Function of Bruton's Tyrosine Kinase during B Cell Development Is Partially Independent of Its Catalytic Activity[1]

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The Tec family member Bruton's tyrosine kinase (Btk) is a cytoplasmic protein tyrosine kinase that transduces signals from the pre-B and B cell receptor (BCR). Btk is involved in pre-B cell maturation by regulating IL-7 responsiveness, cell surface phenotype changes, and the activation of λ L chain gene rearrangements. In mature B cells, Btk is essential for BCR-mediated proliferation and survival. Upon BCR stimulation, Btk is transphosphorylated at position Y551, which promotes its catalytic activity and subsequently results in autophosphorylation at position Y223 in the Src homology 3 domain. To address the significance of Y223 autophosphorylation and the requirement of enzymatic activity for Btk function in vivo, we generated transgenic mice that express the autophosphorylation site mutant Y223F and the kinase-inactive mutant K430R, respectively. We found that Y223 autophosphorylation was not required for the regulation of IL-7 responsiveness and cell surface phenotype changes in differentiating pre-B cells, or for peripheral B cell differentiation. However, expression of the Y223F-Btk transgene could not fully rescue the reduction of λ L chain usage in Btk-deficient mice. In contrast, transgenic expression of kinase-inactive K430R-Btk completely reconstituted λ usage in Btk-deficient mice, but the defective modulation of pre-B cell surface markers, peripheral B cell survival, and BCR-mediated NF-κB induction were partially corrected. From these findings, we conclude that: 1) autophosphorylation at position Y223 is not essential for Btk function in vivo, except for regulation of λ L chain usage, and 2) during B cell development, Btk partially acts as an adapter molecule, independent of its catalytic activity. ***The Journal of Immunology*****, 2003, 171: 5988–5996.**

Bruton's tyrosine kinase (Btk)[3] is a member of the Tec family of cytoplasmic protein tyrosine kinases (PTK) and plays an essential role in B lymphocyte development and function (1, 2). Together with two other types of nonreceptor PTK, Syk and the Src family member Lyn, Btk acts as an important transducer of signals originating from the pre-B cell receptor (pre-BCR) and the BCR.

The pre-BCR complex, which is comprised of μ H chain, the nonrearranging VpreB and λ5 surrogate L chain (SLC) proteins, and the Ig-α/CD79a and Ig-β/CD79b signaling components, is a key checkpoint in B cell development to monitor the expression of a functional Ig μ H chain (3, 4). Pre-BCR expression is essential for the proliferative expansion of cytoplasmic μ H chain-positive pre-B cells and the induction of progression into small pre-B cells in which Ig L chain rearrangement occurs (3, 4). Pre-BCR engagement leads to the formation of a lipid raft-associated calcium signaling module composed of the tyrosine-phosphorylated signaling molecules Lyn, Syk, B cell linker protein (BLNK)/SLP-65, phosphoinositide 3-kinase, Btk, Vav, and phospholipase Cγ2 (PLC-γ2) (5).

The importance of Btk in pre-BCR checkpoint function in humans is evidenced by an almost complete arrest of B cell differentiation due to defective expansion of the earliest cytoplasmic μ H chain-positive pre-B cells in X-linked agammaglobulinemia (XLA) patients with mutations in the Btk gene (6, 7). XLA is characterized by an almost complete absence of peripheral B cells and plasma cells; serum levels of all Ig classes are very low. In contrast, mice with a targeted mutation in the Btk gene or CBA/N mice, which carry an R28C point mutation in the Btk pleckstrin homology (PH) domain, exhibit a milder disorder, x-linked immunodeficiency (*xid*), mainly reflecting poor survival of peripheral B cells (8–10). Btk-deficient mice manifest a specific arrest of peripheral B cell development within the immature B cell pool at the progression of IgM^{high} to IgM^{low} $AA4^+CD23^+$ transitional B cells (8–12). In these mice, the numbers of pre-B cells that are generated in the bone marrow (BM) are normal (8, 9, 13). Nevertheless, Btk is crucially involved in the regulation of the developmental progression of pre-B cells by limiting the IL-7-driven expansion of large cycling pre-B cells (14, 15). In in vitro BM cultures, Btk-deficient pre-B cells showed increased IL-7-driven expansion and reduced developmental progression of large cycling into small resting pre-B cells and subsequently into Ig^+ B cells (14). In addition, we recently demonstrated that Btk cooperates with the BLNK/SLP adapter molecule as a tumor suppressor that limits pre-B cell expansion (15). During the transition of large cycling to small resting cytoplasmic μ^+ pre-B cells in the mouse, Btk-deficient cells fail to efficiently modulate the expression of SLC, the metallopeptidase BP-1, the adhesion molecule CD2, the IL-2R CD25, and the membrane sialoglycoprotein CD43 (14). Btk-deficient cells manifest a specific developmental delay within the small pre-B cell compartment of ~3 h in vivo. The finding of

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[3] Abbreviations used in this paper: Btk, Bruton's tyrosine kinase; BCR, B cell receptor; BLNK, B cell linker protein; BM, bone marrow; MFI, median fluorescence intensity; PH, pleckstrin homology; PLC, phospholipase C; PTK, protein tyrosine kinase; SH, Src homology; SLC, surrogate L chain; TFII-I, transcription factor II-I; TH, Tec homology; TI-II, T cell-independent type II; TNP, trinitrophenol; WASP, Wiskott Aldrich syndrome protein; WT, wild type; *xid*, x-linked immunodeficiency; XLA, X-linked agammaglobulinemia.

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reduced λ L chain usage in Btk-deficient mice implicates Btk in the regulation of the activation of the λ locus for V(D)J recombination in pre-B cells (16).

Btk contains five distinct domains: an N-terminal PH domain, a Tec homology (TH) domain, a Src homology 3 (SH3) domain, an SH2 domain, and a C-terminal catalytic domain (Fig. 1*A*). Upon BCR stimulation, Btk activation is initiated by targeting the kinase to the plasma membrane through interactions of its PH domain with phosphatidylinositol-3,4,5-triphosphate, a second messenger generated by phosphoinositide 3-kinase (17, 18). In concert, the Lyn and Syk kinases are activated, resulting in transphorylation of Btk at position Y551, which promotes the catalytic activity of Btk and subsequently results in its autophosphorylation at position Y223 in the SH3 domain (Fig. 1*A*) (19, 20). Although Y223 phosphorylation has little discernible influence on Btk catalytic activity, it prevents binding to Wiskott Aldrich syndrome protein (WASP) and increases the affinity to Syk (21). Concomitantly, Syk activation results in phosphorylation of BLNK/SLP65, which allows for the association of this adapter molecule with the Btk SH2 domain and with PLC-γ2 (2, 22). These interactions are critical to the activity of Btk and result in PLC-γ tyrosine phosphorylation, inositol triphosphate production, and calcium mobilization.

Although biochemical studies in cultured B cells and fibroblasts have provided important insights into the molecular mechanism of BCR-mediated Btk activation, these systems have not allowed for the investigation of the role of the individual Btk domains in (pre-) BCR checkpoint functions in vivo. In this study, we addressed the functional significance of Btk Y223 autophosphorylation and investigated whether Btk can also act as an adapter molecule, independent of its catalytic activity. Therefore, we generated transgenic mouse strains that express the autophosphorylation site mutant Y223F-Btk and K430R kinase-inactive Btk, under the control of the B cell-specific CD19 promoter region. We crossed these mice onto a Btk null background (10), and examined the in vivo effects of the Y223F and K430R mutations, in particular with respect to Ig λ L chain usage, pre-B cell surface marker modulation, and the proliferative response of pre-B cells to IL-7.

Materials and Methods

Generation of Btk-transgenic mice

Wild-type (WT)-Btk transgenic mice have been described previously (23). The human Btk transgenes consist of a ~6.3-kb genomic fragment containing the CD19 promoter region, a 0.3-kb fragment with the first three exons of human Btk as cDNA sequence, as well as a 27.1-kb genomic DNA fragment, encompassing the Btk exons 3–19 (23). Using double-stranded site-directed mutagenesis (Stratagene, La Jolla, CA), the Y223F and K430R mutations were introduced into the constructs that were previously used to generate the WT-Btk mice (23).

The Y223F mutation was created by replacement of AT by TC in exon 8 of human Btk in a 2.8-kb *Cfr10*I-Asp718 fragment (containing exons 8–11) in pBlueScript, using a mutation primer (5′-GCTGAAAAAGGTT GTGGCCCTTTTCGATTACATGCCAATG-3′) and a 39-bp *Kpn*I-*Bgl*II selection primer. The mutated *Cfr10*I-Asp718 fragment was subsequently cloned into the WT-Btk transgene construct. The A-to-G replacement mutation K430R in exon 14 was created in a 2.1-kb Asp718-*Mun*I fragment encompassing human Btk exon 12–14. A 40-bp mutation primer, 5′-CCAGTAGGACGTGGCCATCAGGATGATCAAAGAAGGCTCC-3′ and the 39-bp *Kpn*I-*Bgl*II selection primer was used. The mutated 2.1-kb Asp718-*Mun*I fragment was ligated to a 9.2-kb *Mun*I-Asp718 fragment, to yield a 11.3-kb Asp718 human Btk fragment, which was used to replace the 11.3-kb Asp718 fragment in the WT-Btk construct (23).

The ~34-kb *Mlu*I-*Not*I inserts from the Y223F-Btk and K430R-Btk constructs were excised from the vector, gel purified, and microinjected into the pronuclei of FVB-fertilized oocytes. Transgenic founder mice were identified by Southern blotting and crossed with Btk null mice (10), which were bred onto a C57BL/6 background for more than eight generations. Sequence fidelity of the mutated fragments was confirmed, both in plasmid DNA before subsequent cloning steps and in genomic DNA obtained from the F_1 transgenic mice generated. All mice were bred and maintained at the Erasmus MC animal care facility under specific pathogen-free conditions.

Mouse genotyping

Tail DNA was analyzed by Southern blotting of *Bam*HI digests using a partial human Btk cDNA probe (bp 133-1153), as described previously (10, 23). Alternatively, the presence of Btk transgenes was evaluated by PCR, using the following primers: *CD19prom*, 5′-TGCAATTAGTGGT GAACAAC-3′, and *hmBtk.65R*, 5′-AGATGCCAGGACTTGGAAGG-3′. Endogenous mouse Btk WT alleles were identified by an exon 9 forward primer (5′-CACTGAAGCTGAGGACTCCATAG-3′) and an exon 10 reverse primer (5′-GAGTCATGTGCTTGGAATACCAC-3′). For Btk knockout alleles, primers were within the *LacZ* reporter (10): forward, 5′-TTCACTGGCCGTCGTTTTACAACGTCGTGA-3′, and reverse, 5′-ATGTGAGCGAGTAACAACCCGTCGGATTCT-3′.

Flow cytometric analyses

Preparations of single-cell suspensions, standard and intracellular flow cytometry, and conjugated mAbs (BD PharMingen, Sunnyvale, CA) have been described previously (10, 14). The anti-SLC hybridoma LM34 (24) was kindly provided by A. Rolink (Basel, Switzerland); Abs were purified using protein G columns and conjugated to biotin according to standard procedures. Mice were analyzed at the age of 8–16 wk. Transgenic Btk expression levels were quantified by intracellular flow cytometry, using affinity-purified polyclonal rabbit anti-Btk (BD PharMingen) and FITC-conjugated goat anti-rabbit Ig (Nordic, Capistrano Beach, CA). Median fluorescence intensity (MFI) values were obtained by CellQuest software (BD Biosciences, San Diego, CA), whereby values for transgenic human Btk and endogenous murine Btk were corrected for background fluorescence, as detected in Btk null mutant mice. Subsequently, the ratio of the MFI values of transgenic Btk over endogenous Btk was calculated.

In vitro (pre-)B cell cultures

IL-7-driven BM cultures and determination of IL-7-dependent proliferative responses of total BM cells have been described previously (14, 16). Mature B cell fractions were purified from spleen, using standard NH_4Cl lysis to deplete erythrocytes, followed by complement-mediated T cell lysis, as described previously (25). The enriched B cell fractions were cultured in vitro in the presence or absence of LPS, 5 μg/ml anti-CD40, or polyclonal goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL), as described previously (16). To measure DNA synthesis, cells were pulsed with [^{3}H]thymidine for 16–20 h, harvested, and counted using standard methods. For apoptosis assays, DNA content was determined in ethanol-fixed cells, using propidium iodide.

B cell stimulation and Western blotting analyses

For analyses of nuclear c-Rel expression, single-cell suspensions from spleen were depleted of erythrocytes by NH_4Cl lysis and enriched for B cells by AutoMACS purification, using biotinylated Abs to Gr-1, Ter119, CD4, CD8, and CD11b and magnetic streptavidin MicroBeads (Miltenyi Biotec, Sunnyvale, CA) for negative selection. Cells were stimulated with 10 μg/ml $F(ab')_2$ of polyclonal goat anti-mouse IgM (Jackson ImmunoResearch, West Grove, PA) in RPMI 1640 at 37°C for 4 h. Total nuclear and cytoplasmic protein extracts were prepared according to Andrews and Faller (26). Samples from equivalent cell numbers were resolved by SDS-PAGE, and proteins were transferred to nitrocellulose membrane, as described (10). Membranes were blocked with 5% milk in TBS/0.05% Tween 20 for 1 h and incubated overnight with anti c-Rel (SC-071; Santa Cruz Biotechnology, Santa Cruz, CA), and subsequently with HRP-conjugated swine anti-rabbit Ig. Densitometry analysis was conducted using ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA).

For analyses of Bcl-x_L and cyclin D2 induction, total splenocytes were depleted of erythrocytes and stimulated with 10 μg/ml polyclonal goat anti-mouse IgM (Southern Biotechnology Associates) in RPMI 1640/10% FCS at 37ºC. Total cell lysates were subjected to SDS-PAGE using standard procedures (27). Immunoblotting Abs used included: anti-Bcl-x_L (2762; Cell Signaling Technology, Beverly, MA), anti-cyclin D2 (M-20; SC-593; Santa Cruz Biotechnology), and anti-extracellular signal-regulated kinase 1/2 (SC-094; Santa Cruz Biotechnology).

For analysis of Btk expression and Btk in vitro kinase assays, single-cell suspensions from spleen were depleted of erythrocytes and enriched for B cells by AutoMACS purification. B cell fractions were stimulated, as described above, for 5 and 10 min. Anti-Btk C-20 (Santa Cruz Biotechnology) was used for immunoblotting, while a polyclonal rabbot anti-Btk

(kindly provided by V. Tybulewicz, London, U.K.) was used for immunoprecipitation. In vitro kinase assays were performed, as described previously (27).

Ig detection and in vivo immunizations

Levels of Ig subclasses in serum were measured by sandwich ELISA, as described previously (25). To measure thymus-independent responses, mice were injected i.p. with 50 μg of trinitrophenol (TNP)-Ficoll in PBS, and TNP-specific IgM and IgG3 were analyzed at day 7 in a TNP-specific ELISA (28).

Results

Expression of Btk mutants in transgenic mice

We have previously described transgenic mice that express WT human Btk under the control of the CD19 promoter region (23). When these mice were mated onto a Btk-deficient background, correction of all *xid* features was observed, indicating that in this system the Btk gene was appropriately targeted to both conventional and $CD5^+$ B-1 cells. We now generated mouse strains, expressing Y223F ($n = 5$) and K430R ($n = 2$) mutant Btk. These mice were crossed onto the Btk null background, and transgenic Btk protein expression was quantified by intracellular flow cytometry. Median fluorescence intensities of transgenic Btk were compared with endogenous Btk in B-lineage subpopulations (Fig. 1*B*; Table I). The different mouse lines exhibited a wide range of Btk protein expression levels, which were directly correlated with the transgene copy number, as estimated by Southern blotting analyses of genomic DNA samples from the mice. The expression of transgenic Btk increased as the B cell progenitors in the BM matured to surface Ig-expressing peripheral B cells (Table I). Expression of transgenic Btk protein was confirmed by Western blotting analysis of BM and spleen cell suspensions, in which Btk protein was visible as a single ~77-kDa band for all transgenic lines generated (data not shown). Because we aimed to investigate the role of Btk in particular at the pre-B cell to immature B cell progression, we mainly focused on those mouse strains that expressed physiological levels of transgenic Btk (1–2 × endogenous levels) in immature B cells in the BM. These transgenic lines, AM-02 (Y223F) and XK-24 (K430R), expressed high Btk levels in mature IgD^+ peripheral B cells (Table I).

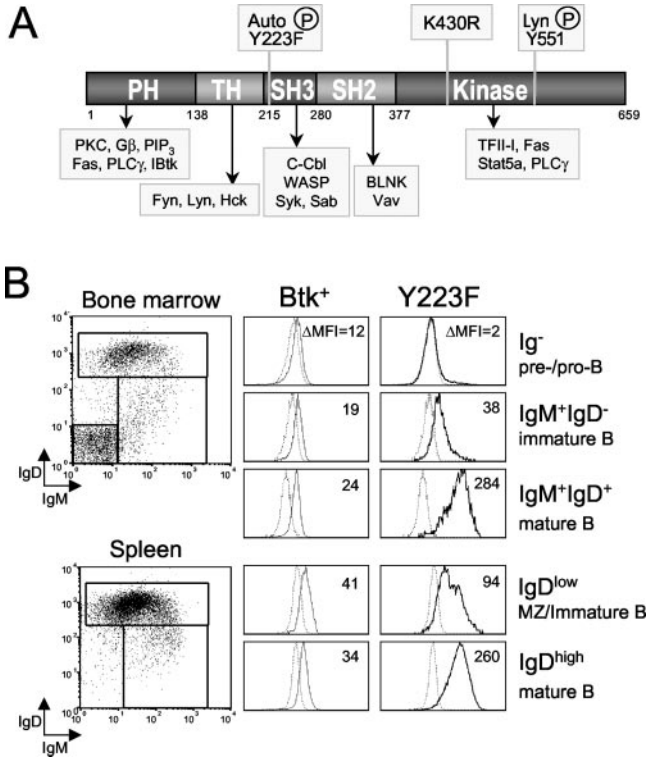

FIGURE 1. Expression of Btk mutant transgenes during B cell development. *A*, Domains of Btk. The positions of the mutations and Btk-associated molecules are shown. The numbers represent the amino acid positions of the domain boundaries. *B*, Intracellular Btk expression in the indicated B-lineage subpopulations in BM and spleen. $B220^+$ cells were gated and B-lineage subpopulations were defined on the basis of surface IgM/IgD expression (*left*) and analyzed for intracellular Btk expression (*right*). The results are displayed as histograms of a wild-type mouse (Btk^+; thin lines) and a Y223F-Btk mutant transgenic mouse (AM-02; bold lines), compared with the background staining, as determined in Btk^- mice (dashed lines). The values for the differences in MFI (ΔMFI) are given. MZ = marginal zone.

Table I. *Overview of transgenic lines expressing mutant Btk*

Btk Mutant	Tg Line	Relative Expression Level[a]		
		Pro-/pre-B	Immature B	Mature B
WT	NR-02[b]	<0.2	1.2	6.9
Y223F	AM-05	<0.2	<0.2	<0.2
	AM-03	<0.2	0.45	2.7
	AM-02	0.37	1.9	10
	TK-20	0.40	2.6	11
	UK-21	5.8	14	37
K430R	YK-25	<0.2	0.20	0.54
	XK-24	<0.2	0.92	17

[a] As determined by intracellular flow cytometry. The median fluorescence intensities in the different B-lineage cell fractions of wild-type mice were set to 1.0.
[b] These mice were described previously (23).

B cell development in Btk mutant mice

To determine the effect of the Btk mutations on B cell development, we examined the size of the B cell subpopulations in spleen, peritoneal cavity, and BM in the mutant mice by flow cytometry. In these experiments, we also included WT-Btk transgenic mice, as well as nontransgenic Btk^+ and Btk^- littermates, which served as controls.

The *xid* phenotype in Btk^- mice is characterized by a specific deficiency of mature $IgM^{low}IgD^{high}$ cells in the spleen and lack of $CD5^+$ B-1 cells in the peritoneal cavity (8–10) (see Fig. 2, *A* and *B*). The only obvious difference between the BM B cell subpopulations of Btk-deficient and control Btk^+ mice is that the subpopulation of mature $B220^{high}$-recirculating cells is reduced and has an aberrant IgM^{high} phenotype (Fig. 2*C*). We previously reported (23) that CD19 promoter-driven human WT-Btk expression restores these defects. We did not detect a significant effect of the Y223 autophosphorylation site mutation, as also the expression of Y223F-Btk appeared to restore B cell numbers completely in spleen, peritoneal cavity, and BM (Fig. 2, *A–C*). Expression of low levels of Y223F-Btk (line AM-05) (<20% of normal in immature B cells, not detectable with our flow cytometric assay) on the Btk null background rescued the presence of peritoneal $CD5^+$ B-1 cells, but not the numbers or surface profile of splenic B cells (data not shown). Considerable overexpression of Y223F-Btk (line UK-21, ~14× in immature B cells) was associated with deletion of IgM^{high} immature B cells in the BM (data not shown), similar to our previous findings in transgenic mice expressing the constitutive active Btk-mutant E41K (23).

Interestingly, reconstitution with kinase-inactive K430R-Btk partially overcame the block in peripheral B cell maturation, when significantly overexpressed. In these mice, the fraction of mature $IgM^{low}IgD^{high}$ B cells in the spleen was partially restored (~16%,

compared with ~7% in Btk$^-$ mice and 30% in WT transgenic mice, Fig. 2*A*), and B220^{high}IgM^{low}-recirculating cells appeared in the BM (Fig. 2*C*). However, we still did not detect CD5$^+$ B-1 cells in the peritoneum (Fig. 2*B*). When present on the Btk$^+$ background, overexpression of K430R-Btk did not appear to result in adverse effects on B cell development (data not shown), indicating that in this respect the K430R-Btk did not act as a dominant-negative mutant.

Collectively, these findings show that expression of Y223F-Btk restored B cell development, indicating that Y223 autophosphorylation is not essential for the function of Btk signaling in directing B cell development. The limited, but detectable, correction of the *xid* phenotype in K430R transgenic mice indicates that Btk function is partly independent of its intrinsic enzymatic activity. Finally, the analyses showed that B-1 B cell development is supported by low levels of Y223F-Btk, but not by kinase-inactive Btk.

Ig λ L chain usage in Btk mutant mice

We previously showed that B cells from Btk-deficient mice have a ~50% reduction in the frequency of Ig λ L chain expression, already at the immature B cell stage in the bone marrow (16). This finding implicated Btk in the regulation of the activation of the λ locus for V(D)J recombination in pre-B cells (16).

To investigate the effects of the Y223F and K430R mutations on the ability of Btk to regulate λ usage, we analyzed immature B220^{low}IgM$^+$IgD$^-$ B cells from the BM of a panel of Btk$^+$, Btk$^-$, and transgenic Btk mutant mice by four-color flow cytometry (Table II). Consistent with our previous report (16), we found reduced λ usage in Btk$^-$ mice, when compared with Btk$^+$ or WT-Btk transgenic mice (Table II). In Y223F-Btk mice, λ usage was still significantly reduced, when compared with Btk$^+$ mice (Table II) or with WT-Btk transgenic mice, which showed normal proportions of λ$^+$ B cells (16). Thus, transgenic expression of physiological levels of Y223F-Btk could only partially correct λ usage in Btk$^-$ mice. Unexpectedly, expression of K430R-Btk fully reconstituted λ usage, as in K430R-Btk mice the proportions of λ L chain-expressing cells were similar to those in control Btk$^+$ mice.

In summary, these findings indicate the differential requirement for Btk domains in the regulation of λ L chain usage: the Y223 autophosphorylation site is important, but kinase activity does not appear to be critical. We conclude that in the context of the control of λ usage in early B cell development, Btk mainly functions as an adapter molecule, independent of its enzymatic activity.

Pre-B cell maturation in Btk mutant mice

We previously showed that during the developmental progression of large cycling into small resting cytoplasmic μ$^+$ pre-B cells, Btk-deficient cells fail to efficiently down-regulate the expression of BP-1 and SLC and up-regulate the expression of surface CD2 and CD25/IL-2R (14). To investigate the requirement for the different Btk domains in the modulation of these phenotypic markers, we analyzed their expression profile in pre-B cells and immature B cells from Btk mutant mice by flow cytometry.

As shown in Fig. 3*A*, Btk-deficient pre-B cells have significantly higher levels of cytoplasmic SLC and BP-1, but lower expression levels of CD2 and CD25. This aberrant marker profile was fully corrected in transgenic mice expressing WT or Y223F Btk (Fig. 3*A*). By contrast, expression of K430R-Btk only partially restored Btk function in pre-B cell differentiation, as the expression levels of BP-1, cSLC, CD2, and CD25 in pre-B cells were intermediate between the values for Btk$^+$ and Btk$^-$ mice (Fig. 3, *A* and *B*). Also, at the next developmental stage of immature B cells, the expression profiles of the BP-1, cSLC, CD2, and CD25 markers were fully corrected in WT and Y223F Btk mice and partially corrected in K430R-Btk mice (data not shown, and Fig. 3*C*).

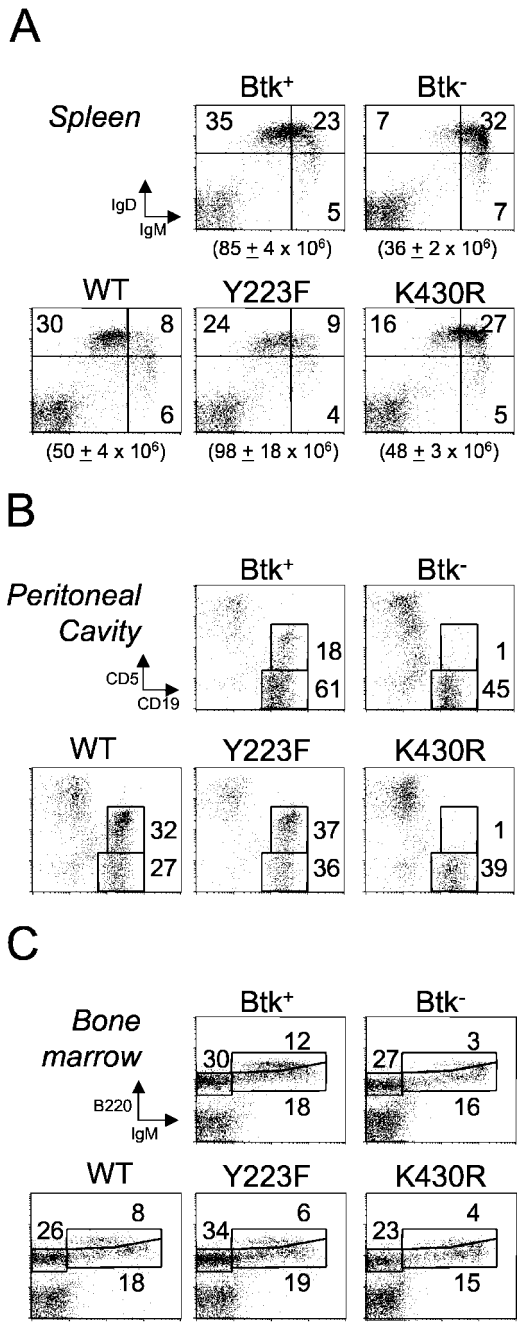

FIGURE 2. Flow cytometric analysis of B cell populations in Btk mutant mice. *A*, Surface IgM/IgD expression in the spleen with total splenic B cell numbers as mean values ± SEM. *B*, Surface CD19/CD5 expression in the peritoneal cavity. *C*, Surface IgM/B220 expression in the BM. Total lymphocytes were gated on the basis of forward and side scatter characteristics. Data are displayed as dot plots, and the percentages of cells within the indicated gates are given. Data shown are representative of 5–20 animals examined within each group.

In summary, these findings demonstrate that the function of Btk in vivo in the induction of cell surface phenotype changes in cytoplasmic μ$^+$ pre-B and immature B cells is not affected by loss of the Y223 autophosphorylation site and is only partially dependent on its kinase activity.

Table II. *Ig λ L chain usage in Btk mutant mice*

Btk Mutant	n^a	Ig λ^+ Immature B Cells (%)	p Values[b]
Btk^+	32	7.7 ± 0.5[c]	
Btk^-	24	2.9 ± 0.3	$<10^{-9}$
Y223F	12	4.9 ± 0.5	0.0017
K430R	19	7.5 ± 0.7	NS

[a] Number of mice per group.
[b] Values of p for difference with the group of Btk^+ mice.
[c] Mean values ± SEM.

Proliferative responses to IL-7 in Btk mutant mice

Btk has an inhibitory effect on the proliferation of B cell precursors in long-term Whitlock-Witte BM cultures and in IL-7-driven cultures of BM or fetal liver cells (14, 29, 30). In [^{3}H]thymidine incorporation experiments, after 5 days of culture in the presence of IL-7, Btk-deficient total BM cells showed significantly higher proliferative responses when compared with Btk^+ cells (Fig. 4*A*). Expression of WT or Y223F Btk exhibited a similar inhibitory effect on IL-7-driven proliferation as intact endogenous Btk. Proliferative responses to IL-7 in K430R mutant mice were only slightly higher than those found in Btk^+ mice. Therefore, we conclude that the function of Btk in down-regulation of IL-7-driven proliferation is not critically affected by loss of Y223 autophosphorylation or kinase activity of Btk.

Developmental progression of Btk mutant pre-B cells in vitro

In Btk-deficient pre-B cells, the increased IL-7-driven proliferation in vitro is accompanied by reduced developmental progression from large cycling $CD2^-$ pre-B cells into noncycling small resting $CD2^+$ pre-B cells, and subsequently into surface Ig^+ B cells (14). To analyze the function of Btk domains in this context, total BM cell suspensions from the panel of mutant mice were cultured in the presence of 100 U/ml IL-7. At day 5, the distribution of $B220^+$ cells over six developmental fractions, μ^- pro-B cells, $CD2^-$ μ^+ large cycling pre-B cells, $CD2^-$ μ^+ small pre-B cells, $CD2^+$ μ^+ small pre-B cells, IgM^+IgD^- immature B cells, and IgM^+IgD^+ mature B cells, was evaluated by flow cytometry (Fig. 4*B*). Consistent with our previous findings (14), we noticed that in the absence of Btk: 1) growth of cytoplasmic μ^- cells was hardly supported, 2) progression from the $CD2^-$ into the $CD2^+$ pre-B cell stage was impaired, and 3) the proportions of IgM^+ immature and mature B cells were reduced, when compared with wild-type mice. The BM cultures from WT and Y223F Btk mice exhibited a subpopulation distribution that was similar to that in Btk^+ control mice. Expression of the K430R-Btk transgene partly restored Btk function, as in the K430R cultures significant fractions of μ^- pro-B cells were found and CD2 expression was more efficiently induced on μ^+ small pre-B cells, when compared with cultures from Btk-deficient mice (Fig. 4*B*).

Taken together, these results show that the role of Btk signaling in the induction of the developmental progression of $CD2^-$ into $CD2^+$ small pre-B cells and subsequently to surface Ig^+ B cells in vitro is completely independent of Y223 autophosphorylation. In this context, kinase-inactive Btk is apparently partially active.

Kinase-independent adapter function of Btk in mature B cells

Because kinase-inactive Btk could partially restore developmental progression of pre-B cells, we examined mature B cell function in K430R-Btk mice to evaluate the overall ability of kinase-inactive Btk to complement other features of the *xid* phenotype.

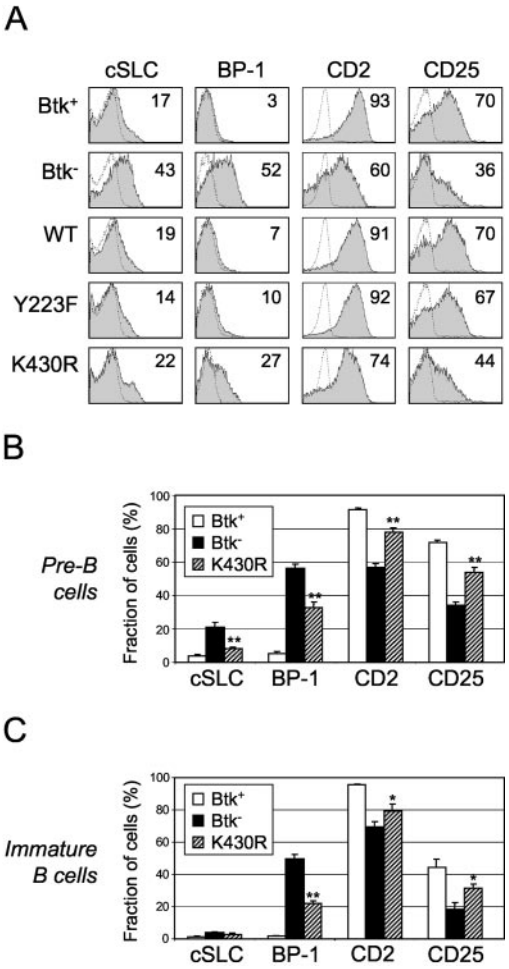

FIGURE 3. Phenotypic characterization of pre-B and immature B cells in Btk mutant mice. *A*, Expression profiles of cytoplasmic SLC, surface BP-1, CD2, and CD25 in pre-B cells of the indicated mice. Flow cytometry data are displayed as filled histograms, with background stainings of $B220^-$ cells (dashed lines) as negative controls. The percentages of positive cells are indicated. Data shown are representative of 5–20 animals examined within each group. *B* and *C*, The K430R-Btk transgene partially corrects the expression of the indicated markers in pre-B cells (*B*) and $B220^{low}IgM^+$ immature B cells (*C*). Differences between Btk^+ and Btk^- pre-B and immature B cells were significant for all markers (*t* test; $p < 0.005$). Asterisks indicate significance of differences between K430R-Btk and Btk^- mice (*, $p < 0.05$; **, $p < 0.0005$). Cytoplasmic SLC and CD2 expression was investigated in $B220^{low}IgM^-c\mu^+$ pre-B cells, while BP-1 and CD25 were analyzed in total $B220^{low}IgM^-$ pro-B/pre-B cell fractions (pro-B cells have very low proportions of $BP\text{-}1^+$ or $CD25^+$ cells).

Btk-deficient mice have severely decreased levels of IgM and IgG3 in the serum and do not mount specific Ab responses to T cell-independent type II (TI-II) Ags in vivo, and *xid* B cells undergo apoptosis instead of proliferation in response to in vitro stimulation (8, 9, 31–33). The K430R-Btk transgene provided a significant correction of the decreased levels of IgM in the serum of Btk-deficient mice, while only a modest increase in IgG3 was observed (Fig. 5*A*). Expression of K430R-Btk in Btk-deficient mice did not reconstitute the in vivo responsiveness of B cells to the TI-II Ag TNP-Ficoll. Moreover, K430R-Btk expression on a wild-type background even showed a dominant-negative effect on the production of TNP-specific IgM (Fig. 5*B*).

When purified splenic B cell suspensions were stimulated in vitro, K430R-Btk completely restored the proliferative responses to anti-CD40 and LPS (Fig. 6*A*), which are known to be affected

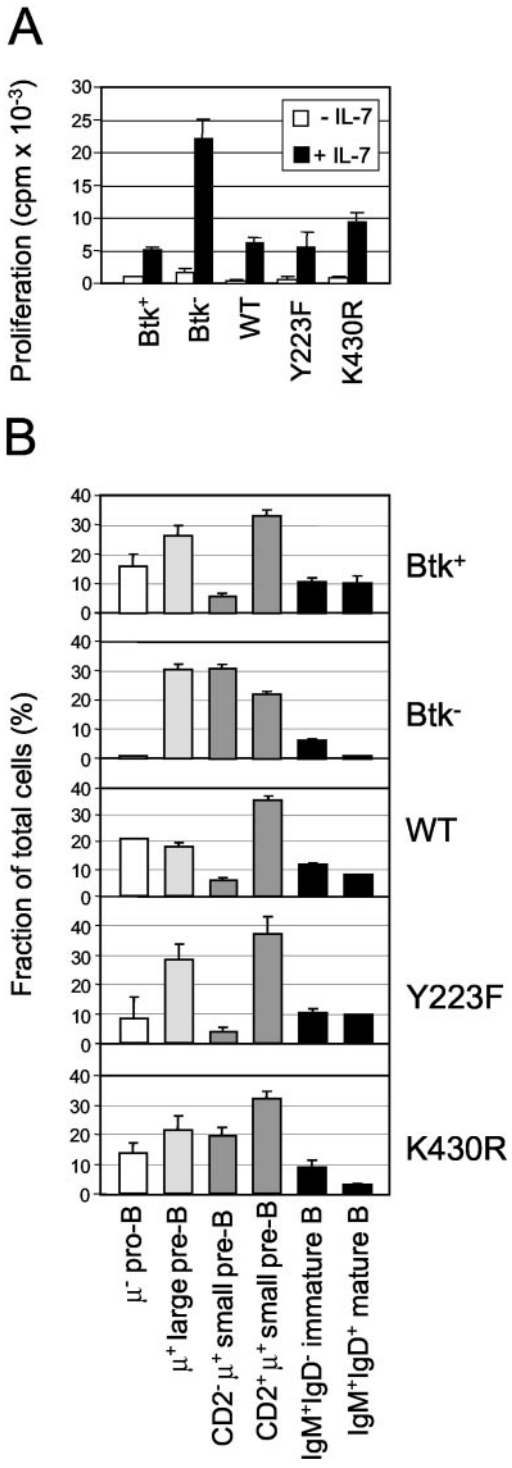

FIGURE 4. Analysis of IL-7-driven BM cultures from Btk mutant mice. *A*, Proliferative response to IL-7, as determined by [^{3}H]thymidine incorporation after 5 days of culture in the presence or absence of 100 U/ml IL-7. *B*, The distribution profile over the indicated B-lineage subpopulations of day 5 IL-7-driven total BM cultures of the Btk mutant mice. Error bars are the SEM values from four to seven mice per group.

by defective Btk function (8, 9, 34). In contrast, the rescue of the proliferative response to anti-IgM stimulation was modest (Fig. 6*A*). To determine the effect of the K430R-Btk transgene on B cell survival, purified splenic B cells from Btk$^+$, Btk$^-$, WT-Btk, and K430R-Btk mice were cultured in vitro in medium alone, or in the presence of anti-CD40, LPS, or anti-IgM. After 40 h, cells were stained with propidium iodide and examined by flow cytometry to determine the fraction of apoptotic cells, i.e., cells with sub-G_1 amounts of DNA. The presence of the K430R-Btk transgene on the Btk null background strongly reduced the proportion of apoptotic cells, in all four culture conditions (Fig. 6*B*). Apoptosis levels of K430R-Btk B cells stimulated with anti-IgM were intermediate between the values for wild-type and Btk-deficient mice. Taken together, these data indicate that kinase-inactive Btk can partially rescue the compromised cell cycle induction and cell survival of Btk-deficient mature B cells.

Kinase-inactive Btk induces NF-κB activation upon BCR stimulation

To explore the mechanism underlying the kinase-independent adapter function of Btk, we further investigated kinase-inactive Btk function in BCR signal transduction. Consistent with a previous report (19), in vitro kinase assays of immunoprecipitated Btk from purified splenic B cell fractions stimulated with anti-IgM Abs confirmed that K430R-mutated Btk did not have any detectable autophosphorylation activity, even though high levels of K430R-Btk were expressed (Fig. 7*A*).

Btk is known to have a critical role in BCR-directed cell cycle induction (32, 33, 35), as well as nuclear translocation of NF-κB, which induces expression of the antiapoptotic survival protein Bcl-x_L (31, 33, 36, 37). We thus evaluated the ability of K430R-Btk to activate NF-κB and induce Bcl-x_L and cyclin D2 expression in response to BCR engagement. Western blot analysis of purified splenic B cells that were stimulated with anti-IgM Abs for 4 h showed that, while induction of nuclear c-Rel was negligible in Btk-deficient B cells, the fold inductions in K430R-Btk were close to those observed in Btk$^+$ control mice (Fig. 7*B*). Under these conditions, the amounts of cytoplasmic c-Rel were similar in all three groups of mice. Furthermore, the induction of Bcl-x_L and cyclin D2 after BCR stimulation, which was blocked in Btk-deficient B cells, was partially restored in K430R-Btk transgenic mice (Fig. 7*C*).

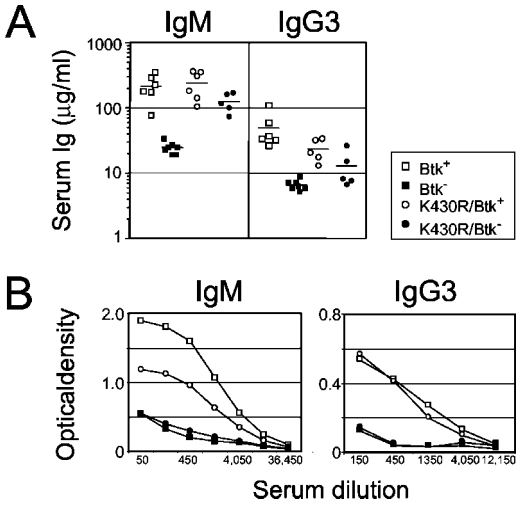

FIGURE 5. Partial correction of mature B cell function in K430R-Btk mice. *A*, Serum concentrations of IgM and IgG3 in Btk$^+$ and Btk$^-$ mice, as well as K430R-Btk mice on the Btk$^+$ and Btk$^-$ background. Mice were 2 mo of age, total Ig levels were determined by ELISA, and each symbol indicates an individual mouse (n = 5–7). *B*, Serum concentrations of TNP-specific IgM and IgG3 7 days after in vivo TNP-Ficoll injection. For each serum dilution, the ODs are shown as mean values from 11–23 mice in each group.

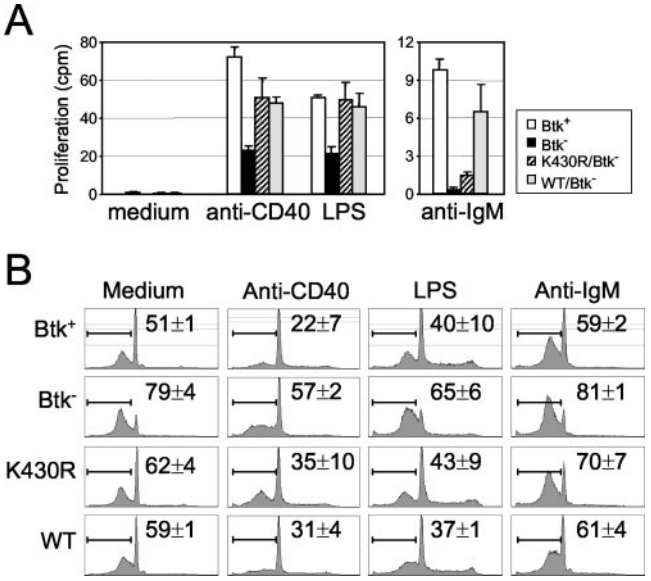

FIGURE 6. Partial correction of mature B cell proliferation and survival in K430R-Btk mice. *A*, Proliferation, determined by [^{3}H]thymidine incorporation, of cells cultured in medium alone, in the presence of LPS, anti-CD40, or anti-IgM. *B*, Propidium iodide DNA content analysis of B cells following stimulation in vitro. Numbers indicate the proportion of cells ± SEM in the sub-G_1 fraction (%) in cell cycle analysis. Data (with mean ± SEM values from two to four mice per group) are representative of three independent experiments.

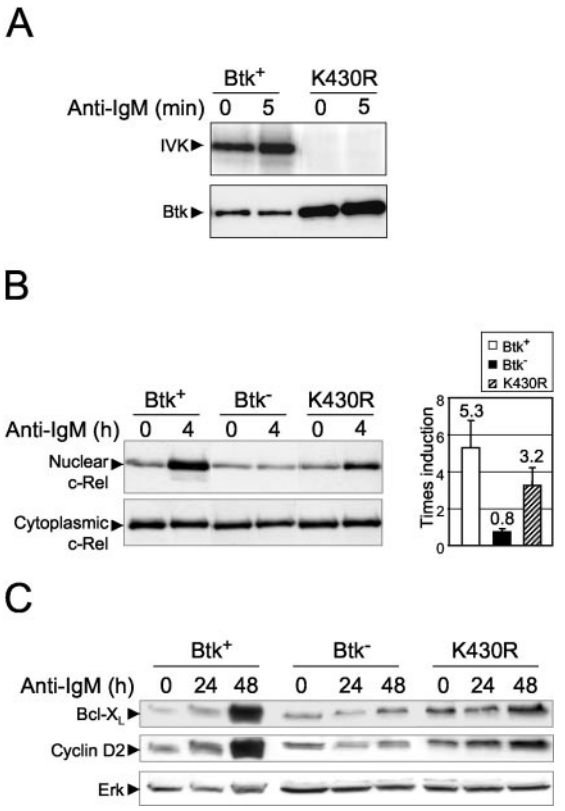

FIGURE 7. K430R-Btk induces NF-κB activation upon BCR stimulation. *A*, SDS-PAGE of in vitro phosphorylated Btk immunoprecipitates from purified splenic B cells (12×10^6) from wild-type and K430R-Btk mice were left untreated or stimulated with anti-IgM for 5 min. For quantification of Btk expression, equal proportions of the immunoprecipitates were analyzed by Western blotting using Btk-specific Abs. In agreement with a previous report (42), wild-type Btk showed detectable kinase activity also in unstimulated splenic B cells. *B*, Western analysis of nuclear and cytoplasmic c-Rel expression. Equal numbers of purified splenic B cells from the indicated mice were left untreated or stimulated with anti-IgM for 4 h (*left*). Induction of nuclear c-Rel was quantified by densitometry, and results are shown as mean values and SEM from four mice per group (*right*). *C*, Induction of Bcl-x_L and cyclin D2. Total splenocytes of the indicated mice were stimulated with anti-IgM and evaluated for the expression of Bcl-x_L and cyclin D2 by immunoblotting using specific Abs. Membrane was reblotted with anti-extracellular signal-regulated kinase. Data are representative of four mice analyzed per group.

In summary, these results show that in mature B cells, K430R-Btk is able to transmit BCR signals that can activate NF-κB and induce Bcl-x_L and cyclin D2 expression, leading to cell cycle entry, proliferation, and rescue from apoptosis.

Discussion

In vivo structure-function analysis for Btk

The characterization of 400 unique human Btk mutations, 155 of which represent missense mutations, in XLA families could in principle have allowed the identification of in vivo structure-function effects of Btk. However, there is phenotypical heterogeneity among patients, even from single XLA pedigrees, and to date no correlation has been described between the type and position of the mutations and clinical or immunological XLA phenotype (38). Furthermore, the almost complete pre-B cell arrest in most XLA patients (6) complicates the analysis of the function of the individual Btk domains in developmental stages beyond the large μ^+ pre-B cell. As a result, the role played by the various Btk domains in vivo in humans remained largely unknown.

Analyses in the mouse to date have focused on the in vivo function of the Btk PH domain, which was shown to be absolutely essential in mature B cells. This was inferred from the finding of the same *xid* phenotype in CBA/N mice with the classical R28C PH domain mutation and in mice with a complete ablation of Btk protein generated by gene targeting (8–10). Apparently, the R28 residue in the PH domain is also essential for Btk function in pre-B cells, as we observed that, comparable to Btk-deficient pre-B cells, also *xid* CBA/N pre-B cells manifested an impaired modulation of the phenotypic markers BP-1, SLC, CD2, and CD25, and an increased proliferative response to IL-7 (S. Middendorp and R. Hendriks, unpublished results).

By using transgenic mice expressing Y223F and K430R mutant Btk, we now demonstrate a differential role of Y223 phosphorylation and Btk catalytic activity in developmental progression of cytoplasmic μ^+ pre-B cells in vivo. In particular, we analyzed λ L chain usage; the modulation of BP-1, SLC, CD2, and CD25 expression; and the IL-7 responsiveness of pre-B cells, all of which were previously shown to be Btk dependent (14, 16). In this study, we demonstrate that: 1) the Y223 autophosphorylation site is not essential for signaling developmental progression of pre-B or B cells, except for the role of Btk in the regulation of λ L chain usage; 2) in pre-B cells, Btk functions to a large extent as an adapter molecule; 3) in mature B cells kinase-inactive Btk can induce the activation of NF-κB upon BCR stimulation.

The Y223 autophosphorylation site in the SH3 domain

Although phosphorylation of Y223F has little discernible influence on Btk catalytic activity, it is generally thought that full activity of Btk is dependent on phosphorylation of Y223. Y223 is located on the surface of the SH3 domain, and its phosphorylation prevents binding to WASP and increases the affinity to Syk (21). The Y223F mutation dramatically potentiates the transforming activity

of the gain-of-function mutant E41K-Btk in fibroblasts (19). Moreover, the SH3 domain has specificity for the N-terminal proline-rich region of the Btk TH domain, and therefore it has been hypothesized that intra- or intermolecular interaction between the TH and SH3 domain might have an important regulatory function (1). With the exception of λ L chain usage, we found that expression of Y223F-mutated Btk fully corrected all features of the Btk-deficient phenotype, including pre-B cell maturation, differentiation of peripheral B cells, and B-1 cell development. Furthermore, we observed reconstitution of B cell function, both in total serum IgM and IgG3 levels, TI-II responses in vivo, and in proliferative responses to anti-IgM stimulation in vitro (R. Hendriks, unpublished). Taken together, these results indicate that Y223 autophosphorylation-dependent interactions are not essential for B cell development and mature B cell function. This is consistent with previous observations that reconstitution of Btk-deficient chicken DT 40 B cells with Y223F and WT Btk resulted in a similar rescue of BCR-induced PLC-γ phosphorylation and calcium flux (39). Our findings may also explain the remarkable absence of missense mutations in the Btk SH3 domain in a total of 155 missense mutations identified to date in XLA families (38), while on the basis of SH3 domain size ~10 of these should be located within the SH3 domain. Nevertheless, the Y223 autophosphorylation site appears to be involved in the regulation of λ L chain usage. In this context, it is tempting to speculate that transcription factor II-I (TFII-I) is involved in the activation of the λ L chain locus for recombination, as the Y223F-Btk mutation also abrogates phosphorylation of a Btk substrate, the NF BAP-135/TFII-I (40). Obviously, additional experiments are required to demonstrate whether TFII-I plays any role in the regulation of gene rearrangements at the λ L chain locus.

It is intriguing that mutation of the Y223 Btk autophosphorylation site partially affected λ L chain usage, while expression of kinase-inactive Btk restored λ usage completely. This may imply that the Y223F mutation does not only change the interaction of Btk with proteins that bind to the SH3 domain when phosphorylated at Y223, but also with proteins that bind to nonphosphorylated Y223, such as c-Cbl and WASP (21). Alternatively, the obtained results could be explained by a contribution of other kinases to phosphorylation of Btk at position Y223 in vivo, once Btk is recruited to the pre-BCR signaling complex.

The kinase-inactive K430R-Btk mutant

The K430R mutation destroys the ATP-binding site, resulting in kinase-inactive Btk (19, 20). Expression of physiological levels of kinase-inactive Btk in early B cell development normalized λ L chain usage and partially reconstituted the impaired pre-B cell differentiation and IL-7 responsiveness in Btk-deficient mice. These findings imply that Btk partially functions as an adapter molecule, independent of its kinase activity, possibly in a larger complex together with other molecules that interact with Btk (Fig. 1*A*). Such a complex might include PTK that either bind directly to Btk, such as Fyn, Lyn, Hck, and Syk, or indirectly through the BLNK/SLP65 linker molecule, which binds to the Btk SH2 domain. It is therefore possible that Syk may partially compensate for kinase-inactive Btk and phosphorylate PLC-γ2 in a complex that contains activated BLNK/SLP65 and Btk. Our data indicate that, especially in the activation of the λ L chain locus for V(D)J recombination in pre-B cells, Btk mainly acts as an adapter molecule. Likewise, in mature B cells, the role of Btk in proliferation and survival upon LPS and anti-CD40 stimulation in vivo was found to be fully independent of Btk enzymatic function. Nevertheless, the (incomplete) induction of NF-κB, Bcl-x_L, and cyclin D2 by K430R-Btk cannot fully rescue B cell development, as is particularly clear from the lack of reconstitution of the B-1 cell compartment and in vivo TI-II responses. In this context, it was recently shown that Btk participates in NF-κB induction by Toll-like receptor 4, whereby K430R-Btk acts as a dominant-negative inhibitor of the LPS-induced activation of NF-κB in an astrocytoma and a monocytic cell line (41). Taken together, these findings illustrate that the importance of the Btk kinase domain varies between the different Btk-mediated signaling pathways during B cell development and between the different signal transduction pathways.

In summary, our findings demonstrate the differential requirement for Btk domains in the developmental progression of cytoplasmic μ H chain-positive pre-B cells. We show that the regulation of Ig λ L chain usage is Y223 dependent, but K430 independent, and that the modulation of pre-B cell marker expression is Y223 independent, but partially K430 dependent. None of the two residues appear to be essential for Btk function in the regulation of IL-7 responsiveness. We therefore conclude that the control of lg λ L chain usage, pre-B cell marker phenotype, and IL-7 responsiveness involve different downstream signaling pathways or molecules interacting with Btk.

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