

Bruton's Tyrosine Kinase Is Required for Activation of I κ B Kinase and Nuclear Factor κ B in Response to B Cell Receptor Engagement

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Abstract

Mutations in the gene encoding Bruton's tyrosine kinase (*btk*) cause the B cell deficiency diseases X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (*xid*) in mice. In vivo and in vitro studies indicate that the BTK protein is essential for B cell survival, cell cycle progression, and proliferation in response to B cell antigen receptor (BCR) stimulation. BCR stimulation leads to the activation of transcription factor nuclear factor (NF)- κ B, which in turn regulates genes controlling B cell growth. We now demonstrate that a null mutation in *btk* known to cause the *xid* phenotype prevents BCR-induced activation of NF- κ B. This defect can be rescued by reconstitution with wild-type BTK. This mutation also interferes with BCR-directed activation of I κ B kinase (IKK), which normally targets the NF- κ B inhibitor I κ B α for degradation. Taken together, these findings indicate that BTK couples IKK and NF- κ B to the BCR. Interference with this coupling mechanism may contribute to the B cell deficiencies observed in XLA and *xid*.

Key words: X-linked immunodeficiency • X-linked agammaglobulinemia • antigen receptor signaling • transcription factor • Tec family tyrosine kinase

Introduction

Biochemical transmission of signals discharged from the B cell antigen receptor (BCR)¹ to downstream transcription factors involves the action of multiple protein tyrosine kinases (PTKs; for review see reference 1). These PTKs include Syk, Lyn, and Bruton's tyrosine kinase (BTK), which are induced after BCR cross-linking (2, 3). Activated BTK transduces signals that are important for the regulation of B cell growth and proliferation (4, 5). For example, BTK acts in concert with Syk to phosphorylate and activate phospholipase C (PLC)- γ 2 (6, 7), which mediates production of the second messengers inositol 1,4,5-triphosphate and diacylglycerol (8). In turn, these second messengers stimulate the activity of protein kinase C (PKC) and increase intracellular calcium levels, resulting in the ac-

tivation of downstream transcription factors (9, 10). However, the identities of specific transcription factors that are controlled by BTK activity remain unknown.

Mutations in the *btk* gene result in the B cell immunodeficiencies X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (*xid*) in mice (for review see references 11 and 12). The severe B cell deficiency underlying XLA is caused by a block in B cell ontogeny at the pre-B cell stage, leading to a dramatic reduction in the levels of serum Igs (13, 14). A less severe form of immunodeficiency occurs in mice harboring either null or point mutations in the *btk* gene (15–18). In *xid* mice, peripheral B cells are reduced in number to ~50% relative to wild type. The *xid* B cells that remain secrete reduced levels of serum IgM and IgG3, fail to respond to T cell-independent type II antigens, and are unable to proliferate in response to BCR stimulation (for review see reference 19). Recent studies indicate that the *xid* phenotype may arise from defects in cell cycle progression and BCR-directed induction of Bcl-X_L, an antiapoptotic protein (4, 5, 20, 21). However, the mechanisms by which BTK regulates these processes remain unclear.

Like BTK, transcription factor nuclear factor (NF)- κ B is activated in response to BCR stimulation and has been im-

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¹Abbreviations used in this paper: BCR, B cell antigen receptor; BTK, Bruton's tyrosine kinase; DTT, dithiothreitol; EMSAs, electrophoretic mobility shift assays; IKK, I κ B kinase; NF- κ B, nuclear factor κ B; PKC, protein kinase C; *xid*, X-linked immunodeficiency; XLA, X-linked agammaglobulinemia.

plicated in the regulation of Bcl-X_L (22). Studies with NF- κ B-deficient B cells have identified defects in BCR-induced survival and proliferation (23–25). Members of the NF- κ B/Rel family of proteins include p50/NF- κ B1, p52/NF- κ B2, RelA, c-Rel, and RelB, which have the capacity to form either homo- or heterodimers (26). In quiescent cells, NF- κ B dimers containing the principle transactivating subunits RelA and c-Rel are found in the cytoplasmic compartment associated with members of the I κ B family of inhibitors, such as I κ B α (26, 27). Upon cellular activation, I κ B α is subject to site-specific phosphorylation, which targets the inhibitor for degradation by the ubiquitin-proteasome pathway. Recent studies indicate that I κ B α is phosphorylated by a multicomponent I κ B kinase (IKK) containing two catalytic subunits (IKK α and IKK β) and one regulatory subunit (IKK γ ; reference 27). The kinase activity of IKK is stimulated by a large set of NF- κ B-inducing agents including the proinflammatory cytokines TNF- α and IL-1. Although it is clear that BCR stimulation leads to the activation of NF- κ B (28), a role for either BTK or IKK in this B cell signal transduction pathway has not been elucidated.

In this study, we investigated the significance of BTK in the BCR/NF- κ B signaling axis using a BTK-deficient B cell line and primary B lymphocytes isolated from *btk*^{-/-} mice. We demonstrate here that BTK is required for BCR-induced degradation of I κ B α and NF- κ B activation in both transformed and primary B cells. Additionally, BCR cross-linking stimulates the activity of IKK in B cells expressing BTK, whereas BTK-deficient B cells are unable to execute this response. We conclude that BTK couples NF- κ B to the BCR via a mechanism involving the action of IKK. Interference with this coupling mechanism may directly contribute to the B cell deficiencies observed in XLA and xid diseases.

Materials and Methods

Mice. The generation of *btk*-deficient mice (null mutant; *btk*^{-/-}) has been described previously (17). These mice have a mixed genetic background of 129/Sv \times C57BL/6. For wild-type controls, 129/Sv \times C57BL/6 or C57BL/6 mice (The Jackson Laboratory) were used. All mice that were used as the source of splenocytes were treated humanely and in accordance with the federal and state government guidelines, and their use was approved by the institutional animal committee.

Cells. The chicken DT40 cell line and DT40 cells made deficient for BTK by homologous recombination (DT40.BTK; reference 6; T. Kurosaki, Riken Cell Bank, Japan) were maintained in RPMI with 10% FCS, 1% chicken serum, 50 μ M 2-ME, 2 mM l-glutamine, and penicillin/streptomycin at 39°C in 5% CO₂. DT40 or DT40.BTK B cells were cultured in low serum media (RPMI with 0.5% FCS, 0.05% chicken serum, 50 μ M 2-ME, 2 mM l-glutamine, and 1% penicillin/streptomycin) for 8–12 h before activation.

Cells were either left unstimulated or stimulated with 1:2 dilution of hybridoma supernatants containing anti-chicken IgM mAb (M4) or PMA and ionomycin, 1 μ M each (Calbiochem). These low serum culture conditions were established to reduce

the levels of constitutive nuclear NF- κ B activity (29, 30) and enhance the detection of the BCR-directed increases in nuclear NF- κ B.

For primary B cell purification, single-cell suspensions were prepared from pooled spleens of control and *btk*^{-/-} mice. The cell suspensions were then depleted of RBCs by density gradient centrifugation on lympholyte-M (Cedarlane Labs.). B cells were purified by a process of negative selection on an affinity chromatography column (Cedarlane). CD4⁺CD8⁺ T cells as well as monocytes and macrophages are removed by the column based on their binding to antibodies against CD4, CD8, and MAC-1. The purity of B cells isolated in this manner was between 90 and 95% for wild type and 85 and 90% for *btk*^{-/-}, as confirmed by FACS[®] analysis using anti-B220 and anti-IgM antibodies (PharMingen). The entire procedure was performed at 4°C, and the cells were used in experiments immediately thereafter.

Purified B cells (3–5 \times 10⁶ cells per sample) were incubated with 10 μ g/ml polyclonal goat anti-mouse IgM F(ab')₂ fragments (Jackson ImmunoResearch) or with PMA and ionomycin (1 μ M each) for 2 h at a cellular density of 2 \times 10⁶ cells per milliliter in culture media (RPMI-1640 supplemented with 10% serum). To monitor any effects of serum on the activation of NF- κ B, cells that were not stimulated were also incubated in medium containing 10% serum for the duration of stimulation.

Electrophoretic Mobility Shift Assays and Western Blot Analysis. Nuclear extracts were prepared by solubilization of cells in lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.4% NP-40, 1 mM dithiothreitol [DTT], 0.5 mM PMSF, 5 μ g/ml antipain, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, 0.5 μ g/ml pepstatin, 7.5 μ g/ml bestatin, 4 μ g/ml phosphoramidon, 5 μ g/ml soybean trypsin inhibitor) to remove the cytoplasmic fraction. Extracts were prepared from nuclear pellets in high salt nuclear extraction buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) containing protease inhibitors (31). For electrophoretic mobility shift assays (EMSA), α -[³²P]dCTP and α -[³²P]dATP and labeled double-stranded oligonucleotide probe derived from the κ B enhancer element of the IL-2R α receptor promoter (5'-CAACG-GCAGGGGAATTCCCCTCTCCTT-3'; κ B binding site underlined) was used (32). To determine the specificity of nucleoprotein binding to DNA, an oligonucleotide (5'-CTACAT-TCATTTCCAGATTCACCTTTCCTGCAGT-3'; κ B binding site underlined and mutations shown in bold) containing two mutant κ B sites derived from sequences within the HIV long terminal repeat (33) was used. To verify equal amounts and integrity of proteins in the nuclear extracts, a control oligonucleotide for NF-Y was used (34). DNA binding reactions were performed on nuclear extracts from equal numbers of cells and 1 μ l of ³²P-labeled probe in a buffer containing 20 mM Hepes, pH 7.9, 50 mM KCl, 5% glycerol, 1 mM EDTA, pH 8.0, 10 mM DTT, 1% NP-40, 1 mg/ml BSA, 2 μ g double-stranded poly(dI-dC), and 2 μ g pd(N)₆ random hexamer. Reactions were incubated on ice for 15 min, and the nucleoprotein-DNA complexes were resolved on a 5% native polyacrylamide gel electrophoresed in 1 \times Tris borate buffer (TBE) at 180 V for 2.5 h and visualized by autoradiography.

For Western blot analysis of various κ B family members, nuclear extracts equivalent to 2 \times 10⁷ cells were denatured in Laemmli reducing buffer by boiling at 95°C for 3 min, and the proteins were resolved by 15% SDS-PAGE. Proteins were electrotransferred onto nitrocellulose membranes and subjected to immunoblotting with rabbit polyclonal antibodies against RelA, c-Rel, or SP1 in blocking solution containing 1 \times TBST (Tris-buffered sa-

line and 0.1% Tween) and 5% nonfat dry milk. The bound antibodies were revealed by horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Zymed Laboratories), followed by enhanced chemiluminescent detection (Pierce Chemical Co.) on autoradiography film.

For I κ B α degradation assays, cells were preincubated for 30 min in medium containing 50 μ M cycloheximide (4×10^6 cells per sample) and then stimulated with anti-IgM or with PMA and ionomycin for 90 min in the continued presence of cycloheximide. After stimulation, whole cell extracts were resolved on a 12% denaturing SDS-PAGE, blotted onto nitrocellulose membranes, and probed with rabbit anti-chicken I κ B α (35).

Plasmid Constructs and Luciferase Assays. The coding sequence of mouse BTK cDNA was amplified using high fidelity Pwo DNA polymerase (Boehringer Ingelheim) using primer 5'-CTGCCATGGCTGCAGTGATACTG-3' containing a NcoI (underlined) with primer 5'-CCGGATCCTCAGGATTCTTCATCCATC-3' containing a BamHI (underlined) site. The PCR product was digested with NcoI and BamHI and cloned into the retroviral vector pMMP (reference 36; a gift of Drs. R. Mulligan and J.-S. Lee, Harvard Medical School, Boston, MA). Ectopic expression of protein of the appropriate size was confirmed by transfection into a *btk*^{-/-} Abelson pre-B cell line followed by Western blotting using rabbit polyclonal anti-BTK antibodies (17). The κ B reporter plasmid encoding firefly luciferase under the control of a promoter containing six consensus NF- κ B binding sites (6 κ B) and a control vector containing a Renilla luciferase gene fused to a thymidine kinase promoter were gifts from Dr. E. Oltz (Vanderbilt University).

DT40 and DT40.BTK were each cotransfected by electroporation (250 V, 960 μ F; Bio-Rad Gene Pulser) with 1.5 μ g of either pMMP.BTK or pMMP (36), 5 μ g of the 6 κ B reporter construct, and 2.5 μ g of the Renilla construct and cultured for 12 h in the medium described above. Cells were also cultured in low serum conditions for 6 h before stimulation and activated for 6 h as described above. Activated cells were then harvested, and levels of both firefly and Renilla luciferase were determined using a Dual-Luciferase Reporter Assay System (Promega Corp.). Levels of firefly luciferase expression were normalized against Renilla as a control for transfection efficiency.

In Vitro Kinase Assays. In vitro kinase assays were performed on the cytosolic protein extracts from $3\text{--}5 \times 10^6$ purified B cells. Resting and stimulated cells were solubilized in lysis buffer containing 1.5 mM MgCl₂, 300 mM sucrose, and the phosphatase inhibitors NaF (2 mM), Na₃VO₄ (100 μ M), and 12.5 mM β -glycerophosphate in addition to its constituents described above. The lysates were cleared at 14,000 rpm in a microcentrifuge for 5 min. Cleared cell extracts from 0.5×10^6 cell equivalents were removed for Western blot analysis, and the remaining cell extract was subjected to immunoprecipitation with anti-IKK α and anti-IKK β antibodies (Santa Cruz Biotechnology) for 1 h on ice and an additional 1 h with rotation after the addition of 20 μ l of protein A-Sepharose beads (Amersham Pharmacia Biotech). The immunocomplexes were washed three times in lysis buffer, followed by one wash in kinase buffer made up of 20 mM Hepes, pH 7.2, 2 mM MgCl₂ and MnCl₂, 1 mM DTT, 20 μ M ATP, and the phosphatase inhibitors described above but without γ -[³²P]ATP and the substrate. The immunocomplexes were then resuspended in 20 μ l of kinase buffer containing 1.0 μ Ci γ -[³²P]ATP and either 50 μ g/ml of wild-type GST-I κ B α substrate or mutant GST-I κ B in which Ser-32 and Ser-36 were replaced with Ala (GST-I κ B α -SS/AA). The reaction was allowed to continue for 30 min at 30°C under agitation and then was ter-

minated by the addition of 4 \times SDS sample buffer. The samples were then boiled for 10 min and resolved by 8% SDS-PAGE. The gel was stained with Coomassie brilliant blue to visualize the GST-I κ B substrate. The gels were dried and exposed to X-ray film to visualize γ -³²P-phosphorylated GST-I κ B.

Results

Btk Is Required for BCR-induced Activation of NF- κ B. Prior studies have demonstrated that NF- κ B is activated in murine B cells after BCR stimulation (28, 37). Furthermore, both BTK- and NF- κ B-deficient B cells exhibit profound defects in BCR-induced survival and proliferation (24, 25). However, a biochemical link between BTK and NF- κ B has not been established. To investigate the role of BTK in the activation of NF- κ B complexes, we employed a BTK-deficient chicken B cell line derived from transformed DT40 cells by homologous recombination. Mutant chicken B cells lacking BTK (DT40.BTK) and parental DT40 B cells were exposed to anti-IgM antibodies that induce BCR signaling. Nuclear extracts were then prepared and analyzed for their content of NF- κ B DNA binding activity in EMSAs. As shown in Fig. 1 A, BCR stimulation of DT40 B cells led to a marked increase in nuclear NF- κ B activity (compare lanes 1 and 3). In sharp contrast, the BCR-inducible activity of NF- κ B in DT40.BTK B cells was negligible (Fig. 1 A, lanes 2 and 4). The observed defect was not apparent when DT40.BTK cells were stimulated with combinations of phorbol ester (PMA) and calcium ionophore (ionomycin), which together mimic the second messengers responsible for PKC activation and calcium mobilization (Fig. 1 A, lanes 5 and 6). These results strongly suggest that BTK is required for BCR-induced activation of NF- κ B.

To extend the prior findings, we next compared the kinetics of NF- κ B activation in DT40 and DT40.BTK B cells over a period of 4 h. As shown in Fig. 1 B, NF- κ B DNA binding activity was clearly evident in the nuclear compartment of DT40 cells after 15 min of stimulation. This activity was still detectable after 4 h. In contrast, DT40.BTK B cells failed to elicit any increase in the NF- κ B DNA binding activity throughout the same time course. As such, the defect in NF- κ B activity observed with DT40.BTK B cells cannot be attributed to a delay in its kinetics of induction.

To confirm this interpretation, we initiated further studies to determine the specificity of the DNA binding proteins detected in EMSA. For these experiments, EMSAs were conducted with the same nuclear extracts as in Fig. 1 A, a radiolabeled κ B consensus sequence, and an excess of unlabeled oligonucleotides containing either wild-type or mutated versions of this κ B sequence (30). As shown in Fig. 1 C, nucleoprotein complex formation was completely blocked in DNA binding reactions containing wild-type κ B oligonucleotides (lanes 3 and 6). In contrast, these nucleoprotein complexes were readily detected in reactions containing equivalent amounts of the mutant κ B oligonucleotide (lanes 4 and 7). These results indicate that the in-

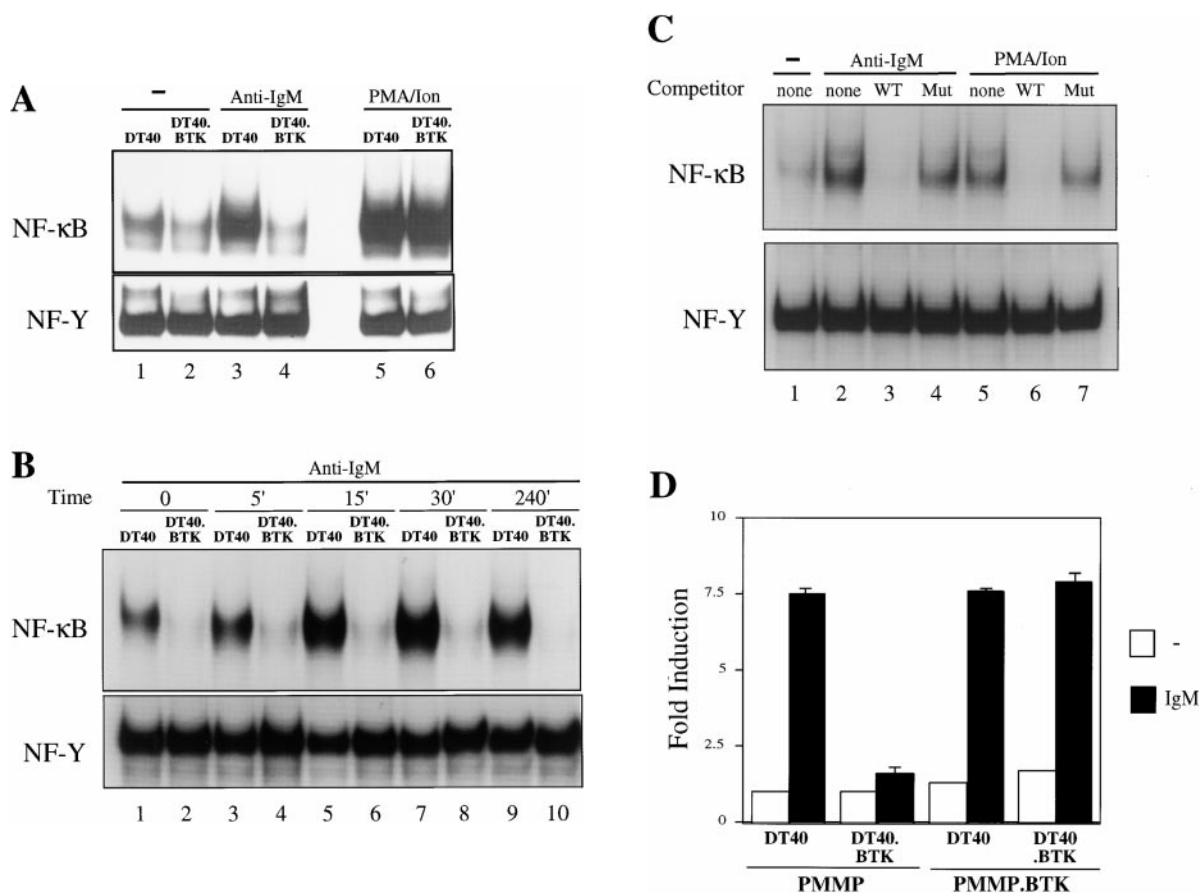


Figure 1. Defective NF-κB activation in BTK-deficient (DT40.BTK) B cells upon BCR stimulation. (A) EMSA analysis of nuclear NF-κB in DT40 and DT40.BTK B cells. Cells were incubated for 2 h in the presence or absence of anti-IgM (lanes 1–4) or PMA/ionomycin (lanes 5 and 6), and nuclear extracts equivalent to 4×10^6 cells were added in a reaction containing a ^{32}P -labeled oligonucleotide probe derived from the κB enhancer element of the IL-2Rα promoter. DNA–nucleoprotein complexes were resolved on nondenaturing polyacrylamide gels. Binding of nucleoprotein complexes to an NF-Υ probe (34) was also performed to verify the integrity and the concentration of proteins in the nuclear extracts (bottom panel). (B) Kinetics of NF-κB activation in DT40 and DT40.BTK B cells upon IgM stimulation. Nuclear extracts were prepared and subjected to EMSA as described in A. (C) NF-κB nucleoprotein complexes in DT40 B cells bind specifically to a consensus NF-κB site. EMSAs were conducted with the same nuclear extracts as in A. Nuclear extracts were either preincubated with 100-fold excess of unlabeled wild-type (lanes 3 and 6), mutant NF-κB probe (Mut; lanes 4 and 7; reference 32), or without the unlabeled probes (lanes 1, 2, and 5) before addition of a ^{32}P -labeled wild-type probe. Protein integrity and concentration in the nuclear extracts was verified with NF-Υ probe (bottom panel). (D) Ectopic expression of BTK restores BCR-directed NF-κB activation in DT40.BTK B cells. DT40 and DT40.BTK B cells were transiently cotransfected with an expression vector encoding wild-type BTK (pMMP.BTK), an NF-κB reporter plasmid (6κB), and a Renilla luciferase plasmid to normalize for transfection efficiency. Control transfections were performed with blank vector (pMMP). After 24-h culture, cells were stimulated with anti-IgM for 6 h and then assayed for reporter gene activity as described in Materials and Methods. Results for these rescue experiments are reported as the mean fold induction of NF-κB-directed transcription relative to unstimulated DT40 cells.

ducible DNA binding activity observed in DT40 B cells after engagement of the BCR is specific for κB core sequences.

To complement these findings, we attempted to rescue the NF-κB defect in DT40.BTK B cells. For these studies, we transfected DT40 and DT40.BTK B cells with an expression vector encoding wild-type BTK along with an NF-κB reporter gene. As shown in Fig. 1 D, expression of the NF-κB-responsive gene was significantly induced in DT40 B cells in the absence of ectopic BTK, whereas DT40.BTK cells failed to elicit this response. However, enforced expression of ectopic BTK fully reconstituted NF-κB directed transcription in DT40.BTK B cells. These rescue experiments indicate that BTK is required for NF-κB activation in response to BCR stimulation.

Prior studies have shown that RelA and c-Rel are the principle transactivator subunits of NF-κB in B cells (38). To analyze the nuclear contents of RelA and c-Rel in DT40 cells, immunoblotting experiments with DT40 nuclear extracts and Rel subunit-specific antibodies were performed. As shown in Fig. 2, nuclear accumulation of both RelA and c-Rel was evident in parental DT40 B cells after stimulation with anti-IgM antibodies (Fig. 2, lane 3, top and center panels), whereas this response was not apparent in DT40.BTK cells (Fig. 2, lane 4, top and center panels). In the absence of BCR stimulation, BTK-deficient B cells expressed lower basal levels of nuclear RelA relative to BTK-expressing controls (Fig. 2, lane 2, top and center panels). These differences could not be attributed to variations in nuclear extract integrity, because similar amounts of

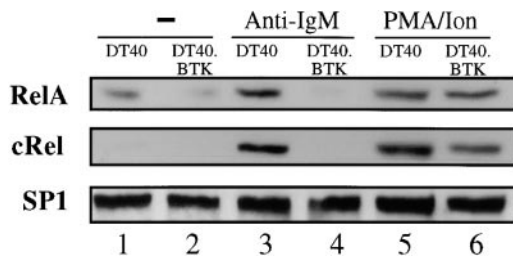


Figure 2. BTK-deficient B cells fail to translocate RelA and c-Rel to the nucleus in response to BCR stimulation. Western blot analysis of RelA and c-Rel in nuclear extracts of DT40 or DT40.BTK B cells. Nuclear extracts were prepared from cells that were either not stimulated (lanes 1 and 2) or stimulated either with anti-IgM (lanes 3 and 4) or with PMA/ionomycin (lanes 5 and 6). Equal amounts of nuclear extracts (2×10^7 cell equivalents per lane) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The relative amounts of RelA and c-Rel were then determined by immunoblotting using antisera directed against either RelA (top panel) or c-Rel (center panel) and visualized by enhanced chemiluminescence detection. The blots were stripped and reprobed for SP1, a constitutively expressed transcription factor to ensure protein integrity and the loading equivalent amounts in each lane (bottom panel).

the constitutively expressed transcription factor SP1 were detected in all samples (bottom panel). Consistent with the results shown in Fig. 1 A, nuclear translocation of RelA and c-Rel in response to PMA/ionomycin was unaffected by the loss of BTK (Fig. 2, lanes 5 and 6). These findings indicate that the κ B-specific DNA binding activity found in the nuclei of DT40 cells after BCR engagement involves BTK-dependent translocation of the RelA and c-Rel transactivating subunits of NF- κ B to this subcellular compartment.

BTK Is Required for BCR-induced Degradation of I κ B α . In resting lymphocytes, NF- κ B is typically found in the cytoplasmic compartment by virtue of its interaction with inhibitory members of the I κ B family of proteins. One of these inhibitors, termed I κ B α , is degraded in response to many NF- κ B-inducing agents (27). Because I κ B α degradation reveals the nuclear localization signal of the Rel dimers, NF- κ B is rapidly mobilized to the nuclear compartment, where it stimulates transcription of many growth-related genes as well as the gene encoding I κ B α . To determine whether BCR-mediated activation of NF- κ B involves the degradation of I κ B α , the stability of this inhibitor in DT40 versus DT40.BTK cells was compared after BCR engagement. In these experiments, cells were first exposed to cycloheximide to prevent de novo synthesis of I κ B α . Translationally arrested cells were then treated with anti-IgM for various periods of time. Consistent with the finding that NF- κ B is activated in response to BCR signaling (Fig. 1), stimulation of wild-type DT40 B cells with anti-IgM resulted in the rapid degradation of I κ B α (Fig. 3, lanes 2–4). In sharp contrast, under the same stimulatory conditions, I κ B α escaped proteolytic breakdown in DT40.BTK B cells (Fig. 3, lanes 7–9). In contrast, treatment with PMA together with ionomycin led to the rapid loss of I κ B α in the DT40.BTK cells (Fig. 3, lanes 5 and 10). Taken together, these findings indicate that BTK mediates the nuclear translocation of RelA and c-Rel via a mechanism involving the proteolytic inactivation of I κ B α .

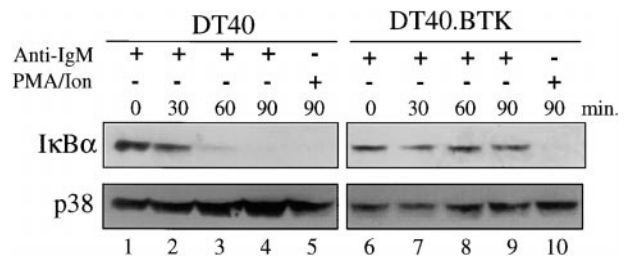


Figure 3. A requirement for BTK in B cells for the degradation of I κ B α in response to BCR cross-linking. Western blot analysis of I κ B α in cytoplasmic extracts from DT40 (lanes 1–5) or DT40.BTK (lanes 6–10). Cells were pretreated with cycloheximide (50 μ M) for 30 min to arrest translation. Translationally arrested cells were stimulated with anti-IgM or PMA/ionomycin for indicated time periods. Equal amounts of cytosolic extracts (4×10^6 cell equivalents per lane) were subjected to immunoblot analysis as described in Fig. 2 with an antiserum against chicken I κ B α (pp40). Blots were stripped and reprobed for a constitutively expressed protein, p38 MAPK, to verify protein integrity and the loading amount of protein in each lane (bottom panel).

Interference with NF- κ B and IKK Signaling in Primary *btk*^{-/-} B Cells. To ascertain the in vivo significance of our findings with transformed chicken B cells, we next monitored the status of NF- κ B in primary B cells derived from *btk*^{-/-} mice. For these studies, wild-type and *btk*^{-/-} B cells were stimulated with anti-mouse IgM F(ab')₂ antibodies, and the corresponding nuclear extracts were analyzed for NF- κ B DNA binding activity using EMSAs. As shown in Fig. 4, NF- κ B activity was substantially increased in primary wild-type B cells after BCR cross-linking (lanes 1 and 3). In contrast, NF- κ B induction in response to BCR engagement was negligible in *btk*^{-/-} B cells (Fig. 4, lanes 2 and 4). Importantly, *btk*^{-/-} B cells were fully competent for NF- κ B signaling after stimulation with combinations of PMA and ionomycin (Fig. 4, lanes 5 and 6). These results with primary B cells fully recapitulate results obtained with

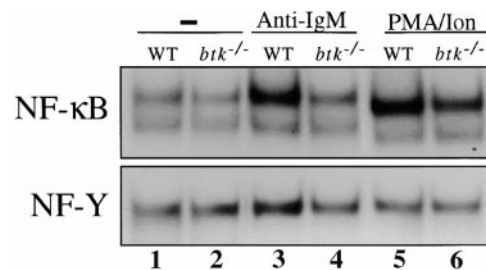


Figure 4. Impaired NF- κ B nuclear translocation in *btk*^{-/-} primary B cells in response to BCR stimulation. EMSA analyses for κ B binding activity in the nuclear extracts from anti-IgM- and PMA/ionomycin-stimulated B cells. Purified splenic B cells from *btk*^{-/-} or C57Bl/6 (WT) mice (WT, 93%; *btk*^{-/-}, 85%; B220⁺/IgM⁺ B cell) were either left unstimulated (lanes 1 and 2) or stimulated for 1 h with anti-mouse F(ab')₂ (lanes 3 and 4) or with PMA and ionomycin (lanes 5 and 6). Equal amounts of nuclear extracts (3×10^6 cell equivalents per lane) were used in each DNA binding reaction. NF-Y binding was used as a control to verify the integrity and concentration of proteins in the nuclear extracts. Based on quantitative analysis of three separate experiments, the mean fold induction of NF- κ B DNA binding activity normalized by their NFY controls in WT versus *btk*^{-/-} cells was 1.7 ± 0.3 and 1.0 ± 0.1 , respectively.

transformed DT40 cells, providing further evidence that the linkage between NF- κ B and the BCR via BTK is physiologically significant.

Experiments shown in Fig. 3 indicate that BTK is required for BCR-induced degradation of I κ B α , a major cytoplasmic inhibitor of NF- κ B. In response to proinflammatory cytokines, I κ B α is phosphorylated at Ser-32 and Ser-36 by a multicomponent IKK. In turn, this phosphorylation event targets I κ B α to the ubiquitin–proteasome pathway. To determine whether IKK is under BTK control in primary B cells, *in vitro* phosphorylation assays were performed using a GST-I κ B α (amino acids 1–54) fusion protein as a substrate (39). Wild-type and *btk*^{-/-} B cells were stimulated with anti–mouse IgM F(ab')₂ antibodies, and en-

dogenous IKK α was immunoprecipitated. These immunocomplexes were then incubated with the GST-I κ B α substrate and γ -[³²P]ATP. As shown in Fig. 5 A, IKK activity was significantly upregulated in primary B cells expressing BTK after BCR cross-linking (lanes 1 and 3). In contrast, *btk*^{-/-} B cells failed to elicit this IKK response (lanes 2 and 4). The lack of IKK response could not be attributed to its absence, changes in the steady state levels (Fig. 5 A, bottom panel), or defective enzymatic activity, as this response was rescued when BTK-deficient cells were treated with PMA and ionomycin (lanes 7 and 8), a combination that potently activates NF- κ B via a BTK-independent mechanism (Fig. 4). Importantly, the IKK activity detected in these experiments was specific for Ser-32 and Ser-36 of I κ B α , because replacement of both sites with Ala in the GST-I κ B α substrate eliminated phosphoryl group transfer (Fig. 5, lanes 5 and 6; reference 31). Similar results were obtained with IKK β immunoprecipitates (Fig. 5 B). However, we cannot discern whether IKK α or IKK β is selectively activated after BCR stimulation, because these catalytic subunits interact in the context of a multicomponent holoenzyme. Collectively, these findings with primary *btk*^{-/-} B cells demonstrate that BTK functions to mediate IKK activation upon BCR engagement, indicating that IKK is positioned downstream of BTK in the BCR signaling pathway.

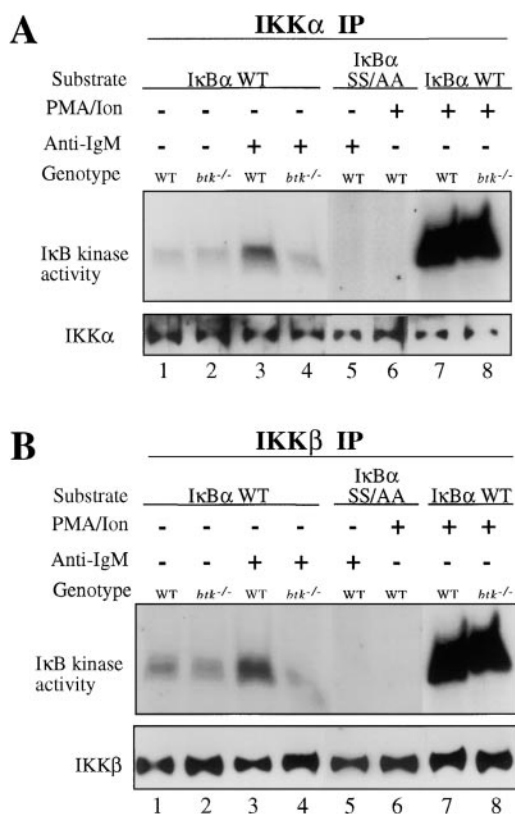


Figure 5. *btk*^{-/-} B cells fail to activate IKK α and IKK β in response to BCR stimulation. (A) primary B cells from spleens of *btk*^{-/-} and C57Bl6 (WT) mice were purified and stimulated as in Fig. 4. Cells were either left unstimulated (lanes 1 and 2) or stimulated for 30 min with anti–mouse IgM F(ab')₂ (lanes 3 and 4) or with PMA and ionomycin (lanes 7 and 8). Cytosolic extracts from 3 × 10⁶ cells per sample were immunoprecipitated with antibodies directed against IKK α (Santa Cruz Biotechnology), and the resulting immunocomplexes were subjected to *in vitro* kinase assay containing γ -[³²P]ATP and 1.0 μ g of GST-I κ B α as the substrate. To determine the specificity of the kinase activity on the I κ B α substrate, a mutant GST-I κ B α in which Ser-32 and Ser-36 were replaced with Ala (SS/AA) was used (lanes 5 and 6). The kinase assays were resolved by SDS-PAGE and visualized by autoradiography. The immunoblots with anti-IKK α (A, bottom panel) were performed on 10% of the cell extract that was used in *in vitro* kinase assay to monitor the integrity and the steady state levels of IKK α protein. (B) IKK β activity was also determined on the same cytosolic extracts as in A, and both the IKK β kinase activity (B, top panel) and the IKK β protein are shown (B, bottom panel).

Discussion

Mutations that inactivate BTK cause the B cell deficiency diseases XLA in humans and *xid* in mice. Prior studies have established that BTK is activated upon BCR stimulation and functions to regulate B cell survival and growth. However, the precise mechanisms by which BTK mediates these biological responses remain unknown. In this regard, recent studies indicate that BCR stimulation also leads to the activation and nuclear translocation of NF- κ B, which controls the expression of multiple growth-related genes at the level of transcription (26, 40). We have discovered that BCR-directed activation of NF- κ B is impaired in both transformed and primary B cells that are deficient for BTK. Importantly, the loss of BTK correlates with a defect in the nuclear translocation of RelA and c-Rel, the primary transactivating subunits of NF- κ B in B cells. Consistent with this, we have found that NF- κ B DNA binding activity accumulates in the nuclei of wild-type B cells after BCR stimulation, whereas this response is significantly reduced in BTK-deficient cells. Furthermore, this activity is restored upon reconstitution with wild-type BTK. These findings strongly suggest that BTK couples NF- κ B to the BCR.

At the present juncture, little is known about the genes downstream of the BCR that function to regulate B cell growth and survival. Our finding that BTK is required for BCR-induced activation of NF- κ B provides new mechanistic insights into this issue. Specifically, BCR-induced upregulation of the antiapoptotic protein Bcl-X_L is impaired in BTK-deficient B cells, which may explain the reduced number of B cells in *xid* mice (our unpublished re-

sults and references 4 and 40). It is well established that this survival gene is under NF- κ B control (4, 22, 41). In addition, recent studies have demonstrated that NF- κ B is required for induction of the gene encoding transcription factor Oct-2 in response to bacterial LPS, a polyclonal B cell mitogen (42). Consistent with the BTK pathways, inactivation of the gene encoding Oct-2 by homologous recombination leads to defects in BCR-directed proliferation (43). This relationship raises the possibility that interference with NF- κ B-directed expression of Oct-2 may also contribute to the observed B cell deficiency in *xid* mice.

Prior biochemical experiments have demonstrated that NF- κ B is regulated by the serine kinases IKK α and IKK β , which constitute the catalytic subunits of a multicomponent IKK complex. In response to proinflammatory cytokines, IKK targets NF- κ B inhibitor I κ B α for proteolysis via site-specific phosphorylation (27). (In the present study, we have established that the IKK complex is activated in response to BCR signaling.) Our studies suggest that IKK is a signal transducer in BCR signaling pathways. However, the activity of IKK is only modestly stimulated in this cellular background, perhaps reflecting IKK-independent mechanisms for NF- κ B activation downstream of the BCR. Consistent with (this finding) an involvement of IKK, we have shown that BCR stimulation also leads to the degradation of I κ B α in cells expressing BTK (Fig. 3). However, both of these signaling events are impaired in BTK-deficient B cells (Figs. 3 and 5), indicating that IKK couples BTK to NF- κ B in the BCR signaling pathway. Because BTK functions as a tyrosine-specific kinase (2, 3), it seems unlikely that IKK is phosphorylated directly by BTK. Instead, we propose that BTK is required for the activation of an upstream IKK kinase (for review see reference 26).

At least three kinases have been implicated in the activation of NF- κ B at the level of IKK phosphorylation. Two of these enzymes, MEKK1 (mitogen-activated protein kinase kinase 1) and NIK (NF- κ B-inducing kinase), are members of the mitogen-activated protein kinase (MAP3K) family (27). The third is a serine/threonine kinase called AKT/protein kinase B (PKB), which binds and phosphorylates IKK (44, 45). Recently, Craxton et al. (46) showed that BCR-mediated AKT activation is impaired in DT40.BTK B cells. Thus, BTK may stimulate IKK activity via an AKT-dependent pathway. In addition, we consider the involvement of PKC and calcineurin in the activation of this upstream kinase likely because inhibition of either PKC activity or calcium influx impairs antigen receptor-induced NF- κ B activation (results not shown; references 47–49). Indeed, both of these enzymes are activated by the second messengers inositol 1,4,5-triphosphate and diacylglycerol, which are generated by PLC- γ 2 in response to the sequential action of Syk and BTK (for review see reference 12). Full resolution of these important missing links in the BTK–NF- κ B coupling mechanism awaits further studies.

In summary, our study demonstrates that BTK-deficient B cells are defective for BCR-induced activation of IKK, which targets the NF- κ B inhibitor I κ B α for proteolysis in

BTK-expressing B cells. In turn, interference with the signal-dependent degradation of I κ B α in BTK-deficient cells prevents the nuclear translocation of RelA and c-Rel, the principle transactivating subunits of NF- κ B. In what may be a related finding, primary B cells lacking c-Rel fail to proliferate in response to anti-IgM stimulation (27). Taken together, these biochemical results indicate that BTK couples IKK, I κ B α , and NF- κ B to the BCR. Interference with this coupling mechanism may contribute to the B cell deficiencies observed in XLA and *xid*.

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