Bruton's Tyrosine Kinase Links the B Cell Receptor to Nuclear Factor \(\kappa B\) Activation

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Abstract

The recognition of antigen by membrane immunoglobulin M (mIgM) results in a complex series of signaling events in the cytoplasm leading to gene activation. Bruton’s tyrosine kinase (BTK), a member of the Tec family of tyrosine kinases, is essential for the full repertoire of IgM signals to be transduced. We examined the ability of BTK to regulate the nuclear factor \(\kappa B/Rel\) family of transcription factors, as the activation of these factors is required for a B cell response to mIgM. We found greatly diminished IgM- but not CD40-mediated \(\kappa B/Rel\) nuclear translocation and DNA binding in B cells from X-linked immunodeficient (xid) mice that harbor an R28C mutation in \(btk\), a mutation that produces a functionally inactive kinase. The defect was due, in part, to a failure to fully degrade the inhibitory protein of \(\kappa B/Rel\), I\(\kappa B\)α. Using a BTK-deficient variant of DT40 chicken B cells, we found that expression of wild-type or gain-of-function mutant BTK, but not the R28C mutant, reconstituted \(\kappa B/Rel\) activity. Thus, BTK is essential for activation of \(\kappa B/Rel\) via the B cell receptor.

Key words: X-linked immunodeficiency • CD40 • B cell receptor • B cell activation • transcription factor

Introduction

Mutation of the gene encoding Bruton’s tyrosine kinase (BTK)1 causes X-linked immunodeficiency (xid) in mice and X-linked agammaglobulinemia (XLA) in humans (1, 2, 2a). XLA patients exhibit a block in early B cell maturation that prevents development of antibody-producing cells (3). Btk mutation in mice reduces peripheral B cells to <50% of wild-type levels and renders them unresponsive to T-independent type II antigens without preventing responses to T-independent type I or T-dependent antigens (4). Xid mice also have low serum IgM and IgG3 and no peritoneal CD5+ B-1a cells (4). Different mutations spanning the human btk gene result in XLA, whereas in mice, a single missense mutation of BTK, R28C, results in X-linked immunodeficiency. This led to speculation that the severity of the human phenotype was due to the location of the mutation in the btk gene. Further experimentation showed that disruption of the btk gene in mice also produced the xid phenotype (5, 6). Conversely, patients with the single basepair mutation producing the R28C amino acid replacement retained the severity of the human disease (7). Together, these data showed that the xid mutation renders mice deficient in essential BTK functions and that the B cell requirement for BTK differs between the murine and human species.

Expression of BTK is limited to B, mast, and myeloid cells. BTK, like other members of the Tec family of cytoplasmic tyrosine kinases, is similar to the Src family kinases in that it contains Src homology (SH)1, SH2, and SH3 domains. However, Tec family members lack the NH\(_2\)-terminal myristylation site and COOH-terminal negative regulatory site found in the Src family kinases. In addition, they have a unique region, termed the Tec homology domain, and an NH\(_2\)-terminal pleckstrin homology (PH) domain (for review see reference 8). BTK is expressed continuously from the late pro-B stage up to the plasma cell stage (9). Loss of BTK function disrupts signaling through the IL-5R, CD38, IL-10R, FceRI, and B cell receptor for an-
tigen (BCR) pathways (10–15). In mature B cells, BTK is tyrosine phosphorylated upon membrane IgM receptor cross-linking (16, 17). In fibroblasts, ectopic expression of Lyn leads to transphosphorylation of BTK at Tyr-551 (18). The kinase responsible for this phosphorylation in B cells has not been identified. Phosphorylation at Tyr-551 leads to BTK autophosphorylation at Tyr-223, membrane localization, and increased kinase activity (19). The PH domain of BTK has a high affinity for phosphoinositide phospholipids, in particular phosphatidylinositol (PI)-3,4,5-triphosphate (PtdIns-3,4,5-P_3) (20). PtdIns-3,4,5-P_3 binding is necessary for the activation-dependent membrane localization of BTK (18, 21). In addition, several proteins have been reported to bind BTK: transcription factor II-I (TFII-I [BAP-135]), protein kinase C (PKC) isoforms, Wiskott-Aldrich syndrome protein (WASP), Ewing’s sarcoma protein (EWS), cbl, SAM 68 (Src-associated in mitosis 68 kD), SLP-65 (SH2 domain–containing linker protein 65 kD; BLNK [B cell linker protein]), and vav (22–27). BTK is critical for the activation of phospholipase (PLC)–γ2, leading to intracellular calcium release, extracellular calcium influx, and PKC activation (28–30).

Wild-type B cells enter cell cycle upon antigen cross-linking of the BCR, whereas xid, or btk<sup>-/-</sup>, cells undergo apoptosis. Although IgM cross-linking of xid B cells does not lead to proliferation, some IgM-mediated signals are successfully transmitted, resulting in upregulation of MHC class II and tyrosine phosphorylation of multiple proteins (29, 31). Further downstream, uncharacterized BTK–dependent pathways lead to Bcl-x<sub>L</sub> (32) and cyclin induction (33). This suggested that IgM–mediated transcription factor activation is compromised in xid B cells.

In primary splenic B cells, IgM cross-linking or CD40 ligation leads to nuclear translocation and DNA binding of the NF-κB family of transcription factors (34–36). Phenotypic analysis of mice deficient in individual NF-κB family members has demonstrated the essential role of these transcription factors in CD40, LPS, and IgM receptor pathways leading to B cell proliferation. In particular, B cells from mice deficient in NF-κB members c-rel or p65 have decreased responses to antigen cross-linking (37, 38). C-rel<sup>-/-</sup> B cells also failed to respond to CD40 ligation (not determined in p65<sup>-/-</sup> mice). Recently, an examination of B cells in mice expressing a transdominant form of IκBα revealed xid-like defects, including a lack of proliferation in response to anti-IgM (39). The CD40 transduction of NF-κB activation has been well characterized (40–42). There is less information about the molecular signaling events connecting the BCR to NF-κB activation.

Recently, several of the events proximal to NF-κB activation have been elucidated (for review see reference 43). In wild-type resting cells, NF-κB family factors are sequestered in the cytoplasm by members of the inhibitory κB (IκB) family of proteins. Activation of IκB kinases results in serine/threonine phosphorylation, subsequent ubiquitination, and the proteolytic degradation of IκB. The nuclear localization sequence of the NF-κB/IκB family proteins is then exposed, and the factors localize to the nucleus.

As BTK and NF-κB are both essential for normal B cell function, we asked whether BTK might be involved in NF-κB activation. We compared wild-type and xid murine B cells with respect to BCR–mediated induction of NF-κB DNA binding, nuclear translocation, transcript induction, and IκB degradation. Reconstitution experiments in the BTK-deficient variant of the chicken B cell line DT40 allowed us to determine the effect of wild-type and mutant BTK proteins on NF-κB activation.

**Materials and Methods**

Preparation and Activation of Splenic Murine B Cells. A BALB/cAnN-xid colony was generated from original breeding pairs derived from BALB/cAnN-xid mice provided by Dr. C. Hansen (National Institutes of Health Genetic Resource Center, Bethesda, MD) and maintained at Tufts University. BALB/cByJ mice were isolated from The Jackson Laboratory. Splenic B cells were isolated from 8- to 12-wk-old mice by complement-mediated T cell lysis as previously described (44). This procedure was modified by addition of a Lympholyte-M Ficoll (Cedarlane) gradient step after treatment with Tris-ammonium chloride–BSA to remove residual RBCs in the lymphocyte populations. Cell populations were 90–99% splenic B cells, as tested by FACS<sup>®</sup> analysis for CD45 (B220) expression.

Cells were pelleted and resuspended for culture at 10<sup>6</sup> cells/ml in RPMI 1640 (Life Technologies) containing 10% heat-inactivated FCS, 20 mM Hepes, pH 7.0, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 mg/ml streptomycin sulfate, and 50 mM 2-ME (called complete medium below). In all assays, cells were left untreated or treated for 4 h with 10 μg/ml F(ab)'<sub>2</sub> goat anti–mouse IgM, μ chain specific (Jackson ImmunoResearch), hybridoma supernatant CD8/CD40L (45) protein at a final dilution of 1:2, or PMA (Sigma-Aldrich) and ionomycin (Sigma-Aldrich) at a final concentration of 30 nM and 1 mM, respectively. (Preliminary time course experiments demonstrated maximal translocation of nuclear c-rel after stimulation for 4 h.) In some experiments, cells were assayed for early signs of apoptosis after B cell stimulation for 4 h, as described (46).

Cell Lines and Constructs. DT40 wild-type and BTK-deficient B cell lines were generated in the murine DT40 line with the addition of 1% heat-inactivated serum. These cells were stimulated (in the absence of anti-IgM) with 10 μg/ml mouse monoclonal anti-chicken IgM, M.4 (generated by Dr. M. Cooper, University of Alabama, Birmingham, AL) and obtained from Dr. E. Clark, Washington University, St. Louis, MO), or 30 nM PMA (Sigma-Aldrich) in combination with 1 μM ionomycin (Sigma-Aldrich). WEHI231 and T220 cell lines were cultured in complete medium.

The pGD-BTK construct was provided by Dr. G. Cheng (University of California at Los Angeles, Los Angeles, CA), R28C (xid) and E41K (gain-of-function) basepair mutations were generated in the murine btk gene by standard PCR mutagenesis and checked by sequence analysis at the Tufts University Sequencing Facility. The wild-type and mutant btk cDNAs were excised from the pGD vector by XhoI and NotI digestion and inserted into EcoRI-I-linearized pBabeApuro vector, which was donated by Dr. T. Kurosaki (47).
The luciferase reporter constructs, containing three NF-κB DNA binding sites or lacking these sites, were provided by Dr. S. Gersh (Yale University, New Haven, CT). A pRLT construct (Promega) served as an internal control in the luciferase experiments.

Electrophoretic Mobility Shift Assays. 4 × 10^7 cells were stimulated, as described above, and nuclear extracts were prepared as previously described (48). The xB site used as a probe in this assay was a 70-bp fragment excised from a plasmid containing the H2K M H gene promoter (49). The fragment was labeled with γ-[32P]dATP (Du Pont) by T4 polynucleotide kinase. 5 μg of nuclear protein or the equivalent of 4 × 10^7 cells was incubated with 10,000 cpm of the NF-κB probe. Electrophoretic mobility shift assays (EMSAs) were performed as described (48).

[35S]Methionine/Cysteine Pulse-Chase Experiments. For each treatment, 2 × 10^7 splenic B cells were cultured in 2 ml of cysteine- and methionine-free RPMI (BioWhittaker), containing 0.5% dialyzed FCS (JRH Bioscience), for 2 h at 37°C. The supernatant was preincubated with Sepharose A beads (Amersham Pharmacia Biotech) and 28 μg of whole rabbit IgG (Sigma-Aldrich) for 45 min at 4°C. These preincubated lysates were incubated overnight with Sepharose A beads and 60 μg of anti-κBα antibody (SC-371; Santa Cruz Biotechnology). The IκBα-bound beads were washed four times with lysis buffer at 4°C. 100 μl of 1% SDS-TNTE buffer lysis buffer was added to the washed beads, and samples were boiled for 5 min. 900 μl of TNTE lysis buffer was added, the beads were pelleted, and the IκBα-containing lysate was transferred to a new tube. The IκBα immunoprecipitation was repeated. The beads were washed four times with lysis buffer, and 50 μl of 2× Laemmli buffer was added to the pelleted beads. The samples were then electrophoresed on 10% SDS-PAGE for 3 h at 30 mA. The gel was fixed for 10 min with 10% acetic acid/50% methanol solution. After multiple washes in water, the gel was placed in a 1 M saline acid/1% glycerol solution for 30 min, rinsed quickly with water, and dried at 50°C for 2 h. The dried gel was exposed in a PhosphorImager (Molecular Dynamics) overnight. Densitometry analysis was carried out using the Bio-Rad Imaging Densitometer and Multi-Analysis Software (Bio-Rad Laboratories).

Western Blot Analysis. Nuclear and cytoplasmic extracts were prepared from wild-type and xid B cells as described above (48). During this lysis procedure, the buffer A supernatant was saved, as it contained the cytoplasmic proteins. Whole cell extracts were prepared by lysis of 10^7 cells in 1× Laemmli buffer. Samples from equivalent micrograms or cell numbers were electrophoresed on 10% SDS-PAGE for 3 h at 30 mA. The gel was fixed for 10 min with 10% acetic acid/50% methanol solution. After multiple washes in water, the gel was placed in a 1 M saline acid/1% glycerol solution for 30 min, rinsed quickly with water, and dried at 50°C for 2 h. The dried gel was exposed in a PhosphorImager (Molecular Dynamics) overnight. Densitometry analysis was carried out using the Bio-Rad Imaging Densitometer and Multi-Analysis Software (Bio-Rad Laboratories).

Results

Reduced DNA Binding and Nuclear Translocation of NF-κB/Rel Transcription Factors in IgM- but not CD40-stimulated XID B Cells. EMSAs showed that xid, but not CD40-stimulated XID B cells, failed to find any differences in mitochondrial membrane potential (early sign of apoptosis) between wild-type and xid B cells. We failed to find any differences in mitochondrial membrane potential (early sign of apoptosis) between wild-type and xid B cells. This suggests that the failure to activate NF-κB is unlikely to be a consequence of the apoptosis of xid B cells. In contrast to IgM cross-linking, CD40 ligation led to an increase of DNA binding activity in both wild-type and xid B cells, indicating that the xid BCR transduced some signals (data not shown).

Next, we asked if the lack of NF-κB DNA binding activity in IgM-activated xid B cells was due to reduced nuclear NF-κB protein. We chose to analyze c-rel, as it is required for B cell proliferation in response to anti-IgM (37). Western blot analysis showed that IgM - but not CD40-induced levels of nuclear c-rel were drastically reduced in xid B cells (Fig. 1B, top panel). Basal nuclear c-rel in xid B cells was consistently less than that seen in wild-type cells. Although CD40-mediated NF-κB/Rel DNA binding and c-rel nu-
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A clear translocation in xid B cells was slightly lower than that in wild-type B cells, the fold inductions were similar (Fig. 1, A and B). These initial studies suggested that BTK plays some role, direct or indirect, in NF-κB activation.

Normal Induction of c-rel Transcript and Normal Cytoplasmic Protein Levels in xid B Cells. The lack of IgM-mediated NF-κB translocation and DNA binding in xid B cells could be due to diminished amounts of total NF-κB protein. Western blot analysis showed that the amounts of c-rel were similar in wild-type and xid B cells under various conditions (Fig. 2 A). In addition, we found that the level of c-rel mRNA increased normally after anti-IgM stimulation of wild-type or xid B cells (Fig. 2 B, top panel, reverse transcriptase [RT]-PCR and bottom panel, Northern blot analysis). This finding suggests that BCR-induced c-rel transcription is not dependent on NF-κB, though it is known that in vitro overexpression of c-rel can increase constitutive activity of the c-rel promoter (51). Given the adequate amounts of cellular c-rel transcript and protein, it was likely that there was a failure to translocate cytoplasmic c-rel protein to the nucleus upon IgM cross-linking.

Inefficient Degradation of IκBα in xid B Cells. In resting cells, NF-κB/Rel transcription factors are sequestered in the cytoplasm by IκB. There are three isoforms of IκB: α, β, and ε (52). We chose to study IκBα, a well characterized member of the inhibitory family. In wild-type splenic B cells, IκBα levels decreased after treatment for 4 h with anti-IgM or CD40 ligand stimulation (Fig. 3 A). In xid B cells, IκBα levels decreased after treatment for 4 h with anti-IgM or CD40 ligand stimulation (Fig. 3 A).

These figures represent one of three experiments.
although the level of IkBα decreased in response to CD40 ligation, it did not decrease after anti-IgM cross-linking. The IkBα protein behaved in a similar fashion (data not shown).

The most plausible explanation for maintenance of IkBα protein levels in xid B cells despite IgM stimulation is that the existing IkBα is not fully degraded. This possibility was examined by 35S pulse–chase experiments. Wild-type and xid splenic B cells were cultured with 35S-labeled cysteine and methionine, and the experiment was carried out as described in Materials and Methods. These 35S-labeled cells were then cultured for 1 h with or without F(ab′)2 anti-IgM. (We chose to stimulate cells for 1 h because we were unable to maintain primary B cells under these labeling conditions for longer times.) The cells were lysed, lysates precleared, 35S-labeled IkBα immunoprecipitated (twice), and the immunoprecipitates electrophoresed on SDS-PAGE. After IgM activation, wild-type B cells showed a 53% decrease in the level of labeled IkBα. In contrast, xid B cells consistently retained IkBα, as only a 10% decrease was detected (Fig. 3 B). These data show that xid B cells failed to fully degrade IkBα downstream of IgM cross-linking.

Reconstitution of IgM-mediated NF-κB activity in DT40 BTK-deficient Cells. DT40, the chicken B cell line, has been a crucial tool in the delineation of the signal transduction events after antigen receptor cross-linking (29, 53, 54). We used DT40 cells to further investigate the role of BTK in NF-κB activation. NF-κB-dependent luciferase reporter activity increased 25-fold after 4-h treatment of

Figure 3. Diminished IkBα degradation in anti-IgM-activated xid B cells. (A) Western blot analysis of IkBα expression (top panel) was performed with 25 μg of cytoplasmic extracts prepared from wild-type or xid splenic B cells cultured for 4 h in medium, F(ab′)2 anti-IgM (10 μg/ml), or CD40 ligand (1:2 dilution). β-actin served as a loading control (bottom panel). This figure represents one of three experiments. (B) Wild-type and xid splenic B cells were cultured with 35S-labeled cysteine and methionine, and the experiment was carried out as described (see text). Mean densitometry values from three independent experiments (shown as percent decrease in 35S-labeled IkBα ± SD) were: wild-type, 55.7 ± 15.1 and xid, 19.0 ± 7.4.

Figure 4. Reconstitution of IgM-mediated NF-κB activity in BTK-deficient DT40 cells by ectopic expression of wild-type (WT) BTK. (A) Transient transfections of WT and BTK-deficient DT40 cells were conducted with NF-κB–driven luciferase reporter and pRLTK constructs, the latter serving as an internal transfection control for the assay. Cells were cultured for 16 h and then stimulated for 4 h with medium, M4 anti-chicken IgM (10 μg/ml; light bars), or PMA and ionomycin (30 nM and 1 mM, respectively; dark bars). Cells were lysed and assayed as described in Materials and Methods. For each sample, luciferase activity was normalized to the pRLTK internal control. The graph shows the fold induction of luciferase activity relative to the luciferase activity detected in the lysates of cells cultured in medium only (= 1). This figure is representative of five experiments. (B) 10 μg of wild-type BTK or vector (pApuro) was transiently transfected together with reporter and internal control constructs as described above (A). Null + Vector lane shows background activity levels. PMA and ionomycin treatment of these cells resulted in similar induction (data not shown). Western blot analysis for detection of BTK expression was performed using lysates (equivalent of 2.5 × 10^6 cells) from this assay. The anti-BTK antibody recognizes both mouse and chicken BTK protein. The reactivity of this antibody for the different BTK species is not known, and the fraction of cells transfected was not determined the expression levels in wild-type and transfected cells cannot be compared. The fold induction values are the mean of three experiments ± SD.
wild-type DT40 cells with anti-IgM (Fig. 4 A). The same luciferase reporter construct, but lacking the three NF-κB sites, was not induced under the same conditions (data not shown). BTK-deficient DT40 B cells failed to induce NF-κB activity after IgM stimulation (Fig. 4 A). Like wild-type cells, an increase of total protein tyrosine phosphorylation was detected after anti-IgM cross-linking of BTK-deficient DT40 cells. In BTK-deficient DT40 cells, NF-κB-dependent luciferase activity was induced by the pharmacological reagents phorbol ester and ionomycin. Expression of BTK in BTK-deficient cells restored the ability of membrane IgM to activate NF-κB (Fig. 4 B). Western blot analysis demonstrated that BTK protein was expressed in BTK-deficient cells in these assays (Fig. 4 B).

The xid mutation diminishes the ability of ectopic BTK to induce NF-κB activation. BTK is similar to Src kinases in that it contains SH1, SH2, and SH3 domains. In addition, it also contains a proline-rich and a unique region (together called the Tec homology domain) and a PH domain. The PH domain has high affinity for phosphoinositide phospholipids (20) and is required for membrane localization of the kinase. The R28C (xid) mutation of the PH domain decreases the affinity for BTK for phospholipids and other ligands (20, 55). The E41K (gain-of-function) mutation in the BTK PH region increases affinity for PtdIns-3,4,5-P3 (56) and results in constitutive membrane association and activity (57). Using ectopic expression of BTK in BTK-deficient DT40 cells, we asked how mutations of the BTK PH domain affected DT40 IgM-mediated NF-κB activity. In these assays, we found that it was necessary to use higher amounts of mutant DNA constructs to express mutant BTK at levels comparable to wild type. It is possible that the mutant proteins are unstable, though we have not examined this. We find that the basal NF-κB activity increased ninefold when the E41K (gain-of-function) mutant was expressed (Fig. 5). No change in basal activity was detected when a similar level of the R28C (xid) mutant was expressed. When comparable amounts of wild-type and mutant proteins were expressed, wild-type BTK allowed a 29-fold increase in NF-κB activity. The increase in reporter activity with the gain-of-function and xid mutants were 34- and 5-fold, respectively.

**Discussion**

NF-κB/Rel function is essential for B cell activation. NF-κB/Rel function is essential for B cell activation. Our studies with murine B cells suggested that BTK function was required for induced degradation of IκB and subsequent NF-κB translocation and DNA binding (Figs. 1 and 3). Mice deficient in individual NF-κB/Rel family members and mice expressing a transdominant form of IκBα demonstrated the importance of these transcription factors in both CD40- and IgM-mediated B cell activation and proliferation (34–36, 39). In particular, expression of a transdominant form of IκBα produced a phenotype strikingly similar to that of xid mice.

Schauer et al. (58) showed that IgM cross-linking of a B cell line, WEH1231, failed to activate NF-κB, leading to apoptosis. When CD40 ligand was used in combination with anti-IgM, these cells activated NF-κB and, in turn, blocked apoptosis (58). Thus, NF-κB proteins serve as...
..."survival" factors, most likely due to their role in the initiation of specific transcription of genes that mediate survival. Previously, we showed that in contrast to wild-type cells, there is no induction of the antiapoptotic protein Bcl-x<sub>L</sub> in IgM-activated xid B cells (32). NF-κB is essential for expression of Bcl-x<sub>L</sub> (59). Thus, in xid B cells, it may be the loss of NF-κB activity that renders the B cells susceptible to apoptosis after IgM cross-linking. It is doubtful that it is solely the loss of induced Bcl-x<sub>L</sub> that is responsible for the xid phenotype, because xid mice expressing a Bcl-x<sub>L</sub> transgene do not correct the total xid phenotype (60).

**BTK Function in Developing versus Mature B cells.** In comparison with circulating, mature B cells in wild-type mice, the B cells in xid mice express lower levels of surface IgD and higher levels of surface IgM (61). This observation suggests that xid B cells are in a transitional stage of B cell development en route to full maturity (62). Thus, the bulk of ex vivo B cells from wild-type and xid mice are at different stages of maturity, making direct comparison of their biochemistry problematic. To distinguish between an indirect, developmental role and a direct role for BTK in NF-κB activation, we turned our analysis to the transformed chicken B cell line DT40. The BTK-deficient variant of DT40 cells was generated previously by homologous recombination (29). These cells, like xid B cells, exhibit a defect in calcium signaling after IgM receptor cross-linking (29, 63). We found that BTK-deficient DT40 cells lacked NF-κB activity after IgM cross-linking, corroborating our findings with xid B cells. Expression of BTK in BTK-deficient DT40 cells restored IgM-mediated NF-κB activation (Fig. 4B). We conclude that BTK function in mature B cells is essential for BCR-induced NF-κB activation.

**The Effect of BTK PH Domain Mutations on NF-κB Activation.** The R28C mutation is known to lower the ability of BTK to interact with and activate TFII-I and to mobilize calcium. In particular, this mutation dramatically reduces the affinity of BTK for the phosphoinositide phospholipid PtdIns-3,4,5-P<sub>3</sub> (20). Recombinant BTK PH fused to green fluorescent protein (GFP) localized to the cell membrane, as did the E41K, gain-of-function PH-GFP. The R28C, xid PH-GFP, failed to localize to the membrane (56). Related work by Gupta et al. showed that decreasing the levels of PIP<sub>3</sub>, by inhibition of PI-3 kinase or membrane SH2 domain-containing inositol 5'-phosphatase (SHIP) expression, led to inhibition of BTK membrane localization and activity (21). This block in BTK activity could be overcome when recombinant BTK was targeted to the membrane. Collectively, these data show that membrane localization of BTK is essential for normal function. We now show that expression of the E41K mutant BTK in BTK-deficient DT40 cells increased basal NF-κB activity ninefold and with anti-IgM cross-linking 34-fold (Fig. 5). In contrast, the equivalent BTK protein expression of the R28C mutant had no effect on basal activity, and with anti-IgM cross-linking only a fivefold increase in NF-κB activity was detected. These results are consistent with previous work showing that an intact PH domain is essential for BTK to function, in this case to activate NF-κB.

The role of BTK in IgM Signaling. Many studies have identified roles for BTK in important signal transduction events downstream of IgM that ultimately lead to cell cycle entry and proliferation, e.g., PLC-γ2 activation, calcium mobilization, IP<sub>3</sub> generation, cyclin activation, and Bcl-x<sub>L</sub> upregulation (29, 30, 32, 33, 64, 65). This study identifies another downstream event controlled by BTK, NF-κB/IκBα activation. Studies of NF-κB activation in other pathways, such as CD40, TNF, and IL-1, have linked IκBα and NF-κB regulation to a signaling cascade that includes the IκBα kinases mitogen-activated protein kinase kinase 1 (MEK1) and NF-κB-inducing kinase (NIK) and TNF receptor-associated factors (TRAFs) (42, 66–68). Previous studies have shown that IκBα is degraded and NF-κB activated as a consequence of IgM signaling, but little else is known (43). Our laboratory is currently focusing on elucidating the molecules that link BTK with IκBα.

Signals Transduced by mIgM and CD 40. There are many events common to CD40 and mIgM ligation, as both lead to activation of activator protein (AP)-1, NF-κB, nuclear factor and activator of transcription (NF-AT), and signal transducers and activators of transcription (STATs), expression of Bcl-x<sub>L</sub>, and entry into cell cycle (32, 36, 69). Yet some differences are clear. These receptors exhibit differential regulation of Fas, CD5, CD23, and mitogen-activated protein (MAP) kinases (70–72). In addition, PKC is necessary for IgM- but not CD40-induced activation of NF-κB (36). Related work by Sen and colleagues has shown differential PKC and calcium requirements for individual NF-κB family members downstream of the BCR (73, 74). In this study, we show that in the absence of BTK, NF-κB can be activated by CD40 ligation but not by IgM cross-linking (Fig. 1). This suggests that after IgM cross-linking, BTK, which is necessary for the phosphorylation of PLC-γ2 (29), is in turn responsible for the activation of PKC and calcium mobilization and the activation of NF-κB.

Data from Bendall et al. showed that expression of a transdominant IκBα blocked IgM- but not LPS-mediated NF-κB activation (39). This study and others (75) suggest that the several B cell activators—CD40 ligand, antigen, and LPS—differentially regulate individual IκB family members. In the cytoplasm of resting cells, IκB family members interact with different NF-κB family members downstream of the BCR (73, 74). Whether CD40 (BTK-independent) and mIgM (BTK-dependent) receptors activate different IκB kinases ultimately leading to distinct gene transcription through activation of different NF-κB dimers will be the subject of future studies. Understanding the specific control of NF-κB activation could provide clues to the differential physiological outcomes of these B cell activation pathways.

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