of PH, SH2, or kinase domain mutant showed an almost flat calcium response (data not shown).

Discussion

Lyn/Syk double-deficient cells exhibited an almost complete defect in induction of tyrosine phosphorylation upon BCR ligation, indicating that activation of Lyn and Syk can account for the vast majority of the initial tyrosine phosphorylation of cellular substrates. Our results demonstrate
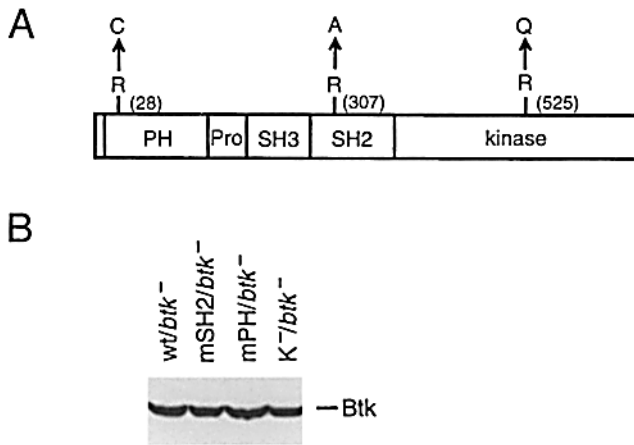
Figure 7. Schematic representation of Btk cDNA constructs (*A*) and expression of transfected Btk in Btk-deficient DT40 cells (*B*). DT40 cells (0.5 × 10⁶ cells/lane) expressing indicated constructs were lysed with NP-40 buffer and electrophoresed on 8% SDS-PAGE gel, transferred to nitrocellulose, and incubated with anti-Btk Ab.

that activation of Lyn is independent of Btk upon receptor ligation. Supporting this concept, Btk has been shown to be activated after the activation of Src kinases (35). Since Btk activation precedes Syk activation in BCR signaling (35), it is speculated that Btk may regulate Syk activation upon receptor stimulation. However, Syk activation, judged by its in vitro kinase activity (Fig. 4 *F*), was not affected by loss of Btk. This conclusion is further supported by no differences of the BCR-induced phosphorylation of Shc between wild-type and mutant cells (Fig. 4 *D*), since we pre-

viously showed that Shc phosphorylation is mediated by Syk upon BCR engagement (46). Because recruitment of Syk to tyrosine-phosphorylated Igα/Igβ ITAM is essential for Syk activation in BCR signaling (26–28, 33), these data also suggest that tyrosine phosphorylation of Igα/Igβ ITAM is not dependent on Btk.

In contrast to Lyn- and Syk-deficient DT40 cells, Btk-deficient cells exhibited almost the same pattern of tyrosine phosphorylation of cellular proteins as the wild-type cells upon BCR cross-linking. This might reflect differences in localization of these kinases for coupling the BCR to downstream substrates. Both Lyn and Syk are associated with the BCR components before and after receptor stimulation, respectively (26–28), whereas no association of Btk with the receptor complex was detected to date.

Involvement of the Syk kinase in BCR-induced PLC-γ2 phosphorylation is certain: Syk-deficient DT40 cells exhibit an almost complete loss of PLC-γ2 tyrosine phosphorylation upon BCR stimulation (38). The data presented here show that Btk also participates in this phosphorylation. In contrast to Syk-deficient cells, the extent of BCR-induced tyrosine phosphorylation of PLC-γ2 in Btk-deficient cells was about threefold less than that of wild-type cells. These results implicate that Syk and Btk may regulate one another in PLC-γ2 phosphorylation. As mentioned above, our data suggest that Syk activation is not dependent on Btk in BCR signaling. Thus, it is possible that Syk regulates Btk in BCR-mediated PLC-γ2 activation. Recent reports by others (51, 52) have shown that in contrast to Src kinase, Syk is incapable of activating Btk kinase in COS cells and fibroblasts. However, since activation of Syk requires the presence of
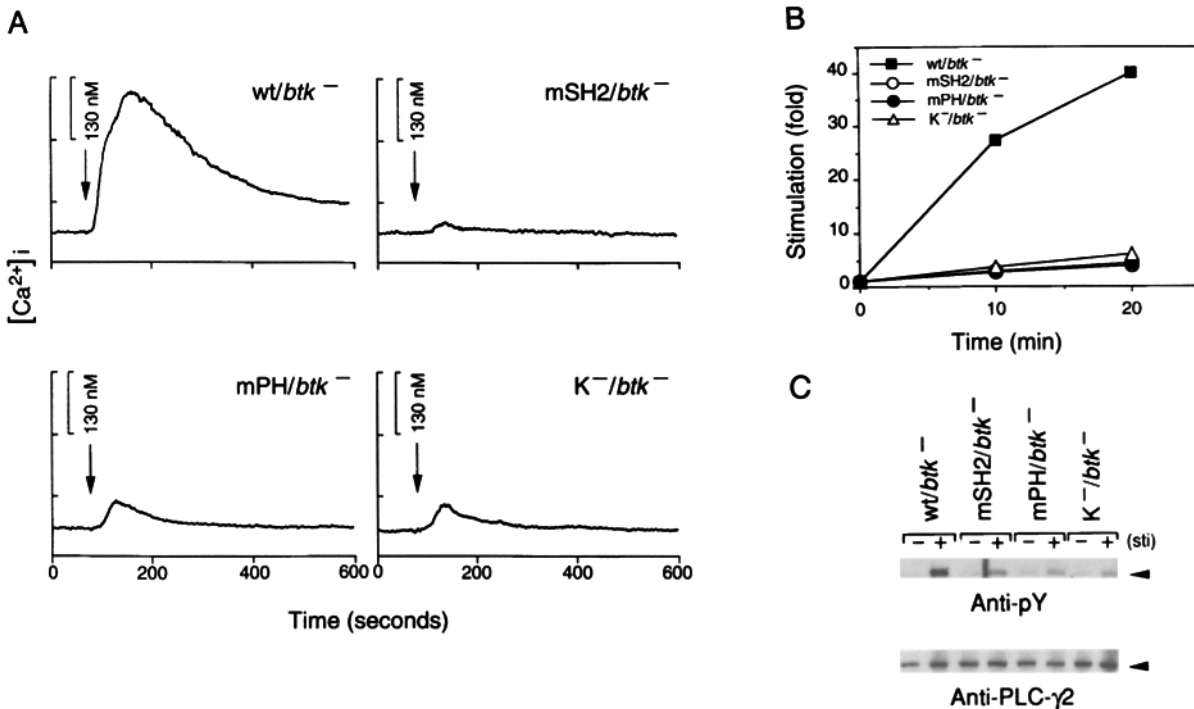
Figure 8. Functional restoration of BCR signaling by various Btk mutants. Calcium mobilization (*A*), IP₃ generation (*B*), and tyrosine phosphorylation of PLC-γ2 (*C*) were examined as described in the legend to Fig. 5. (*C*) Cells were stimulated for 3 min with M4 (4 µg/ml).

Src kinase (45), this differential requirement for Btk activation might reflect relative kinase activity in these cells rather than specificity between Src kinase and Syk. In the context of BCR signaling, it remains possible that Lyn-activated Syk may further enhance Btk kinase activity. Alternatively, activation of Syk and Btk may be mutually independent. Thus, at present, we can only say that these two kinases act in concert for PLC-γ2 tyrosine phosphorylation.

Both Syk and Btk are required for PLC-γ2 activation after BCR stimulation. It is known that three distinct tyrosine phosphorylation sites of PLC-γ1 are required for its activation through growth factor receptors such as platelet-derived growth factor receptor (53). Thus, the most likely explanation is that phosphorylation of these critical tyrosine sites of PLC-γ2 are mediated distinctly by Syk and Btk upon receptor ligation. In contrast to the complete block of BCR-induced PI hydrolysis in Btk-deficient chicken DT40 cells, B cells from *Xid* mice show reduced (40–50%) PI hydrolysis compared with normal B cells (54). This observation implicates that murine Btk is also involved in PLC-γ activation at least to some extent, and that the distinct stringency of Btk requirement for PLC-γ activation may represent a species difference between chicken and mouse. One possible mechanism for this difference is another tyrosine kinase that substitutes for Btk function in *Xid* mice. Alternatively, DT40 cells express exclusively PLC-γ2 isoform (39), whereas both PLC-γ1 and PLC-γ2 may be expressed in mouse B cells. In contrast to PLC-γ2, PLC-γ1 may require only Syk for its activation, obviating a stringent requirement for Btk in mouse as a whole PLC-γ activation.

Our data indicate that Btk activation requires its PH and SH2 domains in BCR signaling, suggesting that interactions of these domains to proteins and/or selected lipids are essential for Btk activation after BCR ligation. Given the importance of SH2 domain in signal transduction (16), Btk may be activated by its recruitment to a phosphorylated molecule. Indeed, in the case of Lyn and Syk, it was reported that binding of their SH2 domains to phosphorylated ITAM increases the kinase activity in vitro (32, 55). The COOH-terminal region of the PH domain has been shown to interact with the β/γ subunits of trimeric G proteins (56, 57). Also, the NH₂-terminal region of several PH domains has been shown to bind the membrane lipid PI-4,5-bisphospate (58), implying a role in membrane localization. Supporting this possibility, a gain-of-function mutant in the PH domain of Btk shows an increase in membrane targeting and an increase in phosphorylation on tyrosine residues (59). Thus, our data suggest that these interactions through the PH domain of Btk is also required for PLC-γ2 phosphorylation, leading to its activation.

Both pre-BCR and BCR associate with Igα and Igβ chains that are involved in initiating signaling cascades (60). Recent genetic evidence that tyrosine residue of Igα/Igβ ITAM is essential for B cell development (61, 62) have implicated a direct link between development and ITAM-induced signaling in B cells. Thus, the requirement of Btk for PLC-γ activation through Igα/Igβ ITAM may offer a biochemical explanation for the defective B cell development in XLA patients and *Xid* mice.

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