Bruton's Tyrosine Kinase Is Essential for Hydrogen Peroxide-Induced Calcium Signaling

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ABSTRACT: Using Btk-deficient DT40 cells and the transfectants expressing wild-type Btk or Btk mutants in either kinase (Arg^{525} to Gln), Src homology 2 (SH2, Arg^{307} to Ala), or pleckstrin homology (PH, Arg^{28} to Cys) domains, we investigated the roles and structure−function relationships of Btk in hydrogen peroxide-induced calcium mobilization. Our genetic evidence showed that Btk deficiency resulted in a significant reduction in hydrogen peroxide-induced calcium response. This impaired calcium signaling is correlated with the complete elimination of IP_3 production and the significantly reduced tyrosine phosphorylation of PLCγ2 in Btk-deficient DT40 cells. All of these defects were fully restored by the expression of wild-type Btk in Btk-deficient DT40 cells. The data from the point mutation study revealed that a defect at any one of the three functional domains would prevent a full recovery of Btk-mediated hydrogen peroxide-induced intracellular calcium mobilization. However, mutation at either the SH2 or PH domain did not affect the hydrogen peroxide-induced activation of Btk. Mutation at the SH2 domain abrogates both IP_3 generation and calcium release, while the mutant with the nonfunctional PH domain can partially activate PLCγ2 and catalyze IP_3 production but fails to produce significant calcium mobilization. Thus, these observations suggest that Btk-dependent tyrosine phosphorylation of PLCγ2 is required but not sufficient for hydrogen peroxide-induced calcium mobilization. Furthermore, hydrogen peroxide stimulates a Syk-, but not Btk-, dependent tyrosine phosphorylation of B cell linker protein BLNK. The overall results, together with those reported earlier [Qin et al. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 7118], are consistent with the notion that functional SH2 and PH domains are required for Btk to form a complex with PLCγ2 through BLNK in order to position the Btk, PLCγ2, and phosphatidylinositol 4,5-bisphosphate in close proximity for efficient activation of PLCγ2 and to maximize its catalytic efficiency for IP_3 production.

Bruton's tyrosine kinase (Btk),[1] a member of the Tec family nonreceptor protein−tyrosine kinases (PTKs), consists of a catalytic domain, Src homology SH2 and SH3 protein interaction domains, and a unique N-terminal Pleckstrin homology (PH) domain capable of directing the protein to interact with the phospholipid (*1*−*4*). One mechanism controlling Btk activation occurs via sequential phosphorylation of Y511 catalyzed by Src family kinases activated after stimulation of the B cell receptor (BCR) and autophosphorylation of Y223 (*5*−*8*). Phosphorylation of Y511, which is located in the kinase domain, dramatically increases the enzymatic activity of Btk. The phosphorylation of Y223 at the SH3 domain occurs after Y511 phosphorylation and exhibits limited catalytic enhancement (*9*). Targeted deletion of Lyn in B cells alters Btk-dependent antigen responses, consistent with Lyn's role as a regulator of Btk function (*5*, *10*, *11*). Btk is also regulated through binding interactions with signaling proteins and second-messenger molecules. Phosphatidylinositol 3-kinase (PI3K) has been shown to be an important coregulator of Btk in receptor signaling pathways (*12*). Membrane targeting of Btk via a phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-P3)/Btk-PH interaction appears to be the key initial step regulating Btk access to Src kinases and activation (*13*−*15*). The X-linked immunodeficiency (XID) allele does not upregulate its signaling function in the presence of enhanced PI3K activity because it binds poorly to PtdIns-3,4,5-P3, whereas alleles constitutively targeted to the membrane have increased activity (*14*, *16*−*18*).

The biological importance of its signaling function was shown by naturally occurring Btk loss of function mutations in human X-linked agammaglobulinemia (XLA) and murine XID syndromes (*19*, *20*). XID results in an alteration of normal B cell development that reduces the total peripheral B cell population by 50% and impairs functional responses to certain T cell-independent antigens, activation of the B cell receptor, the Fcϵ receptor on mast cells, and non-integrin collagen receptors on platelets (*21*−*25*). Btk is an essential component of B cell signaling pathways linking receptor activation to important downstream processes, such as the

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[1] Abbreviations: BCR, B cell receptor; Btk, Bruton's tyrosine kinase; BLNK, B cell linker protein; IP_3, inositol 1,3,5-trisphosphate; PH, Pleckstrin homology; PI3K, phophatidylinositol 3-kinase; PtdIns-3,4,5-P3, phosphatidylinositol 3,4,5-trisphosphate; PTK, protein−tyrosine kinase; SH2, Src homology 2; PLCγ2, phospholipase Cγ2; ROS, reactive oxygen species; Syk, spleen tyrosine kinase; XID, X-linked immunodeficiency.

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control of intracellular free calcium and maintenance of sustained calcium signals (*13*, *14*, *26*). Sustained calcium signals depend on inositol 1,4,5-trisphosphate (IP_3) that is generated from PtdIns-4,5-P2 (*27*). BCR-triggered initial activation of PLCγ is mediated by Syk (*28*). The interaction of Syk with PLCγ occurs through colocalization of these proteins by the tyrosine-phosphorylated scaffold protein BLNK (*29*, *30*). In parallel, BCR-dependent activation of PI3K leads to a concurrent generation of PtdIns-3,4,5-P3. PtdIns-3,4,5-P3 initiates Btk activation in concert with Src kinases by targeting the Btk to the plasma membrane through its PH domain (*14*). Then activated Btk is brought together with PLCγ by an unidentified tyrosine-phosphorylated ligand, most likely tyrosine-phosphorylated BLNK, leading to enhanced tyrosine phosphorylation of PLCγ (*13*, *14*, *26*, *29*−*31*). These combined events result in a rapid accumulation of IP_3 followed by a sustained, low-level accumulation of IP_3. Blockade of PtdIns-3,4,5-P_3 accumulation and/or loss of Btk evokes sustained IP_3 accumulation and turns off the sustained calcium signal (*13*, *14*, *32*).

Reactive oxygen species (ROS) have emerged as physiological mediators of cellular responses. The production of ROS has been detected in a variety of cells stimulated with cytokines (*33*, *34*), with peptide growth factor (*35*, *36*), and with agonists of receptors with seven transmembrane spans (*37*). The generated ROS is correlated to subsequent biochemical responses (*35*, *36*). Thus, studying hydrogen peroxide-stimulated signaling should yield mechanistic information on both receptor-mediated signaling and cellular responses to oxidative stress. When exogenous hydrogen peroxide is applied to cells as one form of ROS, hydrogen peroxide can activate an array of nonreceptor- and receptor-type PTKs in a variety of cell systems (*38*−*43*). Furthermore, hydrogen peroxide stimulates calcium ion mobilization and tyrosine phosphorylation patterns in lymphocytes that are identical to those observed following antigen receptor activation (*44*, *45*), and the B cell receptor complex is required for oxidative stress signaling (*46*). We and others have observed tyrosine kinase-dependent IP_3 production and calcium release in hydrogen peroxide-treated B cells (*47*, *48*), suggesting that hydrogen peroxide mediates IP_3 production and calcium release through the activation of PLCγ2 via tyrosine phosphorylation by hydrogen peroxide-activated tyrosine kinases. We recently demonstrated that activation of PI3K by hydrogen peroxide is required for the maximal catalytic activity of PLCγ2 as reflected by IP_3 production. The requirement of PI3K activation for full PLCγ2 activity is reduced by Btk overexpression (*49*), indicating that Btk may be one of the PTK candidates involved in the regulation of oxidative stress-induced calcium mobilization. Therefore, we have made efforts to investigate the roles and structure−function relationships of Btk in hydrogen peroxide-triggered calcium mobilization. We report here that the majority of hydrogen peroxide-induced calcium responses are Btk-dependent. In addition, site-directed mutagenesis was used to investigate the effects of the catalytic, SH2, and PH domains on the intracellular calcium mobilization pathway induced by hydrogen peroxide.

MATERIALS AND METHODS

Materials. RPMI 1640 medium and fetal bovine serum were purchased from GIBCO Inc. Protein A was from Calbiochem Corp. Fura-2 AM was from Molecular Probes. Anti-phosphotyrosine antibody (4G10), polyclonal anti-Btk antibody, and polyclonal anti-PLCγ2 antibody were from Upstate Biotechnology Inc., Pharmingen (San Diego, CA), and Santa Cruz Biotechnology, respectively. Enhanced chemiluminescence reagents were from Dupont. Assay kits for IP_3 production were from Amersham. The Btk-deficient DT40 cell line was purchased from Riken Cell Bank, Japan (generated by Dr. Kurosaki). Anti-chicken BLNK antibody was a gift from Dr. Kurosaki (Osaka, Japan).

Cell Culture and DNA Transfection. DT40 and DT40-derived cells were maintained in RPMI 1640 medium, supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified 95% air/5% CO_2 atmosphere. For experiments, cells were collected by centrifugation as previously described and stimulated by hydrogen peroxide at 37 °C (*47*). The human Btk cDNA construct and mutant Btk cDNA constructs in PH (Arg^{28} to Cys), SH2 (Arg^{307} to Ala), or kinase (Arg^{525} to Gln) domains were kindly provided by Dr. Kurosaki (Osaka, Japan). These cDNAs were transfected by electroporation using the gene pulser apparatus (Bio-Rad Laboratories) at 550 V and 25 μF, and selected in the presence of 0.5 μg/mL puromycin. Expression of transfected cDNAs was confirmed by Western blotting, and the clones expressing similar levels of Btk were used for the experiments.

Measurement of Intracellular [Ca^{2+}]. Calcium mobilization was measured using the fluorescent indicator, Fura-2, as previously described (*47*). The fluorometer used was a Photon Technology International Quanta Master Model QM-1.

Measurement of IP_3 Levels. After hydrogen peroxide stimulation, IP_3 in chicken B-lymphocytes was extracted by perchloric acid and determined with a highly specific D-*myo*-[^{3}H]IP_3 assay system (Amersham) as described by the supplier. This assay was based on the competition between the unlabeled IP_3 and a fixed quantity of a high specific activity tracer [^{3}H]IP_3 for a limited number of binding sites on a specific and sensitive bovine adrenal binding protein preparation.

Preparation of Cell Extracts. Stimulated cells (1×10^7 cells/mL) were lysed in ice-cold lysis buffer (5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 100 μM Na_3VO_4, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, 50 mM Tris, pH 7.4) after a short centrifugation step. Lysates were clarified by centrifugation at 16000*g* for 15 min at 4 °C.

Immunoblot Analysis. Cell extracts or immunoprecipitates were resolved on SDS−PAGE, transferred electrophoretically onto PVDF membranes, and then immunoblotted with the indicated antibodies. Immunoreactive proteins were visualized by enhanced chemiluminescence.

Immunoprecipitation Kinase Assay. The cell extracts from treated or untreated cells were incubated with anti-Btk antibody for 30 min followed by an additional 1 h incubation with protein A−agarose. The immunoprecipitates were washed three times with lysis buffer, once in 10 mM Hepes, pH 7.8, buffer containing 0.5 M NaCl, and once in the same Hepes buffer without NaCl. The kinase assay was carried out at 30 °C for 10 min by adding 30 μL of kinase assay buffer [10 mM Hepes, pH 7.8, 10 mM $Mg(OAc)_2$, 10 mM $MgCl_2$, and 1 μM cold ATP] containing 10 μCi of [γ^{32}P]-ATP to the Btk containing the immunoprecipitate. The

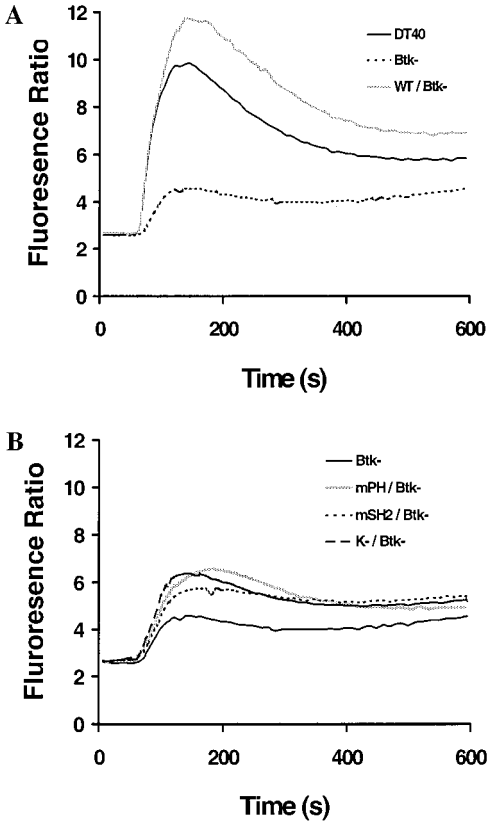

FIGURE 1: Effect of Btk and its mutants on hydrogen peroxide-induced calcium signaling. (A) Indispensability of Btk for hydrogen peroxide-induced calcium signaling. DT40, Btk-deficient DT40, and Btk-deficient DT40 expressing wild-type Btk cells (1.5×10^6/mL) were loaded with 5 μM Fura-2 AM and stimulated with 5 mM hydrogen peroxide. Shown is a representative profile of cells stimulated with hydrogen peroxide. (B) Slight restoration of hydrogen peroxide-induced calcium signaling by K^-, mSH2, and mPH Btk. Fura-2-loaded Btk-deficient DT40 cells and Btk-deficient cells expressing K^-, mSH2, and mPH Btk were stimulated with 5 mM hydrogen peroxide.

reactions were terminated by the addition of 15 μL of SDS sample buffer and boiled for 5 min. Autoradiogram exposure was carried out for ~3 h.

RESULTS

Hydrogen Peroxide-Stimulated Increase in Intracellular Calcium Concentration Depends on Btk, and Restoration of Btk-Dependent Calcium Signal Requires Intact Catalytic, SH2, and PH Domains. To address whether Btk is involved in hydrogen peroxide-induced calcium signaling, intracellular calcium concentrations in DT40 and Btk-deficient DT40 cells were examined using a fluorescent indicator, Fura-2. Hydrogen peroxide stimulated a rapid elevation in intracellular calcium concentration that peaked within 2 min and decreased thereafter in DT40 cells. However, the majority of this calcium response was abrogated when the wild-type cells were replaced with Btk-deficient cells (Figure 1A). This observation indicated that hydrogen peroxide induced both Btk-dependent and -independent calcium signaling. The functional role of Btk in mediating hydrogen peroxide-induced calcium signaling was further confirmed by the fact that Btk-deficient DT40 cells when overexpressed with wild-type Btk exhibited an even more vigorous increase in intracellular calcium concentration.

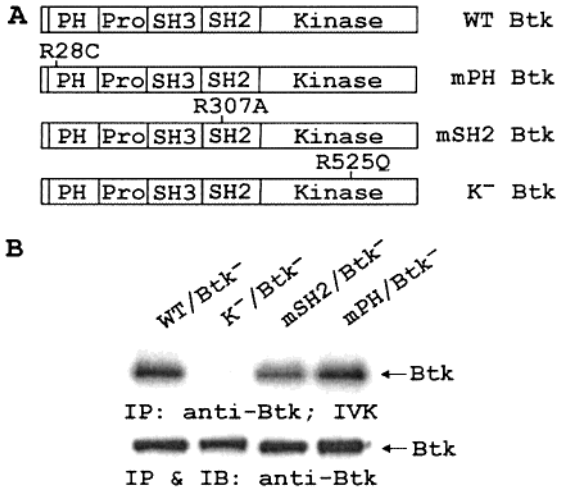

FIGURE 2: Schematic representation of Btk cDNA constructs (A) and expression and kinase activity of mutant Btk in Btk-deficient DT40 cells (B). DT40 cells expressing the indicated constructs were lysed and immunoprecipitated with anti-Btk antibody. Immunoprecipitates were used either for in vitro kinase assay (B, top) or for Western blotting with anti-Btk antibody (B, bottom) as described in Experimental Procedures. The position of Btk is indicated by the arrow.

To investigate the role of the kinase activity and the functions of the SH2 and PH domains of Btk on calcium signaling, we expressed Btk constructs containing inactive mutants in each of these domains [kinase (R525Q), SH2 (R307K), and PH (R28C) (Figure 2A)] and wild-type Btk in Btk-deficient DT40 cells. As shown in Figure 2B, the levels of transfected Btk are similar for the wild-type and the mutant enzymes, and only the mutation on the catalytic domain exhibited no kinase activity. The level of transfected Btk is about three to five times higher than the Btk in the parent cells. Figure 1B shows that none of the mutants, either catalytically inactive K^-/Btk^-, inactive SH2 (mSH2/Btk^-), or inactive PH domain (mPH/Btk^-), restored the calcium mobilization induced by hydrogen peroxide, even though each exhibited a slight increase in calcium signal when compared to that observed in Btk-deficient cells.

Btk Deficiency Eliminates Hydrogen Peroxide-Induced Production of IP_3, and Full Restoration of IP_3 Production Requires Intact Kinase, SH2, and PH Domains. Since PTK-dependent calcium signaling occurs at least in part via IP_3 receptor-gated calcium stores (*47*, *48*), we evaluated whether Btk deficiency would alter IP_3 production. Figure 3A depicts that both DT40 and WT/Btk^- cells generated IP_3 and peaked at about 2 min after hydrogen peroxide stimulation. The quantity of IP_3 in each case decreased to the basal level within 10 min. However, the Btk-deficient cells failed to produce IP_3 in response to hydrogen peroxide. Therefore, the impairment of the calcium signal observed in Btk-deficient cells correlated relatively well with the production of IP_3, suggesting that Btk plays a key role in the regulation of PLCγ2 during oxidative stress signaling.

To investigate the effect of the catalytic activity and the role of intact SH2 and PH domains on hydrogen peroxide-induced IP_3 production, we monitored IP_3 concentrations in the transfectants expressing K^- Btk, mSH2 Btk, or mPH Btk following hydrogen peroxide treatment. The transfectants expressing K^- Btk or mSH2 Btk supported only a negligible amount of hydrogen peroxide-induced IP_3 production. In

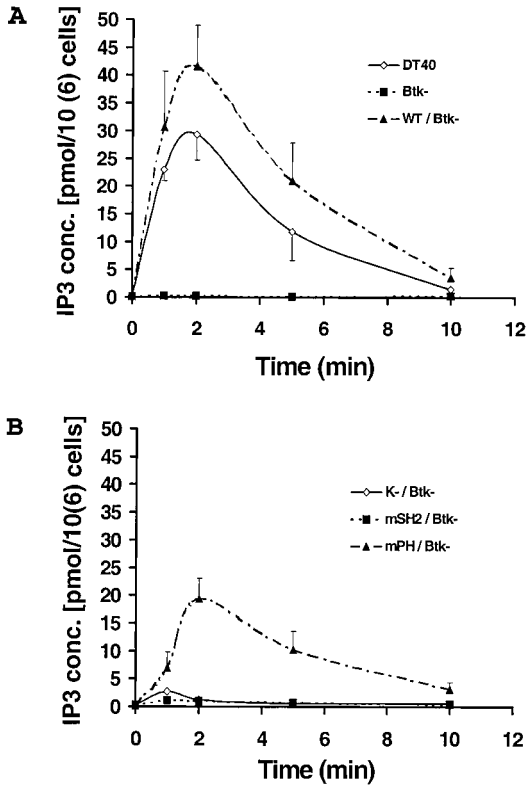

FIGURE 3: Effect of Btk and its mutants on hydrogen peroxide-induced IP_3 production. (A) Control of hydrogen peroxide-induced IP_3 production by Btk. DT40 (Btk^-), Btk-deficient DT40, and Btk-deficient DT40 cells expressing wild-type Btk were stimulated with 5 mM hydrogen peroxide for the indicated times, and IP_3 concentrations in the extracts were assayed as described in Experimental Procedures. (B) Requirement of Btk's kinase activity and intact PH and SH2 domains to restore hydrogen peroxide-induced IP_3. Btk-deficient DT40 cells expressing K^-, mSH2, and mPH Btk were stimulated with 5 mM hydrogen peroxide. At the indicated times, IP_3 levels were determined. The data presented are the mean values of three independent measurements.

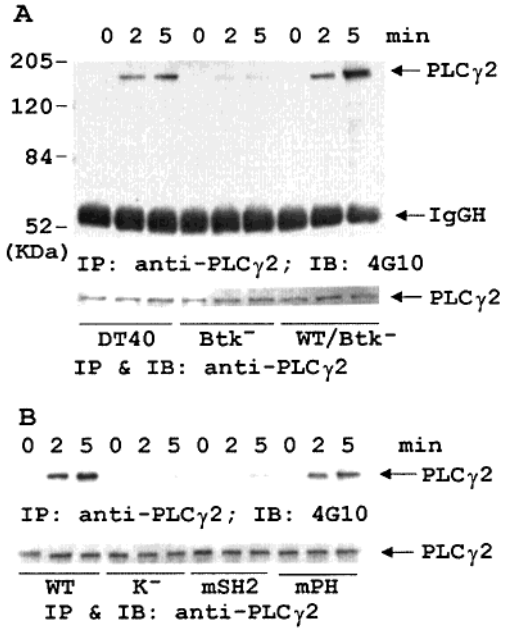

FIGURE 4: Restoration of hydrogen peroxide-induced tyrosine phosphorylation of PLCγ2 by Btk and its mutants. (A) Mediation of hydrogen peroxide-induced PLCγ2 tyrosine phosphorylation by Btk. Anti-PLCγ2 immunoprecipitates from DT40 cells, Btk-deficient DT40 cells, or Btk-deficient DT40 cells expressing wild-type Btk with or without hydrogen peroxide stimulation were immunoblotted with anti-PTyr (top) or anti-PLCγ2 (bottom). Abbreviations: IP, immunoprecipitation; IB, immunoblot. (B) Partial restoration of hydrogen peroxide-induced tyrosine phosphorylation of PLCγ2 by mPH Btk but not by mSH2 Btk or K^- Btk. Btk-deficient DT40 cells expressing K^-, mSH2, or mPH Btk were stimulated with 5 mM hydrogen peroxide. Anti-PLCγ2 immunoprecipitates were immunoblotted with anti-PTyr (top) or anti-PLCγ2 (bottom).

contrast, mPH/Btk^- cells showed a significant accumulation of IP_3 in response to the hydrogen peroxide, although not to the same level exhibited by either DT40 or WT/Btk^- cells (Figure 3B).

Btk Deficiency Inhibits Hydrogen Peroxide-Induced Tyrosine Phosphorylation of PLCγ2, and Full Restoration of Tyrosine Phosphorylation of PLCγ2 Requires Intact Kinase, SH2, and PH Domains. Btk is known to participate in the tyrosine phosphorylation of PLCγ2 following B cell receptor or Fcϵ receptor engagement (*26*), and calcium mobilization as well as IP_3 production is dependent on tyrosine phosphorylation of PLCγ2 (*5*, *49*, *50*). We evaluated whether the effect of Btk on PLCγ2 activation (as assessed by IP_3 production) could account for the effect on PLCγ2 tyrosine phosphorylation. Figure 4A shows that Btk-deficient cells failed to induce tyrosine phosphorylation of PLCγ2 in response to hydrogen peroxide stimulation. Furthermore, expression of wild-type Btk in Btk-deficient DT40 cells restored hydrogen peroxide-induced PLCγ2 tyrosine phosphorylation. The phosphorylation level observed in WT/Btk^- was more pronounced than that found in DT40 cells, most likely due to 3−5-fold higher expression of Btk in WT/Btk^- cells. This is consistent with the enhanced calcium signal and IP_3 production observed in Btk-deficient DT40 cells expressing wild-type Btk. Together, these results indicate that Btk mediates the tyrosine phosphorylation of PLCγ2, which is required for its activation.

Figure 4B shows the effects of catalytic activity, intact SH2, and PH domains of Btk on PLCγ2 tyrosine phosphorylation stimulated by hydrogen peroxide. The data indicate that transfectants expressing K^- Btk or mSH2 Btk in Btk-deficient cells were unable to restore hydrogen peroxide-induced PLCγ2 tyrosine phosphorylation. This observation is consistent with the inability of these Btk mutants to complement them in calcium mobilization and IP_3 production. However, the transfectant expressing mPH Btk could significantly induce the PLCγ2 tyrosine phosphorylation, but clearly at a level lower than that observed in the transfectant expressing wild-type Btk.

Mutations of Btk's SH2 and PH Domains Do Not Affect Btk Activation by Hydrogen Peroxide. The activity of Btk is regulated by tyrosine phosphorylation at Y551 catalyzed by the Src family kinase followed by autophosphorylation at Y223 (*5*, *8*). To investigate whether the impairment of PLCγ2 activation caused by mSH2 and mPH Btk mutants is derived from their effects on Btk activation or translocation, we monitored the extent of Btk tyrosine phosphorylation induced by hydrogen peroxide. Figure 5 shows that hydrogen peroxide stimulates an easily detectable tyrosine phosphorylation of Btk in WT/Btk^-, mSH2/Btk^-, and mPH/Btk^- cells. These results indicate that the SH2 and PH domain-mediated intermolecular interactions are not essential for hydrogen peroxide-induced Btk activation. However, unex-

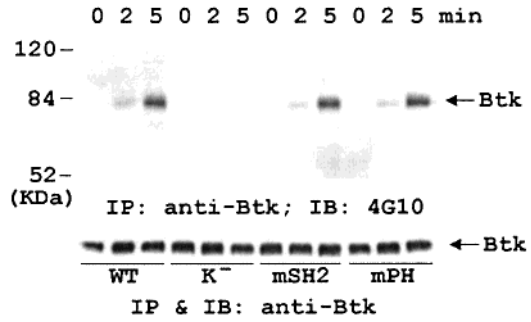

FIGURE 5: Effect of point mutation within the kinase, SH2, and PH domains on hydrogen peroxide-induced Btk activation. Anti-Btk immunoprecipitates from Btk-deficient DT40 cells expressing wild-type Btk or its mutants were immunoblotted with anti-PTyr (top) or with anti-Btk antibody (bottom).

pectedly we found no detectable tyrosine phosphorylation on Btk R525Q after the K^-/Btk^- cells were treated with hydrogen peroxide. This may suggest that substitution of arginine by glutamine at residue 525 inhibits the phosphorylation of Y551, which is generally agreed to be the first phosphorylation site catalyzed by the Src family kinase, or that the hydrogen peroxide-induced Btk tyrosine phosphorylation is mainly mediated by autophosphorylation.

Syk, but Not Btk, Tyrosine Phosphorylates B Cell Linker Protein (BLNK) upon Hydrogen Peroxide Stimulation. In B cell antigen receptor-triggered phosphoinositide−calcium signaling, BLNK has been proposed to play a pivotal role in linking Syk and Btk to the activation of PLCγ2. According to this model, tyrosine-phosphorylated BLNK by Syk provides the docking sites for Btk and PLCγ2, thereby tethering Btk into close proximity with PLCγ2. The activated Btk then phosphorylated PLCγ2, leading to its full activation (*29*, *51*). To test whether this model is applied for oxidative stress-induced calcium signaling, we examined the tyrosine phosphorylation status of BLNK in wild-type, Syk-deficient, and Btk-deficient DT40 cells following hydrogen peroxide stimulation. Cell lysates from these cells with or without hydrogen peroxide were immunoprecipitated by anti-BLNK antibody and immunoblotted with anti-phosphotyrosine antibody 4G10. As shown in Figure 6 (top), BLNK was tyrosine-phosphorylated, peaking around 2−5 min after hydrogen peroxide stimulation in wild-type DT40 cells. This phosphorylation was almost completely abrogated in Syk-deficient DT40 cells. In comparison, tyrosine phosphorylation of BLNK induced by hydrogen peroxide was even stronger in Btk-deficient cells than in wild-type DT40 cells. Anti-BLNK blotting of the same membrane revealed that protein levels of BLNK in all samples were comparable (bottom). These results clearly indicated that Syk, but not Btk, is responsible for BLNK phosphorylation in response to hydrogen peroxide stimulation. A few tyrosine-phosphorylated proteins were also co-immunoprecipitated with BLNK, and their identities are under investigation.

DISCUSSION

In this study, hydrogen peroxide was chosen for its stability and convenience as an exogenous ROS to gain mechanistic information on Btk-dependent oxidative stress signaling events. Our genetic data demonstrate that Btk is essential for hydrogen peroxide-induced calcium signaling in DT40 cells. Btk-deficient cells exhibit a major reduction (~70%)

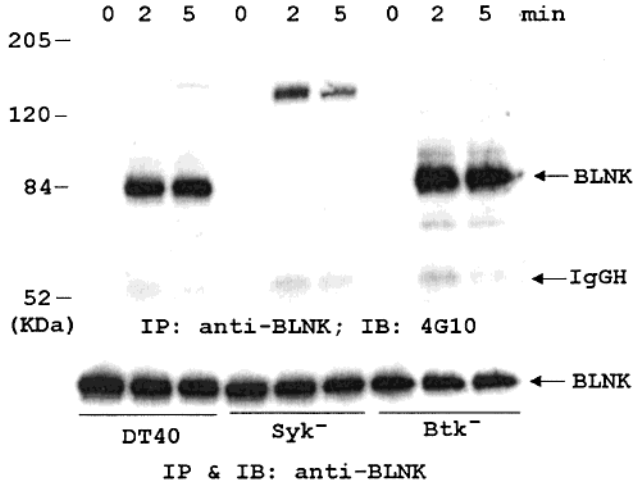

FIGURE 6: Syk-, but not Btk-, dependent tyrosine phosphorylation of BLNK upon hydrogen peroxide stimulation. After 5 mM hydrogen peroxide stimulation for the indicated time, anti-BLNK immunoprecipitates from wild-type, Syk-deficient, and Btk-deficient DT40 cells were immunoblotted with anti-PTyr (top) or with anti-BLNK antibody (bottom).

in hydrogen peroxide-induced calcium mobilization when compared to that observed in wild-type DT40 cells. Ectopic expression of Btk fully restores calcium signaling in Btk-deficient DT40 cells. In parallel to the reduced calcium signaling, Btk-deficient cells show a complete loss of IP_3 production and a very low level of tyrosine phosphorylation of PLCγ2 upon hydrogen peroxide stimulation. Thus, it is likely that, in Btk-deficient cells, impaired activation of PLCγ2 leads to the loss of both hydrogen peroxide-stimulated phosphatidylinositol hydrolysis and calcium mobilization mediated by IP_3. Although overexpression (3−5-fold) of the Btk mutated at its SH2, PH, and catalytic domains in Btk-deficient DT40 cells can slightly restore the hydrogen peroxide-induced calcium release (Figure 1B), this enhancement is not likely mediated by PLCγ2.

Syk has been demonstrated to be involved in hydrogen peroxide-induced tyrosine phosphorylation of PLCγ2 because Syk-deficient DT40 cells exhibit a dramatic reduction in tyrosine phosphorylation of PLCγ2 (*49*). The data presented here show that Btk also participates in hydrogen peroxide-induced tyrosine phosphorylation of PLCγ2 in a similar manner. In contrast to the reduced tyrosine phosphorylation of PLCγ2, either Syk or Btk deficiency leads to a complete loss of hydrogen peroxide-induced IP_3 production, clearly suggesting that both Syk and Btk are required for PLCγ2 activation upon hydrogen peroxide stimulation.

Although in vitro PLCγ is a substrate for most tyrosine kinases tested (*52*), our results and those of others suggest that Btk is the primary tyrosine kinase that phosphorylates PLCγ2 in intact B cells (*13*, *26*, *49*). The dual requirement for both Syk and Btk in hydrogen peroxide-induced tyrosine phosphorylation of PLCγ2 is consistent with the notion that Syk exerts its effect via tyrosine phosphorylation of a linker protein, BLNK, which then forms a complex with both PLCγ2 and Btk, thus facilitating the phosphorylation of PLCγ2 by Btk (*51*, *53*, *54*). BLNK is known to be phosphorylated by Syk and yields high-affinity binding sites for the SH2 domains of PLCγ2 and Btk (*29*), which is consistent with the results showing that the intact SH2 of Btk is required for IP_3 generation (Figure 3B). Therefore,

both Syk and Btk are needed for effective tyrosine phosphorylation stimulated by hydrogen peroxide.

Btk is known to be activated by the phosphorylation of its Y551 followed by autophosphorylation of Y223 mediated by Lyn (*5*, *7*, *9*). However, the mechanism for the phosphorylation of Y551 has not yet been worked out. Since the functional SH2, PH, and catalytic domains of Btk have been implicated as requirements for this activation, Lyn may not directly phosphorylate Y551. Our data indicate that both the SH2 and PH domains are not essential for the hydrogen peroxide-induced Btk activation (Figure 5). However, a functional catalytic domain is required for the phosphorylation of Btk, suggesting that Btk may not be the direct target of Lyn phosphorylation or that the mutant Btk R525Q is not a substrate for Lyn. The activated Btk is insufficient to mobilize the release of intracellular calcium since this process required functional SH2 and PH domains, suggesting that the activated Btk has to bind to a protein that contains a SH2 domain binding site. As discussed above, the most likely candidate for this linker protein is BLNK, which possesses multiple SH2 binding sites capable of binding the SH2 domains of both Btk and its substrate, PLCγ2. This idea is consistent with the observation that the mSH2/Btk$^-$ mutant failed to phosphorylate PLCγ2 (Figure 4B) and generate IP_3 (Figure 3B). It is interesting to note that Btk-deficient cells expressing mPH/Btk can support a significant level of hydrogen peroxide-induced tyrosine phosphorylation of PLCγ2 (Figure 4B) and IP_3 production (Figure 3B), although these levels are clearly lower than those observed for the DT40 cells. Fluckiger et al. also demonstrated that the Btk SH2 domain is required and the PH domain is not necessary for Btk (activated by coexpression with Lyn) to phosphorylate PLCγ2 when they are coexpressed in fibroblasts in the absence of receptor stimulation (*13*). Our data suggest that an intact PH domain is required to achieve a proper conformation of the Btk−PLCγ2−BLNK complex for efficient activation of PLCγ2 and for positioning the active PLCγ2 near its substrate site. The fact that mPH/Btk$^-$ cells failed to cause a significant intracellular calcium release (Figure 1B) may be due to the low level of IP_3 formed being insufficient to meet the "threshold" value needed to trigger intracellular calcium mobilization since this is a highly cooperative process (*55*).

Our genetic studies also demonstrate that functional roles of Btk in BCR-induced and hydrogen peroxide-mediated phosphinositide−calcium signaling are distinctive as evidenced from Btk activation and different effects of Btk on the regulation of IP_3 production and calcium mobilization. Calcium mobilization is completely dependent on Btk in BCR signaling (*26*), whereas there is Btk-independent calcium mobilization in oxidative stress signaling (Figure 1A). mPH/Btk can significantly restore hydrogen peroxide-induced tyrosine phosphorylation of PLCγ2 (Figure 4B) and IP_3 production (Figure 3B), even though lower than those observed for the DT40 cells. However, mPH/Btk fails to do so in BCR signaling. In addition, the intact binding motif within the SH2 domain appears not to be required for hydrogen peroxide-induced Btk activation (Figure 5), whereas this motif is essential for antigen receptor-induced Btk activation (*13*, *56*). Furthermore, it should be pointed out that calcium mobilization inhibited by the PI3K specific inhibitor, wortmannin, was observed in both hydrogen peroxide and antigen receptor signaling. However, overexpression of Btk can overcome this inhibition only for hydrogen peroxide-mediated signaling (*49*). To explain these observed differences, it is essential to fully understand how cells sense oxidative stress and subsequently PTKs such as Btk and Syk are activated. Currently, hydrogen peroxide-induced activation of PTKs, particularly those activated by tyrosine phosphorylation, is believed to be mediated via its ability to inhibit protein−tyrosine phosphatases. However, there are two reports showing that Ltk (*57*) and Fyn (*58*) can be activated by the oxidation of their sulfhydryl moieties.

In summary, our results reveal the following: (i) In DT40 cells Btk is the major tyrosine kinase that phosphorylates and activates PLCγ2 in response to hydrogen peroxide stimulation and leads to intracellular calcium mobilization. The drastically reduced intracellular calcium release observed with Btk-deficient DT40 cells can be fully restored by reexpressing wild-type Btk with the intact kinase, SH2, and PH domains. (ii) The catalytic domain, but not the functional SH2 and PH domains, is required for Btk activation induced by hydrogen peroxide. (iii) The requirement for the intact SH2 domain for Btk to phosphorylate PLCγ2 and induce IP_3 production and calcium mobilization implies that Btk exerts its effect via a complex formed with a linker protein, which is most likely the BLNK (*51*, *53*, *54*). BLNK is phosphorylated by Syk and explains the inability of Syk$^-$ DT40 cells to induce a reasonable level of PLCγ2 phosphorylation that is required to mobilize intracellular calcium in response to hydrogen peroxide treatment (*49*). (iv) An intact PH domain is required to position the Btk, PLCγ2, and phosphatidylinositol 4,5-bisphosphate in close and proper proximity to achieve maximal PLCγ2 activation and IP_3 generation induced by hydrogen peroxide.

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