Bruton's tyrosine kinase targets NF-κB to the *bcl-x* promoter via a mechanism involving phospholipase C-γ2 following B cell antigen receptor engagement

James B. Petro, Iris Castro, John Lowe, Wasif N. Khan*

Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232-0146, USA

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Abstract Disruption of Bruton's tyrosine kinase (BTK) function leads to x-linked immunodeficiency (*xid*) in mice. BTK-deficient ($btk^{-/-}$) B cells are defective for survival. Prior studies show that BTK is required for the induction of Bcl-x_L following B cell antigen receptor (BCR) engagement. However, the mechanism underlying Bcl-x_L induction in response to BCR ligation remains unresolved. We now demonstrate that BTK regulates *bcl-x* expression by transcriptional control in response to BCR engagement. BTK targets nuclear factor-κB (NF-κB) to activate the *bcl-x* promoter via a phospholipase C-γ2 (PLC-γ2)-dependent mechanism. Perturbation of the BTK/PLC-γ2/NF-κB signaling axis likely contributes to the defective expression of *bcl-x* and compromised survival of *xid* B cells.

Key words: B cell antigen receptor; X-linked immunodeficiency; Bruton's tyrosine kinase; Nuclear factor-κB; Bcl-x_L; Phospholipase C-γ2

1. Introduction

The B cell antigen receptor (BCR) transmits biochemical signals via the activation of protein tyrosine kinases (PTK) including the cytoplasmic PTKs Lyn, Syk, and BTK (Bruton's tyrosine kinase) [1–3]. Of these, naturally occurring mutations in the *btk* gene cause the B cell deficiency disorders X-linked agammaglobulinemia in humans and x-linked immunodeficiency (*xid*) in mice [4,5]. *Xid* B cells undergo apoptosis instead of proliferation in response to BCR stimulation [6–8], suggesting a defect in survival and cell cycle progression [7,9]. Although prior studies demonstrate that *xid* B cells fail to increase the levels of pro-survival protein Bcl-x_L upon BCR stimulation [6,8], the role of BTK in this response remains largely undefined.

In response to BCR stimulation, BTK phosphorylates and activates phospholipase C-γ2 (PLC-γ2) [10,11]. Active PLC-γ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate, producing the two lipid second messengers, inositol 1,4,5-triphosphate and diacylglycerol. These second messengers trigger calcium and protein kinase C signaling modulating transcription factors and their target genes [12,13]. We have recently demonstrated that BTK is critical in the BCR-directed nuclear translocation of nuclear factor-κB (NF-κB) via a PLC-γ2/IκB kinase (IKK)-dependent pathway [14]. However, the significance of this signaling axis in the BCR-responsive modulation of gene expression remains unclear. It is also not known whether BCR executes this effect by directing *bcl-x* transcription or enhancing the post-translational stability of Bcl-x_L. In keeping with the established link between BTK, PLC-γ2 and NF-κB, we asked whether BTK facilitates Bcl-x_L induction at the level of transcription. Here we demonstrate that BTK and PLC-γ2 regulate *bcl-x* expression via NF-κB following BCR engagement.

2. Materials and methods

2.1. Mice, cells, and reagents

The generation of *btk*-deficient mice (null mutant; $btk^{-/-}$) has been described previously [15]. The chicken DT40 cell line and DT40 cells deficient for BTK or PLC-γ2 [10,16] were kind gifts of Dr. Tomohiro Kurosaki (Riken Cell Bank, Japan). Primary B lymphocytes were purified by AutoMACS (Miltyni Biotech) by a negative selection protocol, cultured, and stimulated as described [14,17]. The purity of B cells isolated in this manner was approximately 90–95% as verified by fluorescence-activated cell sorting (FACS) analysis using anti-B220 and anti-IgM antibodies (Pharmingen, San Diego, CA, USA). Except where indicated, DT40 and primary mouse B cells were stimulated with anti-chicken IgM monoclonal antibody (M4) and goat anti-mouse IgM respectively or phorbol myristate acetate (PMA) and ionomycin 1 μM each (Calbiochem-Novabiochem, La Jolla, CA, USA). For the pharmacological inhibition of NF-κB, cells were incubated with the indicated concentrations of pyrrolidine dithiocarbamate (PDTC, Sigma, St. Louis, MO, USA) and anti-IgM antibodies. FITC-anti-Bcl-X_L, FITC-IgG_3 isotype control and PE-anti-B220 antibodies were purchased from Pharmingen and anti-phospho-IKK from Cell Signaling (Beverly, MA, USA).

2.2. Northern blotting analyses

For Northern analysis of *bcl-x* mRNA, 20×10^7 splenocytes were activated for 8 h at 37°C and total RNA was extracted via tri-Pure Reagent (Boehringer Mannheim, Indianapolis, IN, USA). 10 μg of RNA was subjected to Northern blotting and hybridized with an α[^{32}p]CTP-labeled probe consisting of a 0.6 kb fragment of murine *bcl-x* cDNA (Gift of A. Ma, University of Chicago, Chicago, IL, USA). The blots were exposed to a phosphoimager plate for signal quantification. The blot was then stripped and reprobed with a α[^{32}p]CTP-labeled probe murine Cμ cDNA.

2.3. Western blotting analyses

For Bcl-x_L studies whole cell extracts were resolved by 12% SDS–PAGE, and blotted on to Immobilon (Millipore, Bedford, MA, USA) membranes as described [14,17]. The membranes were probed with

*Corresponding author. Fax: (1)-615-343 7392.
E-mail address: wasif.khan@mcmail.vanderbilt.edu (W.N. Khan).

Abbreviations: BTK, Bruton's tyrosine kinase; BCR, B cell antigen receptor; PLC-γ2, phospholipase C-γ2; NF-κB, nuclear factor-κB; xid, x-linked immunodeficiency

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rabbit anti-Bcl-x_L (BD Transduction Labs, Cockeysville, MD, USA) or anti-Bcl-2 (Santa Cruz, Santa Cruz, CA, USA). For IκBα degradation assays, 4×10^6 cells/sample were preincubated for 30 min in medium containing 10 μM cycloheximide and various concentrations of PDTC, then stimulated as indicated. Cell extracts were resolved by SDS–PAGE as described [14]. For IKK phosphorylation, whole cell extracts were resolved by 8% SDS–PAGE, and blotted on to Immobilon membranes as described [14,17]. The membranes were probed with rabbit anti-phospho-IKK antibodies that recognize phosphorylated Ser 180 and Ser 181 in IKKα and IKKβ respectively (Cell Signaling Technology) or anti-Bcl-2 (Santa Cruz). The bound antibodies were revealed by horseradish peroxidase-conjugated goat anti-rabbit antibodies and detected by enhanced chemiluminescent detection (Pierce, Rockford, IL, USA) on autoradiography film.

2.4. FACS

For flow cytometric analyses (FACS) of Bcl-x_L protein levels, splenocytes were activated for 24 h in the presence of anti-IgM. 1×10^6 cells/sample were stained for surface expression of B220 and intracellular levels of Bcl-x_L was achieved by with FITC-conjugated anti-Bcl-x_L antibodies in the presence of Fix and Perm (Caltag Labs). Samples were stained in parallel with a FITC-conjugated IgG_3 antibody as a background control. Samples were assayed on a FACStar™ fluorescent cell sorter and the data was analyzed using CELLQuest™ software (Becton Dickinson, Cockeysville, MD, USA).

2.5. Plasmid constructs and luciferase assays

Reporter plasmids containing 0.6 or 1.2 kb of the mouse *bcl-x* promoter fused to a firefly luciferase gene (BclxLuc0.6/BclxLuc1.2) were the kind gift of G. Nunez. Additionally, a NF-κB reporter plasmid encoding firefly luciferase under the control of a NF-κB-responsive promoter (6 κB), a *Renilla* luciferase control and a dominant-negative IκBα ($I\kappa B\alpha^{ss/aa}$) expression vector were [14,17] cotransfected with indicated plasmids into 1×10^7 DT40 cells and analyzed for luciferase activity 6 h after treatment with anti-IgM or PMA/ionomycin as described [14,17].

3. Results

3.1. BTK is critical for BCR-responsive induction of bcl-x mRNA

To begin to define the molecular mechanisms underlying defective induction of Bcl-x_L protein in $btk^{-/-}$ B cells, we analyzed *bcl-x* mRNA levels in BCR-stimulated cells. Northern analyses showed that BCR crosslinking results in a significant increase in the steady state levels of *bcl-x* transcript in wild type but not $btk^{-/-}$ B cells (Fig. 1, upper panel, compare lanes 1 and 2 with 5 and 6). The failure of $btk^{-/-}$ B cells to induce *bcl-x* transcription following stimulation via the BCR is not due to an intrinsic defect in *bcl-x* gene expression, as stimulation via CD40 or pharmacological agents PMA and ionomycin induced similar levels of the *bcl-x* transcript in both $btk^{-/-}$ and wild type B cells (compare lanes 1 and 5 with 7 and 8). These results indicate that BTK is essential for BCR-responsive induction of *bcl-x* mRNA suggesting that BTK signaling pathways may directly regulate Bcl-x_L protein levels.

3.2. Inhibition of NF-κB prevents BCR-directed increases in Bcl-x_L

To elucidate the mechanism by which BTK regulates *bcl-x* levels, we investigated whether inhibition of BCR-induced NF-κB blocks *bcl-x* expression. The effect of the selective NF-κB inhibitor PDTC [18–20] on BCR-responsive *bcl-x* expression was examined. PDTC blocked BCR-directed Bcl-x_L protein induction as determined by FACS or Western blotting (Fig. 2A,B). To ascertain that PDTC inhibited the NF-κB pathway, we assayed for the BCR-directed degradation of

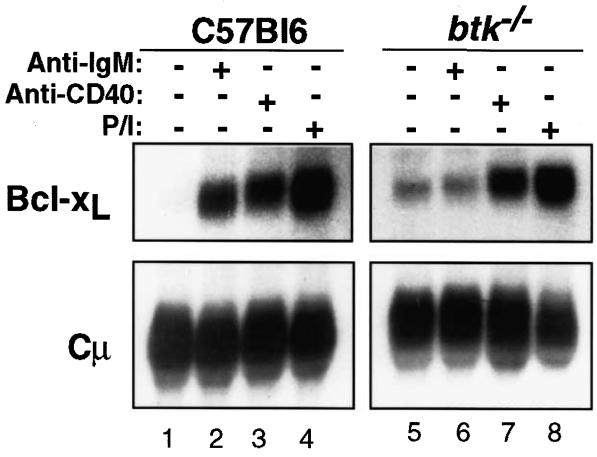

Fig. 1. BCR-responsive expression of *bcl-x* mRNA is BTK-dependent. Northern analysis of RNA extracted from splenocytes isolated from wild type or $btk^{-/-}$ mice. Cells were either left unstimulated (lanes 1 and 5) or activated for 8 h with 10 μg/ml anti-mouse IgM $F(ab')_2$ (lanes 2 and 6), 10 μg/ml anti-mouse CD40 antibodies (lanes 3 and 7), or PMA/ionomycin (lanes 4 and 8). 10 μg of total RNA from each sample was used in Northern analyses with α[^{32}p]CTP-labeled murine *bcl-x* cDNA as a probe (upper panel). The blot was stripped and reprobed with a α[^{32}p]CTP-labeled murine Cμ cDNA probe to verify the integrity and quantity of B cell RNA in each sample (lower panel).

the NF-κB inhibitor IκBα, a required step in the activation of this transcription factor. Consistent with its proposed inhibitory function at the proteasome level, PDTC blocked BCR-induced degradation of IκBα (Fig. 2C). In contrast, PDTC did not block BCR-induced phosphorylation of IKKα and β which lie directly upstream of IκBα in NF-κB pathway (Fig. 2D). These experiments suggest that in primary B cells NF-κB regulates *bcl-x* expression following activation via the BCR.

3.3. BTK, PLC-γ2, and NF-κB are required for activation of the bcl-x promoter

To extend our findings, we investigated whether the BTK/NF-κB signaling axis targets the *bcl-x* promoter directly. BCR stimulation resulted in a two-fold increase in *bcl-x* promoter activity in DT40 (Fig. 3, lane 1) but not in cells deficient for either BTK or PLC-γ2 (lanes 2 and 3). This activity was completely restored in DT40.BTK cells reconstituted with human BTK cDNA (DT40.BTKR) or DT40.PLC-γ2 reconstituted with human PLC-γ2 cDNA (DT40.PLC-γ2R), indicating that BTK and PLC-γ2 expression are necessary for BCR-directed transcription of the *bcl-x* reporter gene (lanes 4 and 5). Furthermore, PMA and ionomycin induced similar levels of reporter expression in all cell types, indicating that downstream signaling pathways are intact and function independently of BTK and PLC-γ2.

To test whether NF-κB is functionally important for BCR-responsive transcription of *bcl-x*, DT40 cells were cotransfected with the *bcl-x* reporter plasmid and an expression construct encoding a dominant negative form of the NF-κB inhibitor IκBα ($I\kappa B\alpha^{ser32/36ala}$), which has been demonstrated to block nuclear translocation of NF-κB [21]. Consistent with our hypothesized role for NF-κB in BCR-directed transcription of *bcl-x*, ectopic expression of $I\kappa B\alpha^{ser32/36ala}$ completely blocked both BCR- and PMA/ionomycin-directed activation

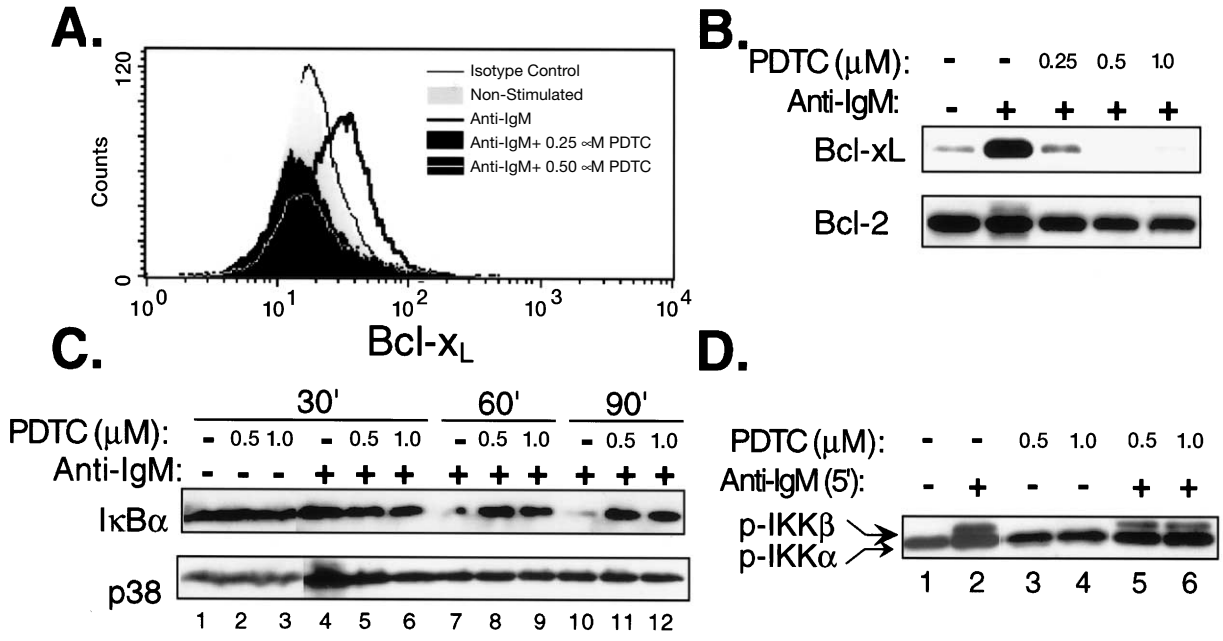

Fig. 2. The NF-κB inhibitor PDTC blocks BCR-induced Bcl-x_L production. A: FACS analysis of Bcl-x_L in B cells activated with 20 μg/ml anti-IgM for 24 h in the presence or absence of PDTC. Expression of Bcl-x_L in $B220^+$ B cells is displayed. B: Purified splenic B cells (95% as determined by FACS) were incubated with or without 20 μg/ml of anti-IgM and PDTC for 16 h and total cellular extracts (13 μg/lane) were analyzed by SDS–PAGE immunoblotting. The blots were probed sequentially with anti-Bcl-x_L (top panel) and anti-Bcl-2 for protein loading control (bottom panel). C: PDTC interferes with IκBα degradation. Cells were preincubated with cycloheximide (to arrest protein synthesis) and PDTC for 30 min prior to stimulation as indicated. Cellular extracts were analyzed by Western blotting. The blots were probed with anti-IκBα, stripped and reprobed with anti-p38 antibodies to verify the protein content and integrity. D: PDTC does not prevent BCR-induced IKK phosphorylation. Western blot analysis of protein extracts from B cells treated as indicated. Blots were probed sequentially with anti-phospho-specific IKKα and β and anti-p38 for protein content and integrity.

of the *bcl-x* reporter gene (Fig. 3, lane 6). This result implicates the BCR-directed activation of NF-κB in the transcriptional activation of the *bcl-x* promoter.

4. Discussion

We have previously reported a requirement for BTK in the BCR-directed activation of NF-κB [17]. Furthermore, we and others have identified functional NF-κB binding sites in the *bcl-x* promoter [22,23]. As such, we hypothesized that BTK-dependent activation of NF-κB may promote BCR-responsive transactivation of *bcl-x*. Here we demonstrate that in response to BCR signaling BTK mediates Bcl-x_L induction by targeting NF-κB to *bcl-x* promoter. This finding is consistent with a role for NF-κB in *bcl-x* regulation in response to CD40 ligation [23]. Furthermore, these data validate our hypothesis at multiple levels. First, BTK is indeed required for BCR-directed transcriptional activation of *bcl-x* (Fig. 1). Second, a BTK substrate PLC-γ2 that we previously showed to be essential for NF-κB activation is also required for *bcl-x* promoter activity (Fig. 3). Third, pharmacological inhibition of BCR-induced NF-κB interferes with *bcl-x* expression (Fig. 2). Fourth, a dominant negative inhibitor of NF-κB ($I\kappa B\alpha^{ss/aa}$) completely blocks BCR-dependent activation of *bcl-x* promoter (Fig. 3). Together these findings provide mechanistic insights as to the necessary role for BTK signal transducers (PLC-γ2 and NF-κB) in the transcriptional regulation of *bcl-x*, explaining in part why *xid* B cells are impaired in BCR-directed Bcl-x_L induction. Interference with this survival mechanism caused by loss of BTK function may also explain the paucity of mature B cells observed in *xid* mice.

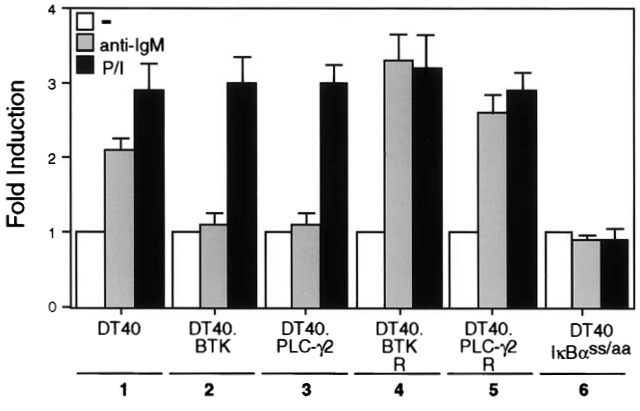

Fig. 3. BTK, PLC-γ2, and NF-κB are required for BCR-directed transcription from the *bcl-x* promoter. DT40 chicken B cells and DT40 cells deficient for BTK (DT40.BTK) or PLC-γ2 (DT40.PLC-γ2) or DT40.BTK cells reconstituted with human BTK cDNA (DT40.BTKR) or DT40.PLC-γ2 cells reconstituted with human PLC-γ2 cDNA (DT40.PLC-γ2R) (1×10^7 B cells/sample) were co-transfected with 15 μg of a plasmid containing the firefly luciferase gene downstream of a 1.2 kb region of the murine *bcl-x* promoter (Bcl-xLuc1.2) and 1 μg of a *Renilla* luciferase expression vector to normalize for transfection efficiency. Also, 1×10^7 DT40 cells were transfected with the addition of 6 μg of a dominant-negative IκBα expression vector (DT40/$I\kappa B\alpha^{ss/aa}$). Cells were incubated for 18 h post-transfection and activated for 6 h with anti-IgM or PMA/ionomycin (P/I). Cytoplasmic extracts were obtained and luciferase was quantified. Results are reported as the mean fold induction of luciferase in stimulated relative to unstimulated cells. Data are representative of three experiments.

Our results mirror those presented in a prior study describing a role for NF-κB in the negative regulation of *bcl-x* in $CD4^+/CD8^+$ double positive thymocytes isolated from transgenic mice harboring a dominant negative form of IκBα [24]. However, Bcl-x_L levels were not affected in transgenic thymocytes stimulated via the T cell antigen receptor. In contrast, data presented here demonstrate positive regulation of the *bcl-x* gene by NF-κB in both primary B cells and an immature B cell line (DT40, Figs. 1 and 3). This positive regulation of the *bcl-x* promoter by NF-κB is further supported by our observation that overexpression of Rel subunits is sufficient to drive transcription of a luciferase gene under *bcl-x* promoter control in vitro (data not shown). Within lymphocytes, *bcl-x* gene transcription is thus likely to be regulated in a tissue- and stage-specific manner. Our observations regarding *bcl-x* expression in B cells are consistent with the recent findings that B cells isolated from mice deficient for NF-κB subunits c-Rel or RelA or express a dominant inhibitor of NF-κB display defects in BCR-directed survival and proliferation similar to those of $btk^{-/-}$ B cells [25–28]. Thus, a block in BTK-dependent NF-κB activation impairs BCR-directed induction of *bcl-x* transcription, likely contributing to the compromised survival and proliferation of *xid* B cells. Although it is likely that NF-κB cooperates with other transcription factors to promote *bcl-x* gene expression, the BTK/PLC-γ2/NF-κB dependence of *bcl-x* transcription reveals a critical and non-redundant role for NF-κB in this activity.

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