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# Defective B Cell Development and Function in *Btk*-Deficient Mice

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## Summary

**Mutations in the Bruton's tyrosine kinase (*Btk*) gene have been linked to severe early B cell developmental blocks in human X-linked agammaglobulinemia (XLA), and to milder B cell activation deficiencies in murine X-linked immune deficiency (*Xid*). To elucidate unequivocally potential Btk functions in mice, we generated mutations in embryonic stem cells, which eliminated the ability to encode Btk pleckstrin homology or kinase domains, and assayed their effects by *RAG2*-deficient blastocyst complementation or introduction into the germline. Both mutations block expression of Btk protein and lead to reduced numbers of mature conventional B cells, severe B1 cell deficiency, serum IgM and IgG3 deficiency, and defective responses in vitro to various B cell activators and in vivo to immunization with thymus-independent type II antigens. These results prove that lack of Btk function results in an *Xid* phenotype and further suggest a differential requirement for Btk during the early stages of murine versus human B lymphocyte development.**

## Introduction

X-linked agammaglobulinaemia (XLA) is an inherited X chromosome–linked humoral immunodeficiency disease (Bruton, 1952). Afflicted males have a severe deficiency of mature B cells and circulating immunoglobulins of all isotypes. The XLA defect is intrinsic to the B lineage (Conley et al., 1986) and is manifested at several stages of B cell development. An early developmental block is evidenced by an increase in pro-B cells and inefficient expansion and proliferation of pre-B cells in the bone marrow (BM) (Pearl et al., 1978; Campana et al., 1990; Milili et al., 1993). There is also a severe deficiency of peripheral B cells due to a block in development at the pre-B stage; peripheral B cells that are present usually have an immature, (immunoglobulin $M^{hi}$ [$IgM^{hi}$]/$IgD^{lo}$) phenotype (reviewed by Rosen et al., 1984; Conley, 1985; Cooper et al., 1993).

Murine X-linked immunodeficiency (*Xid*, Amsbaugh et al., 1972; Scher et al., 1975, 1979) shares many features with human XLA. Like XLA, the *Xid* defect (reviewed by Scher, 1982) is X-linked and intrinsic to B cells. In CBA/N (*Xid*) mice, the overall number of peripheral B cells is usually 50%–60% of normal (Scher et al., 1975; Janeway and Barthold, 1975); in addition, the $IgM^{lo}$/$IgD^{hi}$ B cell population (population 1) in the spleen is severely reduced (Hardy et al., 1982, 1983; Forrester et al., 1987), $CD5^+$ B cells (B1 cells) are not detected (Hayakawa et al., 1986), and levels of serum IgM and IgG3 are low (Perlmutter et al., 1979). Furthermore, *Xid* mice are unable to respond to thymus-independent type II (TI-II) antigens (Scher et al., 1975; Amsbaugh et al., 1972) and to some thymus-dependent (TD) antigens (Boswell et al., 1980; Press, 1981; Press and Giorgetti, 1986). In vitro studies also have shown that *Xid* B cells do not proliferate when triggered through their surface IgM receptor (Mond, 1982; Rigley et al., 1989) and show hyporeactivity to lipopolysaccharide (LPS) stimulation (Amsbaugh et al., 1972; Huber and Melchers, 1979).

A recently identified X-linked gene that encodes a cytoplasmic tyrosine kinase, denoted Bruton's tyrosine kinase (*Btk*), has been shown to be mutated in humans with XLA (Vetrie et al., 1993; Tsukada et al., 1993) and mice with *Xid* (Thomas et al., 1993; Rawlings et al., 1993) mutations. Btk, along with Tec (Mano et al., 1993) and Itk (Siliciano et al., 1992), comprise the Tec/Btk subfamily of Src-related tyrosine kinases. Btk contains src homology (SH) domains, including the SH1 (kinase), SH2, and SH3 domains (reviewed by Pawson and Gish, 1992); a unique N-terminal region, which is comprised of a pleckstrin homology (PH) domain (Clark and Baltimore, 1993; Haslam et al., 1993; Mayers et al., 1993; Musacchio et al., 1993; Shaw, 1993); and a Tec homology (TH) domain (Vihinen et al., 1994) (Figure 1). The PH domains have been found in many proteins involved in intracellular signaling pathways and, although of unknown function, have been speculated to be involved in protein–protein interactions. Likewise, the function of the TH domain is unknown. Unlike the Src protein, Btk protein lacks a negative regulatory tyrosine residue at the C terminus and a myristylation signal for membrane anchoring at the N terminus (reviewed by Mustelin and Burn, 1993).

*Btk* is expressed in B lymphocytes as well as in myeloid and erythroid cells. The gene is expressed in most stages of B cell development, except the terminally differentiated plasma cell stage (de Weers et al., 1993; Smith et al.,

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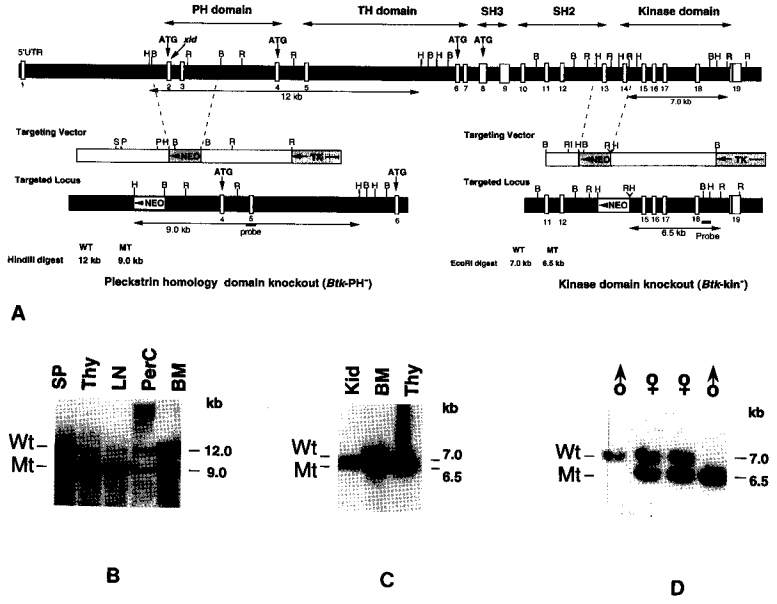



Figure 1. Disruption of Murine *Btk* Gene

(A) Physical map of the *Btk* locus (top). Exons are represented by boxes. Targeting vector for PH domain knockout and predicted structure of the mutated locus (bottom left). Targeting vector for kinase domain knockout and predicted structure of the mutated locus after homologous recombination (bottom right). Probes and expected sizes of DNA fragments from the endogenous and after homologous recombination are shown. TK; thymidine kinase gene; Neo, neomycin resistance gene. Restriction endonucleases: B, BamHI; R, EcoRI; H, HindIII; S, SacI; and P, PstI.

(B) Contribution of the PH⁻ ES cells to lymphoid tissues as indicated in PH⁻/*RAG2*$^{-/-}$ somatic chimeric mouse. DNA (10 μg) was digested with HindIII and assayed for hybridization to a 700 bp PvuII fragment shown in (A) (bottom left). The endogenous locus gave a band of about 12.0 kb and the mutant locus gives a band of about 9.0 kb. Male ES cells were used in which, due to X chromosome linkage of *Btk*, only a single targeting event was necessary to get inactivation of *Btk* for injection in *RAG2*-deficient blastocyst complementation system.

(C) Contribution of the kinase-negative ES cells to lymphoid tissues, as indicated, in *Btk*–Kin⁻/*RAG2*$^{-/-}$ somatic chimeric mouse. DNA (10 μg) was digested with EcoRI and assayed for hybridization to the 700 bp BamHI–HindIII fragment shown in (A) (bottom right). The endogenous locus gave a band of about 7.0 kb and the mutant locus gives a band of about 6.5 kb. Male ES cells were used in which, owing to X chromosome linkage of *Btk*, only a single targeting event was necessary to get inactivation of *Btk* for injection in *RAG2*-deficient blastocyst complementation system.

(D) Germline transmission of the *Btk* kinase mutation analyzed by Southern blotting of mouse tail DNA. DNA was digested with EcoRI, separated in a 0.7% agarose gel bloted to a zeta probe membrane, and hybridized with random-primed $^{32}$P-labeled BamHI–HindIII 700 bp DNA fragment shown in (A) (bottom right). From left, DNA from a normal littermate male (X/Y) (used as a wild-type [WT] control), two *Btk–kin*$^{-/+}$ heterozygous (X/X$^{M}$) females and a *Btk–kin*$^{-}$ (X$^{M}$/Y) male are shown.

1994; Khan et al., 1995). The pattern of *Btk* expression coupled with the defects observed in the context of the XLA and *Xid* mutations, led to the notion that Btk may serve critical signaling functions at various stages of B cell development. In this context, a number of recent studies have suggested a potential function for Btk in signaling from the pre-B cell receptor and from the mature B cell receptor (Saouf et al., 1994; Aoki et al., 1994), as well as in the response of B cells to stimulation via the CD40, interleukin-5 (IL-5), IL-10, and CD38 pathways (Hasbold and Klaus, 1994; Go et al., 1990; Sato et al., 1994; Santos-Argumendo et al., 1995).

Mutational analyses of *Btk* in XLA patients to date have not been helpful in correlating different domains to function. XLA patients carry mutations in the *Btk* gene that include deletions, insertions, and point mutations leading to amino acid substitutions or premature stop codons in virtually all the domains (reviewed by Sideras and Smith, 1995). However, most patients show the typical severe XLA phenotype outlined above. The putative murine *Xid* mutation involves a single amino acid substitution of a highly conserved residue in the PH domain (Thomas et al., 1993; Rawlings et al., 1993). However, a different substitution mutation of the same amino acid residue mutated in *Xid* mice was found to be associated with the severe XLA phenotype in humans (de Weers et al., 1994). In this context, the *Xid* mutation also leads to a severe block in B cell development when introduced into the homozygous nude (nu$^{-}$/nu$^{-}$) background (Karagogeos et al., 1986). Likewise, within families of XLA patients, the disease phenotype can be highly variable in severity (reviewed by Rosen et al., 1984). Together, these findings suggest that other factors may greatly influence the phenotype of *Btk* mutations.

There are several proposed explanations for the milder phenotype of the murine *Xid* as compared with the human XLA mutations, assuming that both involve only the *Btk* gene. One is that the function affected by the *Xid* mutation would only interfere with Btk activities required at later stages in the context of some forms of B cell activation, whereas more severe inactivation of the Btk protein in XLA could block a required function in early B cell development (Thomas et al., 1993; Rawlings et al., 1993). Other possibilities suggested from the apparently differential effects of similar mutations in *Xid* and certain cases of XLA, are that mice have a redundant signaling pathway in early B cell development, or that genetic background may have a major influence on the phenotypic severity of *Btk* mutations, or both.

In this report, we describe the use of gene-targeted mutation in embryonic stem (ES) cells to elucidate the essential roles of Btk in B cell maturation and function, to distinguish the various models proposed to explain differences in XLA and *Xid* phenotypes, and to establish a Btk-deficient mouse model.

## Results

### Generation of PH and Kinase Domain *Btk* Mutations in ES Cells and Mice

The *Btk* genomic locus is encoded by 19 exons (Sideras et al., 1995; Figure 1A). There are four potential translation

initiation codons in exons 2, 4, 6, and 7, which upon in vitro translation give proteins of 77, 66, 58, and 50 kDas. However, only the 77 kDa protein starting at the first ATG is detectable in vivo (Tsukada et al., 1993). We introduced two separate mutations into the *Btk* gene. For the first, we replaced, in CCE ES cells, the first two coding exons (which encode 80 of the 117 aa of the PH domain) with a neomycin resistance (*neo*$^r$) gene (Figure 1A, left, *Btk*–PH$^-$ mutation; Figure 1B). This mutation deletes the first translation initiation codon in exon 2 and should either permit generation of a truncated Btk protein without a PH domain or should completely ablate synthesis of the Btk protein. For the second mutation, we replaced, in J1 ES cells, exons 13 and 14 (which encode the first two exons of the kinase domain) with a *neo*$^r$ gene (Figure 1A, right, *Btk–kin*$^-$; Figure 1C). The latter mutation disrupts the *Btk* open reading frame by introducing a stop codon that inactivates the entire C-terminal coding region.

There is only one copy of the *Btk* gene in the male-derived CCE and J1 ES cells. Thus, *Btk*–PH$^-$ ES cells were directly used to generate chimeras by injection into recombination activating gene-2 (*RAG2*)-deficient blastocysts. *RAG2*-deficient mice lack mature lymphocytes due to their inability to initiate VDJ recombination (Shinkai et al., 1992); therefore, all the mature lymphocytes in chimeric mice are mutant ES cell derived (Chen et al., 1993). The *Btk–kin*$^-$ ES cells were similarly used to generate somatic chimeras in *RAG2*-deficient blastocyst complementation system. In addition, to compare results obtained for lymphocyte-intrinsic versus germline mutations, the *Btk–kin*$^-$ mutation was also introduced into the mouse germline (*Btk–kin*$^-$/GL) (Figure 1D).

### Lack of *Btk* Expression in PH and Kinase Domain Mutant B Cells

As assayed by *RAG2*-deficient blastocyst complementation (*Btk*–PH$^-$ or *Btk–kin*$^-$ mutations) or germline transmission (*Btk–kin*$^-$/GL), neither *Btk* mutation had an obvious effect on T cell development and population distribution (Tables 1, 2, 3; data not shown). Contribution of *Btk*–PH$^-$ and *Btk–kin*$^-$ ES cells to mature lymphocytes generated by *RAG2*-deficient blastocyst complementation was further confirmed by Southern blot analysis, which demonstrated nearly full contribution of the mutant allele to thymic DNA (Figures 1B and 1C). Likewise, neither mutation led to severe blocks in the generation of conventional B cells in this system, although both had similar effects on B cell development and function (see below).

To confirm the predicted effects of the introduced mutations on *Btk* gene expression, we assayed for potential *Btk* transcripts and proteins in both splenic B cells and A-MuLV transformed pre-B cells carrying each respective mutation. In *Btk*–PH$^-$ B cells, *Btk*-specific transcripts from the mutated loci were detectable by Northern blotting analyses (Figure 2A). These transcripts hybridized to probes derived both from the 5′ and 3′ ends of the *Btk* cDNA but not to the *neo*$^r$ gene probe (Figure 2A). As the predicted size of the *Btk*–PH$^-$ transcripts is not substantially different from that of wild type, we cloned *Btk* cDNA from *Btk*–PH$^-$ B cells by reverse transcription–polymerase chain reaction (PCR), utilizing primers in exons 1 and 6. PCR products of the expected size (Figure 2B, right, 600 bp) in the normal and *Btk–kin*$^-$ B cells could be seen (Figure 2B, left). Also, an expected reduced-size PCR product, resulting from deletion of exons 2 and 3, was detected in *Btk*–PH$^-$ B cells (Figure 2B, right, 340 bp). The nucleotide sequence of the PCR products was determined through the regions containing the PH and TH domains. Exon 1 was found spliced correctly to exon 4, deleting the intraintronic *neo*$^r$ gene, which was inserted by gene targeting (data not shown). Northern blot analyses of RNA from *Btk–kin*$^-$ A-MuLV-transformed lines revealed a larger low abundance transcript that hybridized to 5′ end and 3′ end fragments of *Btk* cDNA and to the *neo*$^r$ gene probes (Figure 2A), indicating that *Btk*-specific transcripts from the mutated locus were generated in which *Btk* sequences both 5′ and 3′ to the insertion were fused to the *neo*$^r$ gene (Figure 2A).

To assay for Btk protein production, Western blotting experiments were performed on extracts from normal and mutant A-MuLV transformants with affinity-purified antibodies raised against the C-terminal region or N-terminal region of Btk. The normal *Btk* gene encodes a 77 kDa protein, which is readily detectable in extracts from a *RAG2*$^{-/-}$ A-MuLV transformant (Figure 2C). The anti-C terminus anti-sera should also detect potential truncated proteins generated by utilizing the second or third ATGs in the *Btk* gene. However, no Btk protein of normal or reduced size was detected in extracts from *Btk*–PH$^-$ A-MuLV transformants (Figure 2C), indicating that the truncated transcript detected in these lines either is not translated from these ATGs or that the resulting proteins are highly unstable. Likewise, no Btk protein was detected by this method in *Btk*–Kin$^-$ A-MuLV transformants, although this does not rule out presence of a truncated protein that lacks the C terminus. Therefore, to investigate whether a truncated protein is synthesized from the *Btk–kin*$^-$ locus, anti-N-terminal antibodies raised against GST–PH/TH fusion proteins were employed in immunoprecipitation experiments with extracts from $^{35}$S-labeled *Btk–kin*$^-$ A-MuLV-transformed cells. However, Btk protein was not detected in the extracts from *Btk–kin*$^-$ cells by this method from the *Btk–kin*$^-$ allele (Figure 2D).

### Flow Cytometric Analysis of Lymphocytes from *Btk*$^-$ and *Xid* Mice

B lineage cells in the BM can be identified by the expression of the B220 surface antigen. Subpopulations representing different stages of early B cell development can be further distinguished on the basis of expression of additional surface markers (Hardy et al., 1991). The normal BM contains B220$^{lo}$/CD43$^+$ pro-B cells, B220$^+$/CD43$^-$ pre-B cells, B220$^+$/IgM$^+$ newly generated B cells, and B220$^{hi}$/IgM$^+$/IgD$^+$ B cells that are mostly recirculating B cells from the periphery (Kantor et al., 1995; Allman et al., 1992, 1993). Flow cytometric analyses for the expression of these markers clearly demonstrated the presence of the pro-B, pre-B, and newly generated surface immunoglobulin-positive B cell subsets in the BM of *Btk*–PH$^-$/*RAG2*$^{-/-}$ and *Btk*–Kin$^-$/*RAG2*$^{-/-}$ chimeric mice. However, there appeared to be a small increase in relative size of the B220$^+$/CD43$^+$ pro-B cell population compared with 129Sv mice,

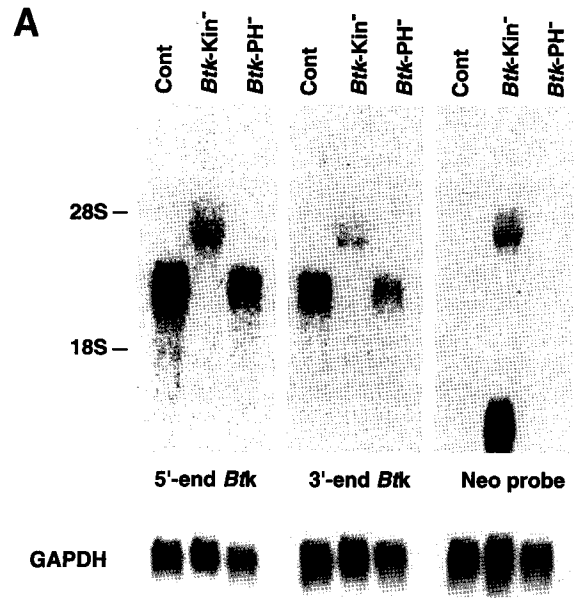
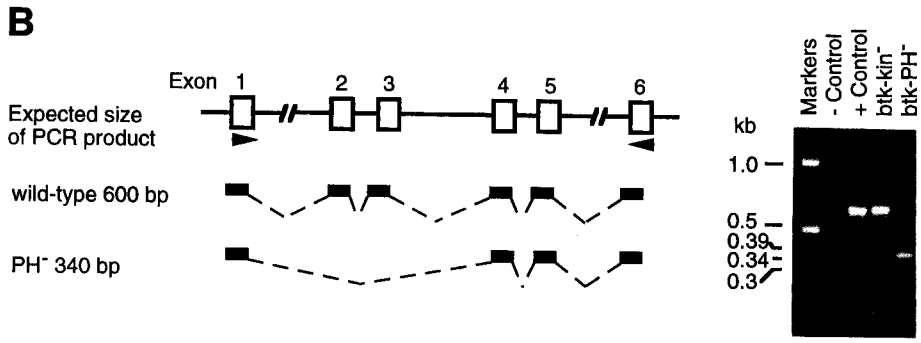


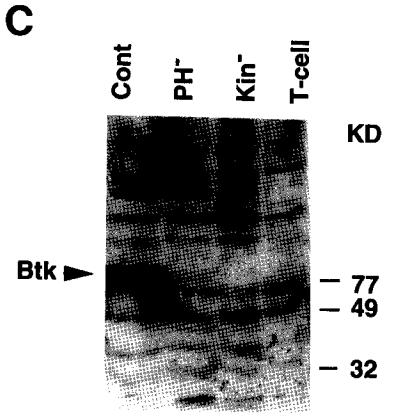

Figure 2. *Btk* mRNA and Protein Analyses from *Btk*–PH⁻ and *Btk–kin*⁻ Targeted Loci

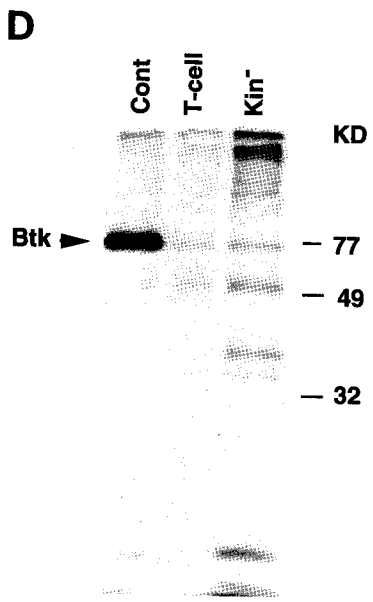
(A) Northern blot analysis of cytoplasmic RNA from *Btk*–PH⁻, *Btk–kin*⁻, and the *RAG2*$^{-/-}$ A-MuLV cell line 63–12 (indicated as control). Total cytoplasmic RNA (10–30 μg) was electrophoresed in formaldehyde agarose gel and transfered to zeta probe membrane and hybridized with a 5′-end 1.4 kb EcoRI *Btk* murine cDNA fragment (top left), a 3′-end 1.5 kb EcoRI *Btk* murine cDNA fragment (top middle), and neomycin resistance gene fragment (Pst–Pst 600 bp) (top right). For comparison of RNA quantities in different lanes, the blots were stripped and rehybrized to a GAPDH probe (bottom).

(B) Structure of the *Btk*–PH⁻ transcript. In *Btk*–PH⁻ B cells, a smaller (340 bp) PCR product was detected due to deletion of exons 2 and 3. RT–PCR was performed on RNA from purified B cells of *Btk*–PH⁻, *Btk–kin*⁻, and normal mice; T cells were used as negative control. Position of primers and expected fragment sizes are depicted in the cartoon (top left). PCR products were electrophoresed in a 1.2% agarose gel and stained with ethidium bromide.

(C) Western blot analysis of *Btk*–PH⁻ A-MuLV cell lines. Lysates from approximately 10 × 10$^6$ cells were electrophoresed in 8% SDS–PAGE and transfered to immobilon membrane and allowed to react with immunoaffinity-purified anti-Btk raised against the C terminus. The antibodies bound to filter were detected by second antibody and chemileuminiscene detection system. Mobility of the molecular weight markers is shown on the right side of the blot.




(D) Immunoprecipitation and SDS–PAGE analysis of *Btk*–Kin⁻ A-MuLV cell line. Lysates from approximately 10 × 10$^6$ [$^{35}$S]methionine/cysteine-labeled cells were subjected to immunoprecipitation with antibodies to the N terminus of Btk, eluates were electrophoresed in 8% SDS–PAGE and exposed to X-ray film for 5 hr. *RAG2*$^{-/-}$ cell line, 63–12, was used as positive control and T cell line, D010, was used as negative control. To detect any small quantities of truncated protein as indicated by weak signal in Northern analysis or due to instability of the truncated protein for *Btk–kin*⁻ cell line, we used extract from 30 × 10$^6$ cells. The bands seen in the *Btk–kin*⁻ A-MuLV cell line were also visible in positive control upon longer exposure and therefore represent nonspecific background. Mobility of the molecular weight markers is shown on the right side of the autoradiogram.

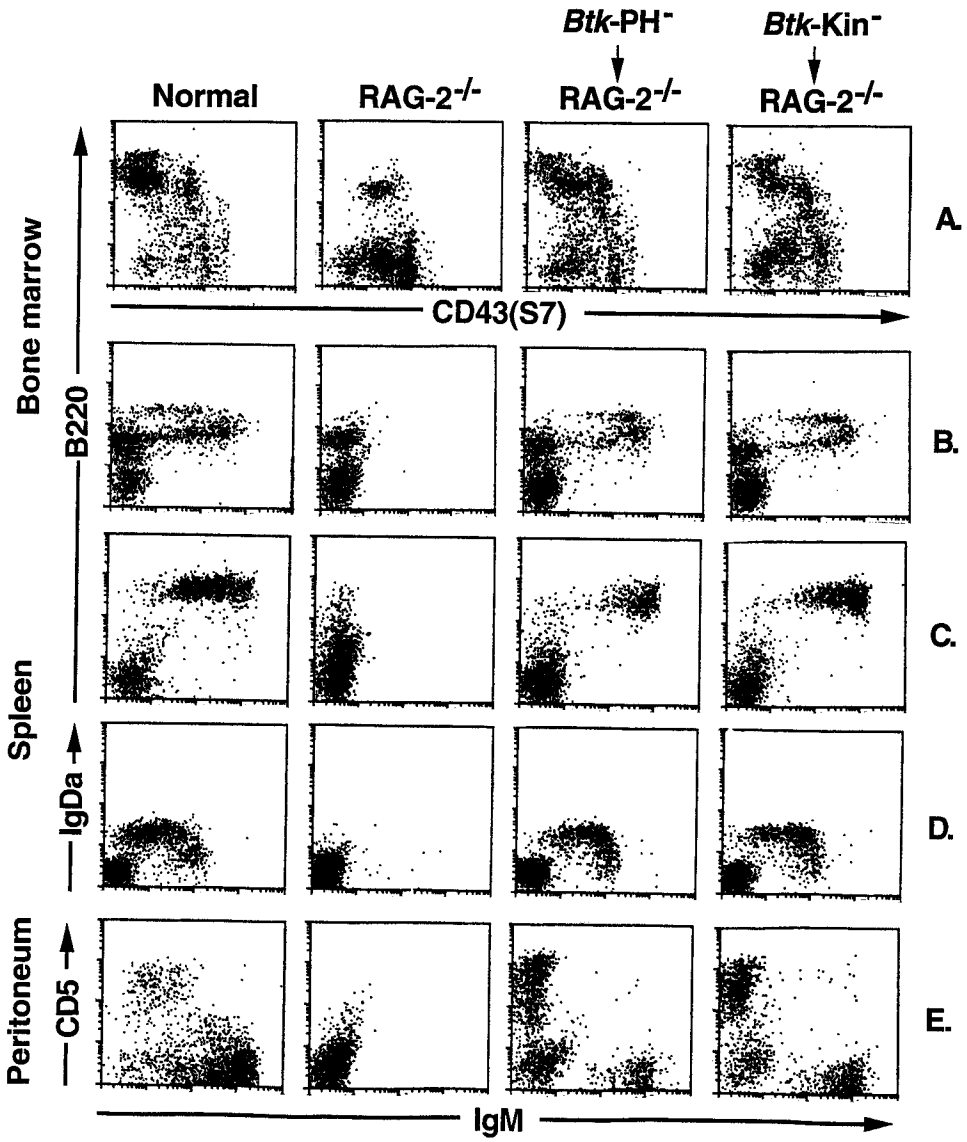



Figure 3. Flow Cytometric Analysis of 8- to 10-week-old *Btk*–PH$^-$/*RAG2*$^{-/-}$ and *Btk*–Kin$^-$/*RAG2*$^{-/-}$ Chimeric Mice, *RAG2*$^{-/-}$, and 129Sv Mice

Dot plots after excluding dead cells (propidium iodide-bright) are displayed.

(A) Early B lineage lymphocytes reperesenting different stages of maturation (Hardy et al., 1991) in the BM were analyzed by double staining with PE–B220 and FITC–CD43.

(B) Mature B lymphocytes in the BM were revealed by staining with PE–IgM and FITC–B220.

(C) B lymphcytes in the spleen were analyzed by double staining with PE–anti-IgM and FITC–anti-B220.

(D) Three populations of B lymphocytes expressing different levels of IgM and IgD expression, IgM$^{lo}$/IgD$^{hi}$ (population 1), IgM$^{hi}$/IgD$^{hi}$ (population 2), and IgM$^{hi}$/IgD$^{lo}$ (population 3) cells in the spleen were revealed by double staining with PE–anti-IgM and FITC–anti-IgD.

(E) IgM$^+$ and CD5$^+$ (B1a) B lymphocytes in peritoneal cavity were analyzed by double staining with PE–anti-CD5 and FITC–anti-IgM. Dot plots of lymphocytes gated by forward scatter and side scatter are displayed.

which were used as normal control (Figure 3A). Since B cell development in the BM of *RAG2*$^{-/-}$ mice is blocked at the B220$^+$/CD43$^+$ pro-B cell stage (Shinkai et al., 1992), the apparent increase in the size of the pro-B population in *Btk*–PH$^-$/*RAG2*$^{-/-}$ and *Btk*–Kin$^-$/*RAG2*$^{-/-}$ chimeric BM is difficult to interpret unequivocally but was further resolved by analyses of germline *Btk–kin*$^-$ mutant mice (see below).

To investigate the effect of *Btk*–PH$^-$ and *Btk–kin*$^-$ mutations on the peripheral B cell compartment, comparative flow cytometric analyses were performed on splenocytes of *Btk*–PH$^-$/*RAG2*$^{-/-}$ and *Btk–kin*$^-$/*RAG2*$^{-/-}$ chimeric mice (Figure 3C). Compared with normal mice, the two *Btk* mutant chimeras exhibited fewer IgM$^+$ B cells (60%–70% of control) as well as a decrease in the IgM$^{lo}$/IgD$^{hi}$ mature B cells (Figure 3D). A substantial number of B cells present in the peritoneal cavity of normal mice are B1 B cells. A subset of B1 cells, B1a cells, can be distinguished from conventional (B2) IgM$^+$ B cells by their expression of CD5

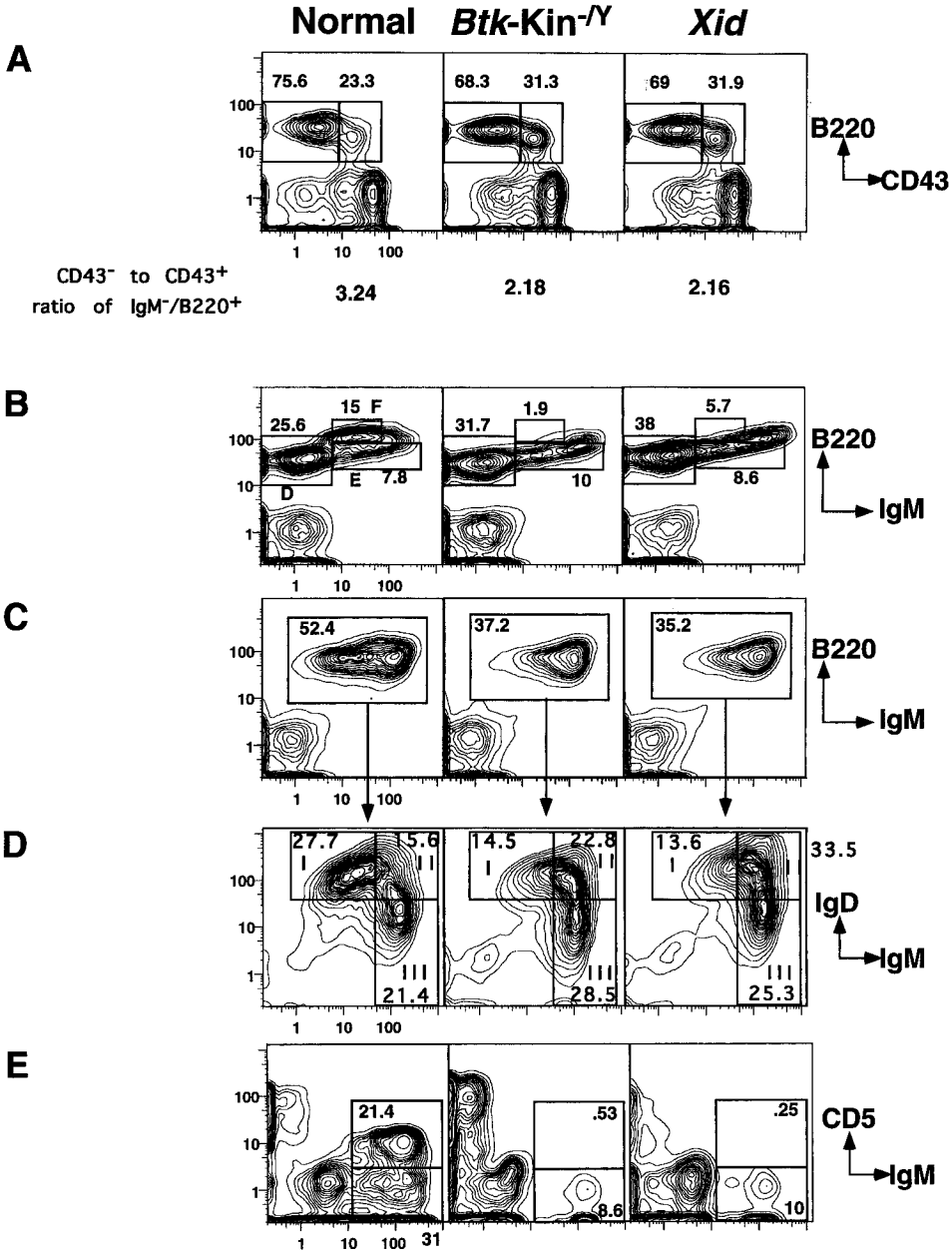



Figure 4. Flow Cytometric Analysis of a Normal Littermate Mouse (*Btk–kin*$^{+/Y}$ Sibling of a *Btk–kin*$^{-/Y}$ Mouse), a *Btk–kin*$^{-/Y}$ Mutant, and a *Xid* (CBA/N X$^{xid}$Y) Mouse Are Compared

The mice shown are representative of the mice examined.

(A) BM cell suspensions from 9- to 12-week-old mice were stained with CD43/S7(PE), B220/6B2(APC), and IgM/331(FITC). IgM$^+$ cells, as well as dead cells (propidium iodide-bright) and high obtuse scatter cells, are excluded by software gating (29%–31% of the total cells collected). The percent of B220$^+$, IgM$^-$ cells that are B220$^+$ and CD43$^+$ and the percent of B220$^+$, IgM$^-$ and CD43$^-$ cells is indicated. The ratio of IgM$^-$, B220$^+$ cells that are CD43$^-$ to IgM$^-$, B220$^+$ CD43$^+$ cells is shown.

(B) BM cell suspensions from 10- to 13-week-old mice were stained with CD43/S7(PE), B220/6B2(APC), and IgM/331(TR-AV). CD43$^-$ cells are displayed (29%–33% of total cells; dead cells and high obtuse scatter were also excluded). The percent of cells displayed that fall within fraction D, fraction E, and fraction F (as described by Hardy et al., 1991) are indicated. Because fractions E, F, (and a small population of B220-bright, IgM-bright cells that are not included in either fraction) are not well resolved, it is likely that (especially for the *Btk–kin*$^-$ and *xid* mice) the percentages indicated for fraction F may be an overestimation of this population.

(C) Spleen cell suspensions from 10- to 13-week-old mice were stained with IgD/11–26(TR-AV), IgM/331(FITC), and B220/6B2(APC) (20%–23% of the cells were excluded as dead or high obtuse scatter). The percent of cells displayed that are B220$^+$ is indicated.

(D) B220$^+$ cells from the above samples (C) are displayed. The percent of cells displayed that are IgD$^+$ and IgM$^{lo}$ (population 1), IgD$^+$ and IgM$^+$ (population 2), or IgD$^{lo}$ and IgM$^+$ (population 3) (Hardy et al., 1982, 1983) is indicated.

(E) Cell suspensions from peritoneal washes from 10-to 13-week-old mice were stained with IgM/331(FITC) and CD5/53–7(APC). After excluding cells by propidum iodide stain and obtuse and forward scatter, 42% of cells from the normal mouse are displayed in this panel, 13.2 % from the knockout, and 10.8% from the *xid*.

(Herzenberg et al., 1986; Herzenberg and Kantor, 1993). Notably, the *Btk*–PH$^-$/*RAG2*$^{-/-}$ and *Btk–kin*$^-$/*RAG2*$^{-/-}$ appeared to lack B1a (CD5$^+$) cells in their peritoneum and have reduced numbers of conventional B cells (Figure 3E).

The *RAG2*-deficient blastocyst complementation method examines only the consequence of lymphocyte-intrinsic defects, as other cell types can be variably contributed by the host blastocyst. As *Btk* is known to be expressed at low levels in other cell types, we considered the possibility that a more marked phenotype, such as that observed in typical XLA cases, might arise as a result of complete ablation of Btk expression in all cell lineages. Therefore, we determined the affects of the *Btk–kin*$^-$ mutation after introduction into the mouse germline. Mutant males and homozygous mutant females had identical phenotypes, which were similar to the aberrations in BM and peripheral B cell populations observed by *RAG2*-deficient blastocyst complementation. These abnormalities included decreased numbers of peripheral splenic B cells, decreased numbers of IgM$^{lo}$/IgD$^{hi}$ splenic B cells, decreased numbers of peritoneal B cells, substantial diminution of B1 cells, and no apparent defects outside of the B lineage (Figure 4; Tables 1, 2, 3; data not shown).

Examination of germline *Btk–kin*$^-$ mutant mice (*Btk–kin*$^-$/GL) allowed us to assess the potential affects of this mutation on early B cell development in the BM in more detail. We clearly observed an increase in the size of the pro-B (B220$^+$/CD43$^+$) cell population in both *Btk–kin*$^-$/GL and *xid* mice as compared with normal mice (Figure 4A; Table 1, legend). The pro-B cell compartment can be subdivided into three subfractions (A, B, and C), representing increasing levels of maturation (Hardy et al., 1991). Analysis of the relative proportions of these fractions in the pro-B compartment of *Btk–kin*$^-$/GL mice revealed no obvious differences from the distribution observed in normal animals (data not shown). Finally, as observed with both types of *RAG2*-deficient chimeras, both *Btk–kin*$^-$/GL and CBA/N mice showed a markedly reduced population of B220$^{hi}$/IgM$^+$/IgD$^+$ recirculating mature B cells (fraction F) (see Figure 3B; Figure 4B; Tables 1, 2, 3; data not shown).

The B cell populations in the periphery of *Btk*–PH$^-$/*RAG2*$^{-/-}$ and *Btk–kin*$^-$/*RAG2*$^{-/-}$ and *Btk–kin*$^-$/GL mice appeared to be very similar to that previously described for CBA/N (*Xid*) mice (Scher et al., 1975; Hardy et al., 1982, 1983; Hayakawa et al., 1986; Figure 4; Tables 1, 2, 3). In the BM, *Btk*-deficient mice, like *Xid* mice, have, at most, a modest early developmental arrest in B cell development.

### *Btk*-Deficient Mice Have Reduced Levels of Serum Immunoglobulins

Serum levels of all immunoglobulin isotypes were determined by enzyme-linked immunosorbent assay (ELISA) in *Btk*–PH$^-$/*RAG2*$^{-/-}$ and *Btk–kin*$^-$ germline mutants and compared with those of normal littermates, CBA.*Xid*/HHW (Wortis et al., 1982) and CBA/CaHN-*Xid*/J (Jackson Laboratories) mice ranging in age from 8 weeks to 6 months in age. All of the mutant mice had severely decreased levels of IgM and IgG3 (Figure 5); in addition, the *Btk–kin*$^-$/GL, *Btk*–PH$^-$/*RAG2*$^{-/-}$ chimeric and the *Xid* mice had

Table 1. Frequencies of Lymphocyte Populations in *Btk–kin*$^{-/Y}$, CBA/N.*xid*, and C57BL/6.*xid*

| Bone Marrow | | | Total B lineage Cells (B220$^+$) | | Pro-B cells (fractions A, B, C)[b] | | Pre-B cells (fraction D)[a] | | IgM$^+$B220$^{int}$ (fraction E)[c] | | IgM$^+$B220$^{hi}$ (fraction F)[c] | | Total B cells (IgM$^+$) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Type of Mouse | Number of mice | Recovered cells in millions | Percent | Number ($10^6$) | Percent | Number ($10^6$) | Percent | Number ($10^6$) | Percent | Number ($10^6$) | Percent | Number ($10^6$) | Percent | Number ($10^6$) |
| Wild type | 16 | 80 ± 18 | 24 ± 6 | 19 ± 6.3 | 2.7 ± 1.1 | 2.1 ± 1.1 | 10 ± 3.3 | 8.2 ± 2.9 | 5.4 ± 2.9 | 4.4 ± 3.4 | 3.3 ± 1.8 | 2.7 ± 1.9 | 11 ± 4.3 | 8.6 ± 5 |
| *Btk* knockout | 8 | 74 ± 20 | 20 ± 6 | 14 ± 4.6 | 6.3 ± 1.7 | 4.3 ± 1.4 | 11 ± 2.6 | 7.5 ± 1.5 | 3.7 ± 1.7 | 2.6 ± 1.3 | 0.5 ± 0.2 | 0.4 ± 0.2 | 6 ± 2.1 | 4.4 ± 1.5 |
| *xid* | 5 | 66 ± 28 | 30 ± 3 | 20 ± 9.4 | 5.3 ± 1.4 | 3.6 ± 2.1 | 14 ± 2.7 | 9.6 ± 5.5 | 4.7 ± 1.5 | 3 ± 1.6 | 1.0 ± 0.7 | 0.7 ± 0.6 | 9 ± 0.7 | 5.7 ± 2.3 |
| B6.*xid* | 7 | 114 ± 37 | 14 ± 3 | 16 ± 5.3 | 1.2 ± 0.6 | 1.2 ± 0.3 | 8 ± 1.8 | 9.1 ± 3.3 | 3.2 ± 1.1 | 3.4 ± 1.3 | 0.5 ± 0.1 | 0.6 ± 0.4 | 4.6 ± 0.1 | 5.1 ± 1.5 |

The phenotype of lymphocyte populations was determined by flow cytometry as described in the legend to Figure 4 and in Experimental Procedures. The percentages of the total cells collected that had the indicated phenotype were determined for each mouse, and the mean value and standard deviation were calculated for each group. The absolute number of live cells recovered from each tissue was determined by counting, using acridine orange and ethidium bromide to exclude dead cells. Using a Kruskal–Wallis rank sum test to compare the frequencies of pro-B cells (CD43$^+$B220$^+$), both *Btk–kin*$^{-/Y}$ mutant and *Xid* (CBA/N X$^{xid}$Y) mice had a significant increase ($p < .02$) as compared with normal littermates (i.e., percent of pro-B cells/total cells for *Btk–kin*$^{-/Y}$ mice = 7.5, 4.2, 8.8, 7.3, 4.4, 6.6, and 5.2 [$p = 0.0018$]; for *Xid*(CBA/N X$^{xid}$Y) mice = 3, 6.7, 5.7, 5.9, 5.3 [$p = 0.019$]; for normal littermates (*Btk–kin*$^{+/Y}$) = 4.2, 2.7, 3.8, 4.4, 2, 3.8, 2.5, and 2.4). Within the wild-type group, eight were *Btk–kin*$^{+/Y}$ siblings of a *Btk–kin*$^{+/Y}$ mouse, and the rest were inbred mice (BALB/c, C57BL/6, CBA, or BAB.25). There were no significant differences between the different types of wild-type mice.

[a] Wild-type mice (9) and three *Btk–kin*$^{-/Y}$ mice were analyzed.

[b] Wild-type mice (15) and seven *Btk–kin*$^{-/Y}$ mice were analyzed.

[c] Wild-type mice (15).

Table 2. Frequencies of Lymphocyte Populations in *Btk–kin*$^-$ Mice, CBA/N.*xid*, and C57BL/6.*xid*

| Spleen | | | Total B Cells (B220$^+$) | | Total IgM$^+$ cells | | CD4$^+$ T cells[a] | | CD8$^+$ T cells[a] | |
|---|---|---|---|---|---|---|---|---|---|---|
| Type of mouse | Number of mice | Recovered cells in millions | Percent | Number ($10^6$) | Percent | Number ($10^6$) | Percent | Number ($10^6$) | Percent | Number ($10^6$) |
| Wild type | 17 | 87 ± 34 | 43 ± 11 | 37 ± 17 | 37 ± 10 | 32 ± 15 | 18 ± 3.3 | 18 ± 5.6 | 12 ± 1.7 | 11 ± 3.4 |
| *Btk* knockout | 8 | 59 ± 27 | 35 ± 10 | 22 ± 16 | 29 ± 4.7 | 18 ± 8.6 | 19 ± 1.8 | 14 ± 5.7 | 13 ± 2.6 | 9.2 ± 3.4 |
| *xid* | 9 | 34 ± 13 | 33 ± 9.0 | 11 ± 4 | 31 ± 8.6 | 10 ± 3.7 | 24 ± 2.2 | 8.4 ± 3.2 | 14 ± 2.8 | 4.6 ± 1.2 |
| B6.*xid* | 7 | 96 ± 29 | 43 ± 14 | 41 ± 19 | 46 ± 3.7 | 44 ± 13 | 15 ± 1.6 | 14 ± 5.3 | 9.3 ± 1.6 | 8.7 ± 2.6 |

See legend to Table 1.
[a] Wild-type mice (11) and seven *Btk–kin*$^{-/Y}$

relatively decreased, but variable levels of IgG1, IgG2a, IgG2b, and IgA isotypes (Figure 5). Notably, the deficiency of all isotypes was even more pronounced in the *Xid* than in either of the two *Btk* mutant animals generated by gene targeting. However, the severity of immunoglobulin deficiency also was found to be variable with respect to the two different genetic backgrounds on which the *Xid* mutation was analyzed (Figure 5).

### Defective Proliferation of *Btk*-Deficient B Cells

To assess the capacity of *Btk* mutant B cells to respond to mitogenic signals, purified splenic *Btk*–PH$^-$/*RAG2*$^{-/-}$, *Btk*–Kin$^-$/*RAG2*$^{-/-}$, *Xid*, and normal B cells (generated by injecting normal ES cell into *RAG2*$^{-/-}$ blastocysts) were treated with anti-IgM and different concentrations of LPS. Flow cytometric analyses, after 12 hr of anti-IgM or LPS treatment, demonstrated the induction of the expression of early activation markers including CD69 and IL-2Rα on both normal and mutant B cells (data not shown). Measurement of proliferation by quantitating [$^3$H]thymidine incorporation after 60 hr of anti-IgM treatment indicated that *Btk*–PH$^-$, *Btk–kin*$^-$, and *Xid* B cells did not proliferate compared with normal B cells (Table 4). Similarly, the proliferative response of *Btk*–PH$^-$, *Btk–kin*$^-$ B cells to LPS treatment was comparable to that of *Xid* and was significantly lower than that of normal B cells, particularly at lower concentrations (Figure 6). In contrast, phorbol ester and ionomycin stimulation of *Btk*–PH$^-$ B cells resulted in proliferative responses comparable to those of normal B cells (Table 4).

In a separate set of experiments, purified splenic B cells from *Btk–kin*$^-$/GL mice also were compared with those of normal littermates or CBA/CaHN (*Xid*, Jackson) mice for responsiveness to anti-IgD–dextran treatment or anti-CD40 treatment with or without added IL-4. Anti-IgD–dextran has been shown to be a stronger stimulator of B cells than soluble anti-IgD or anti-IgM (Mond et al., 1983; Mandler et al., 1993), while IL-4 enhances the survival of B cells and induces proliferation when used as costimulant with B cell receptor or CD40 stimulation (Howard et al., 1982; Gold and Defranco, 1994; reviewed by Noelle and Show, 1992). Responses to anti-IgD–dextran and CD40 ligation were diminished in *Btk–kin*$^-$/GL compared with control B cells and were similar to those of *Xid* B cells; however, the stimulatory effect observed in the mutant cells was enhanced by IL-4. Our finding with the *Xid* cells are in accord with those of a previous report that *Xid* B cells show some proliferation in response to sepharose-conjugated anti-immunoglobulin antibodies (Mond et al., 1983) and defective response to anti-CD40 antibodies (Hasbold and Klaus, 1994).

### Defective Humoral Immune Responses in *Btk*-Deficient Mice

To evaluate TI-II responsiveness, *Btk*-PH$^-$/*RAG2*$^{-/-}$, normal ES cell–derived *RAG2*$^{-/-}$ chimeric mice and CBA/N mice were immunized with the TI-II antigen trinitrophenol (TNP)–Ficoll, and TNP-specific serum antibody titers were measured by ELISA. The results showed that *Btk*–PH$^-$/*RAG2*$^{-/-}$ chimeric mice and *Xid* mice (as previously described: Scher et al., 1975; reviewed by Scher, 1982) did not respond to this TI-II-dependent antigen (Figure 7A). In another set of experiments, germline *Btk–kin*$^-$ mutant and CBA/N mice were also found to be unresponsive to immunization with TNP–Ficoll (Figure 7B).

To investigate TD responses in *Btk*–PH$^-$/*RAG2*$^{-/-}$, *Btk*–Kin$^-$/*RAG2*$^{-/-}$ chimeric mice, *Btk–kin*$^-$/GL, *Xid* mice and normal control mice were challenged with optimal doses of the TD antigen TNP–keyhole limpet hemocyanin (KLH) and primary and secondary antibody responses to TNP and KLH were measured by ELISA. The primary antibody responses to both TNP and KLH were poor in all the *Btk*-defective animals for all tested isotypes (data not shown). On the other hand, the secondary TNP-specific responses were relatively normal with respect to levels of total antigen-specific immunoglobulin, IgG1, IgG2a, and IgG2b, although antigen-specific IgM and IgG3 levels were still significantly reduced (Figure 8). Carrier (KLH)-specific total immunoglobulin, IgG1, IgG2a, and IgG2b levels were also comparable between mutant and normal animals (data not shown). The TNP–KLH responses observed in the *Btk*-deficient mice are consistent with those previously reported for *Xid* mice (Figure 8; Scher et al., 1979; Boswell et al., 1980).

## Discussion

### Effects of *Btk* Null Mutations in Mice

To test directly whether Btk deficiency results in an XLA- or *Xid*-like phenotype and to elucidate the role of Btk in B cell development and function, we have introduced two different mutations into the *Btk* gene in murine ES cells and assayed their effects on general development, lymphocyte

Table 3. Frequencies of Lymphocyte Populations in *Btk-kin*⁻ Mice, CBA/N.*xid*, and C57BL/6.*xid*

| Peritoneum | | | Total B lineage cells ($IgM^+$) | | $IgD^+IgM^+$ B cells | | $IgD^{lo}IgM^+$ B cells | | B1a cells ($CD5^+$ $IgM^+$) | | T cells | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Type of mouse | Number of mice | Recovered cells in millions | Percent | Number ($10^3$) | Percent | Number ($10^3$) | Percent | Number ($10^3$) | Percent | Number ($10^3$) | Percent | Number ($10^3$) |
| Wild type | 17 | 5 ± 1.2 | 25 ± 15 | 1330 ± 1000 | 10 ± 5.8 | 510 ± 330 | 15 ± 11 | 820 ± 790 | 12 ± 11 | 650 ± 750 | 5.2 ± 6.2 | 260 ± 350 |
| *Btk* knockout | 9 | 4 ± 2.6 | 2.6 ± 1 | 100 ± 100 | 2.2 ± 1 | 88 ± 93 | 0.4 ± 0.2 | 16 ± 13 | 0.2 ± 0.1 | 6.6 ± 3.8 | 9.6 ± 8.2 | 270 ± 200 |
| *xid* | 9 | 3 ± 1 | 1.5 ± 0.6 | 44 ± 14 | 1.4 ± 0.5 | 39 ± 14 | 0.2 ± 0.1 | 4.7 ± 2.6 | 0.07 ± 0.04 | 2.2 ± 1.2 | 1.1 ± 0.4 | 32 ± 15 |
| B6.*xid* | 7 | 4.5 ± 1.6 | 11 ± 6 | 520 ± 330 | 9.4 ± 6 | 460 ± 330 | 1.6 ± 1.6 | 57 ± 39 | 2.2 ± 3.3 | 86 ± 110 | 16 ± 5.5 | 780 ± 49 |

See legend to Table 1.

development, and B cell function. Both mutations were designed to eliminate specific functional domains of Btk (the PH and kinase domains); however, both appeared to result in the general absence of expression of any detectable immunoreactive Btk protein and, thus, apparently represented null mutations. Our analyses of these introduced *Btk* mutations clearly establish a role for this tyrosine kinase in B cell development and function. However, the studies also demonstrate that complete elimination of Btk function did not result in the severe early B lymphocyte developmental arrest attributed to *Btk* mutations in human XLA. Rather, *Btk*-deficient mice had a murine *Xid*-like phenotype that manifests mainly as defects in peripheral B cell responses and populations. In addition, the phenotype of mice deficient for *Btk* in their germline appeared identical to that of chimeras generated from *Btk*⁻ ES cells via *RAG2*-deficient blastocyst complementation; this shows that *Btk* has no obvious role in cells other than those of the B lineage.

### Signaling Defects in B Lineage Cells of Btk-Deficient Mice

Pre-B cells express a surface molecule that is composed of the Igμ heavy chains plus the λ5 (Sakaguchi and Melchers, 1986) and $V_{pre\text{-}B}$ (Kudo and Melchers, 1987) surrogate light chains (Karasuyama et al., 1993; Tsubata and Reth, 1990). This complex is thought to generate signals that are involved in the pro-B to pre-B cell transition (Takemori et al., 1990). Correspondingly, inactivation of the λ5 gene leads to a developmental block at the pre-B cell stage, which resembles that observed in XLA patients (Kitamura et al., 1992). In this context, Btk was found to be constitutively phosphorylated in murine pre-B cells (Aoki et al., 1994), leading to the suggestion that it may have a role in signaling from the pre-B cell receptor (Aoki et al., 1994). Our current results show that Btk is not essential for generating the signals necessary for the various transitions observed in early murine B cell development, as we do not see a major early B cell developmental block in *Btk* deficient animals. In fact, the most obvious difference between the BM B cell profiles of *Btk*-deficient and normal mice is the lack of recirculating mature B cells, which most likely reflect B cells selected in the periphery (Kantor et al., 1995; Allman et al., 1992, 1993). However, as we do see a small, but apparently significant, increase in the size of the pro-B cell population in *Btk*-deficient animals, it remains possible that Btk does play some role in generating signals involved in the transition from the pro-B to pre-B cell.

The mature B cell receptor (BCR) complex is composed of two immunoglobulin heavy chains (μ or δ) and two immunoglobulin light chains plus the invariant Igα and Igβ chains that are involved in signal transduction (Campbell and Sefton, 1992; Burkhardt et al., 1991; Venkitaraman et al., 1991). Cross-linking of IgM on normal splenic B cells induces early activation markers (e.g., CD69 and IL-2Rα), followed by induction of DNA synthesis and proliferation (Testi et al., 1989; reviewed by Pleiman et al., 1994; Weiss and Littman, 1994; Janeway and Bottomly, 1994). Cross-linking of *Btk*-deficient B cells did not induce DNA synthe-

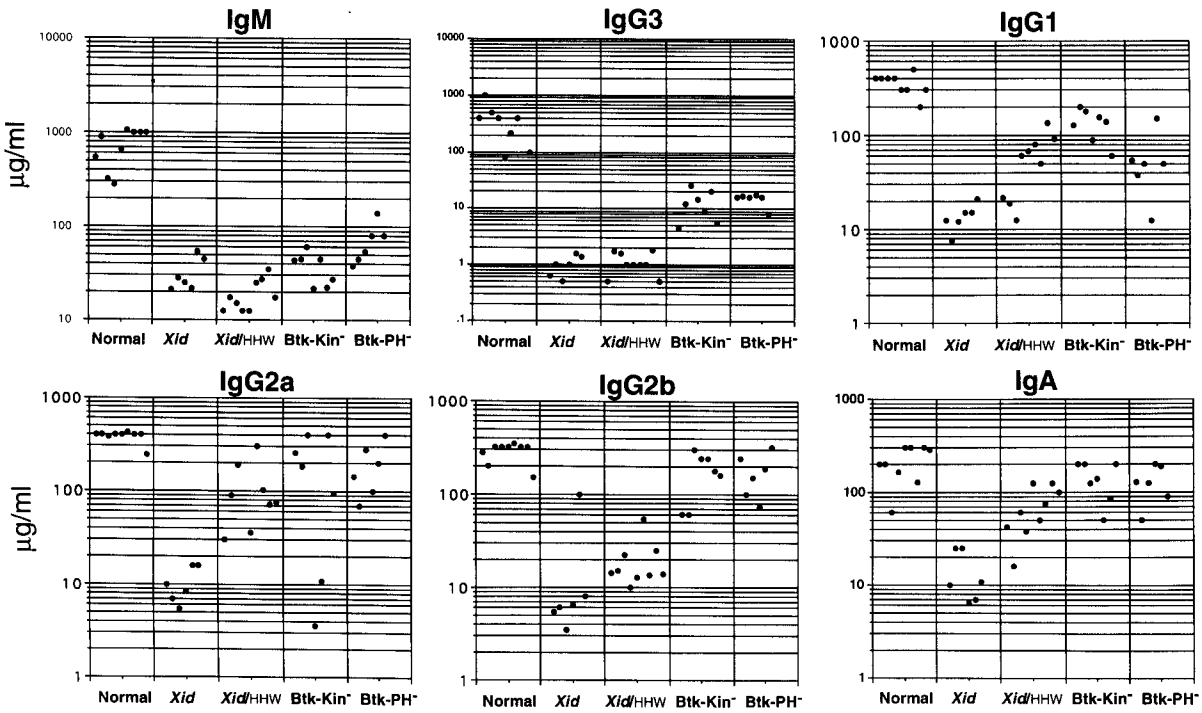



Figure 5. Decreased Serum Immunoglobulin Levels in *Btk*-Deficient Mice

Serum immunoglobulin levels in normal littermates (*Btk–kin*$^{+/Y}$), *Xid*; (CBA/CaHN.*Xid*/J, Jackson Laboratories) Xid/HHW; (CBA.*Xid*/HHW, Wortis et al., 1982); and *Btk*–PH$^{-}$/*RAG2*$^{-/-}$ somatic chimeras were screened by ELISA for immunoglobulin isotypes.

sis or proliferation, but still induced early activation marker expression. Therefore, some IgM-triggered events appear to require Btk as a downstream effector, while others do not. *Btk*-deficient cells also had a greatly diminished proliferative response to anti-IgD–dextran stimulation compared with normal cells, implicating Btk in signaling through the IgD BCR as well. However, some proliferation was observed in response to this treatment. Conceivably, the prolonged and extensive surface immunoglobulin cross-linking generated by immobilized anti-immunoglobulin, which leads to activation of tyrosine kinases by *trans*-phosphorylation and recruitment of effector molecules (reviewed by Pleiman et al., 1994), can stimulate B cell proliferation through a non-Btk-dependent pathway. Btk phosphorylation and activation have been shown to occur following BCR cross-linking but with delayed kinetics relative to that of several BCR-associated nonreceptor PTKs (Aoki et al., 1994; Saouf et al., 1994; reviewed by Pleiman et al., 1994). As several of the latter PTKs (e.g., Fyn, Lyn, and Hck) specifically interact with Btk, it has been suggested that Btk may function downstream from them in BCR signaling (Cheng et al., 1994). In this context, it will be of interest to determine the relative dependence of Btk-dependent and independent signaling events on the activities of the putative upstream PTKs.

*Btk*$^{-}$ B cells showed deficiencies in additional signaling pathways. Thus, treatment of Btk$^{-}$ B cells with anti-CD40 antibodies did not lead to the strong proliferative response observed in normal cells. Furthermore, *Btk*$^{-}$ cells did not respond well to LPS treatment, exhibiting significant proliferation only at high doses. Significantly, LPS-induced signaling has been shown to differ from that of the BCR in that it involves a protein kinase C(PKC)–independent pathway (reviewed by Klaus, 1988; Sigal and Dumont, 1992). Expression of early activation markers was observed in response to both of these signaling pathways. Thus, *Btk* is not essential in early activation of B cells as induced by signaling through either the BCR or by polyclonal mitogens, but is required for proliferative responses in the context of all of these pathways. As phorbol ester and ionomycin stimulation of *Btk*$^{-}$ B cells resulted in normal proliferative responses, it further seems that Btk functions upstream of PKC activation and calcium mobilization in the affected pathways. Finally, defective signaling in *Btk*$^{-}$ mice was also manifested by failure to mount a response to a classical TI-II antigen and a reduced primary response, but relatively unaffected secondary response, to a classical TD antigen.

A potential signaling defect in *Btk*$^{-}$ mice is also suggested by the marked reduction of CD5$^{+}$ (B1a) cells. Absence of CD5$^{+}$ B cells was also noted in *vav*$^{-/-}$ mice (Zhang et al., 1995; Tarakhovsky et al., 1995). Notably, the *vav*$^{-/-}$ mice also have defective signaling through their BCR but, unlike *Btk*$^{-}$ mice, appear to respond relatively normally through the LPS and CD40 pathways. The differences in signaling responses in *Btk*- and *vav*-deficient B cells suggests a required role for Btk that is broader than *vav* with respect to B cell signaling pathways. However, the com-

Table 4. Splenic B Cell Proliferation to Anti-μ, Anti-δ, Anit-CD40 with or without IL-4

| Experiments | Mouse | RPMI | IL-4 | Anit-μ | Anti-δ-dex | Anti-CD40 | Phorbol ester + Ion + IL-4 | Anti-μ + IL-4 | Anti-δ-dex + IL-4 | Anti-CD40 + IL-4 |
|---|---|---|---|---|---|---|---|---|---|---|
| Experiment 1[a] | B6/CBA | 2.1 ± 0.3 | – | 34.2 ± 4.0 | – | – | 110.1 ± 8.7 | – | – | – |
| | *Btk*–PH⁻ | 1.8 ± 0.4 | – | 3.2 ± 0.7 | – | – | 120.6 ± 10.9 | – | – | – |
| | CBA/N | 1.6 ± 0.3 | – | 4.0 ± 0.9 | – | – | – | – | – | – |
| Experiment 2 | 129Sv | 6.2 ± 0.2 | – | 58.4 ± 11.1 | – | – | – | – | – | – |
| | *Btk*–PH⁻ | 2.0 ± 0.7 | – | 3.1 ± 0.2 | – | – | – | – | – | – |
| | *Btk–kin*⁻ | 1.7 ± 1.3 | – | 0.9 ± 0.4 | – | – | – | – | – | – |
| | CBA/N | 0.9 ± 0.29 | – | 2.8 ± 0.2 | – | – | – | – | – | – |
| Experiment 3[b] | Normal | 1.6 ± 0.6 | – | 22.8 ± 2.3 | 265.8 ± 2.2 | 197.8 ± 26.7 | – | 39.8 ± 10.4 | 287.9 ± 11.8 | 196.3 ± 8.7 |
| | *Btk–kin*⁻ | 0.9 ± 0.3 | – | 0.4 ± 0.04 | 4.6 ± 0.759 | 3.5 ± 1.3 | – | 2.1 ± 0.8 | 14.6 ± 0.7 | 20.3 ± 3.5 |
| | CBA/N | 1.2 ± 0.1 | – | 1.8 ± 0.1 | 26.9 ± 0.9 | 13.8 ± 1.3 | – | 4.4 ± 0.4 | 36.0 ± 1.1 | 53.9 ± 7.3 |
| Experiment 4[c] | Normal | 6.0 ± 0.1 | 7.3 ± 3.2 | 26.1 ± 2.7 | 316.3 ± 50.7 | 161.7 ± 16.7 | 39.4 ± 2.3 | 59.0 ± 8.6 | 297.8 ± 38.2 | 240.2 ± 8.7 |
| | *Btk–Kin*⁻ | 0.7 ± 0.3 | 3.0 ± 0.3 | 1.1 ± 0.3 | 0.850 ± 0.3 | 9.0 ± 0.8 | 13.2 ± 0.8 | 3.7 ± 1.7 | 7.7 ± 0.4 | 169.0 ± 4.6 |
| | CBA/N | 0.6 ± 0.1 | 3.5 ± 0.1 | 2.4 ± 0.5 | 26.7 ± 2.6 | 26.0 ± 2.5 | 27.9 ± 3.1 | 7.6 ± 0.3 | 63.1 ± 4.3 | 188.1 ± 10.6 |

Purified splenic B cells from mice as indicated were cultured for 48 or 60 hr with various stimuli and proliferation was measured as incorporation of [³H]thymidine under an incubation period of 8 hr. Data are presented as mean cpm × $10^{-3}$ (± SEM) triplicate samples.

[a] In this experiment, phorobol 12,13-dibutyrate (PDBu) was used.

[b] In this experiment, 2.0 μg/ml anti-CD40 was used.

[c] In this experiment, phorbol 12-myristate 13-acetate (PMA) was used, and the concentration of anti-CD40 was 10.0 μg/ml.

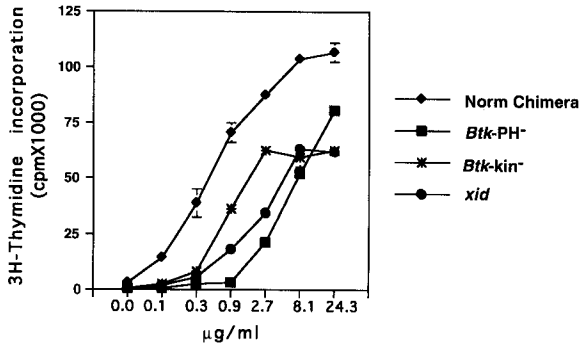



Figure 6. Defective Proliferation of *Btk*–PH⁻ and *Btk–kin*⁻ or CBA/N B Cells in Response to the Mitogenic Activity of LPS

Purified B lymphocytes were cultured in media alone or in the presence of different concentrations of LPS. Proliferation was determined at 48 hr by [³H]thymidine incorporation for 8 hr. Data are ploted as mean ± SD.

mon defect in BCR signaling and reduction of CD5⁺ cells is also consistent with the possibility that signaling through the BCR or a closely linked pathway may be important for the generation of these cells (Ying-zi et al., 1991; Haughton et al., 1993). It is also notable that *Btk*-deficient mice have low serum IgM levels, whereas *vav*$^{-/-}$ mice have normal serum IgM levels (Zhang et al., 1995; Tarakhovsky et al., 1995), indicating that this parameter does not directly relate to presence or absence of CD5⁺ cells.

## Comparison of the *Btk*-Deficient versus *Xid* and XLA Phenotypes

In the human disease XLA, B cell development is affected at several developmental stages. There is an increase in pro-B cells and a decrease in pre-B cells (Campana et al., 1990) and there is often a major block in maturation from the pre-B cell stage resulting in a severe deficiency of mature B cells. B cells that do appear in the periphery mostly have an immature phenotype and are incapable

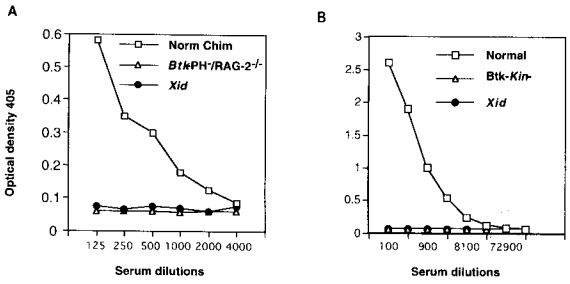



Figure 7. Lack of Serum Antibodies Response to TI-II Antigens in *Btk*-Deficient Mice

(A) No serum antibody response to TI-II antigen TNP–Ficoll immunization in *Btk*–PH⁻/*RAG2*$^{-/-}$ somatic chimeras and CBA/N mice. Normal ES cell/*RAG2*$^{-/-}$ somatic chimeras were used as control.

(B) No serum antibody response to TI-II antigen TNP–Ficoll immunization in *Btk–kin*⁻/GL and CBA/N mice. Normal littermates were used as control. Serial dilutions of serum were analyzed for TNP-specific total immunoglobulin by ELISA. Results are expressed as OD405 of anti-κ-specific ELISA using TNP–Ficoll as capture reagent.

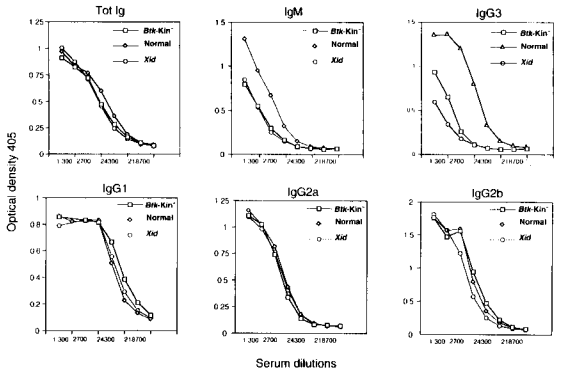



Figure 8. Secondary Antibody Response to TD Antigen TNP–KLH Immunization in *Btk–kin⁻*/GL, Normal Littermates, and CBA/N Mice

Serial dilutions of serum were analyzed for TNP-specific immunoglobulin isotypes by ELISA. Results are expressed as OD405 of anti-isotype-specific ELISA using TNP–bovine serum albumin as capture reagent. A representative experiment is shown; the same results were obtained with three mice in each group.

of responding to TI-II antigens, although some cells do mature to antibody-secreting plasma cells (reviewed by Conley, 1985). The murine *Xid* defect, on the other hand, does not have a severe early impairment in B cell development but does have similar defects in peripheral B cell activation and function as observed in XLA. In all aspects, the phenotype of *Btk⁻* mice is essentially identical to that of *Xid* mice, unequivocally proving that lack of Btk function can result in an *Xid* phenotype. This is striking because *Btk⁻* mice do not express any detectable *Btk* gene products, while *Xid* mice express *Btk* mRNA and intact protein kinase activity (Rawlings et al., 1993). Therefore, the *Btk* point mutation attributed to the *Xid* defect apparently generates the same loss of function phenotype as complete ablation of Btk protein synthesis. In this context, it is possible that the *Xid* mutation disrupts a functional domain(s) required for all *Btk* function or, conversely, leads to the generation of a protein with a dominant-negative activity.

The *XLA* phenotype was suggested to result from mutations of the Btk protein, which eliminated all function or at least functions required for early B cell development (Thomas et al., 1993; Rawlings et al., 1993). However, this possibility was challenged when the same amino acid mutated in murine *Xid* was found mutated in a man ($Arg28^{His}$) (de Weers et al., 1994), but in association with the typical XLA phenotype. This finding led to the notion of a less stringent requirement for *Btk* for early murine B cell development than that of humans. However, even in humans, subjects carrying the same *Btk* mutation may present a variable phenotype, and the characterization of XLA the phenotype tends to be biased by those who show severe clinical symptoms. Another case of such variability was recently reported in case of IL-2R(γ) chain knockout and human XSCID (Cao et al., 1995). These observations raise the possibility that other genetic factors, not linked to the *Btk* gene, may affect the phenotypic severity of the mutation. Indeed, we note that the *Xid* mutation confers a notably less severe B cell defect when bred into the genetic background of C57BL/6 mice (Tables 1, 2, 3). Quite conceivably, genetic backgrounds might be found that further enhance the severity of the murine mutations or reduce the severity of some human mutations. However, our findings do not rule out the additional possibility that some mutations of Btk may result in dominant-negative proteins, which could cause a more severe phenotype than complete lack of Btk protein (Conley et al., 1994).

In any case, it appears that Btk function is accompanied in mice by a compensatory mechanism that operates during early B cell development to rescue B cell maturation. One possible mechanism is a PTK that substitutes for Btk activity. Another is the specific spatio–temporal expression of a cytokine or its receptor (i.e., a factor produced by murine, but not human, stromal cells or T cells) that can mediate the transition of precusors to mature B cells in mice, obviating a stringent requirement for Btk. In this context, when the *xid* and *nude* (a mutant mouse strain with a severly deficient thymus) defects are expressed together, B cell development is blocked at the pro-B stage (Karagogeos et al., 1986). This phenotype resembles the block in B cell development of human XLA. The nude defect is not mediated at the level of stromal cells (Dong and Wortis, 1994). In fact, *Xid* BM cells do not repopulate the periphery of thymectomized mice unless T cells are provided (Sprent and Bruce, 1984). The gene responsible for the nude defect, designated *whn* (Nehls et al., 1994), encodes a member of the winged-helix domain family of transcription factors and is specifically expressed in thymus and in skin. Together, these findings suggest that T cells or T cell–derived factor(s) play a role in facilitating the transition of pro-B cells to pre-B cells.

## Future Prospects

*Btk⁻* mice should enable studies of signal transduction in the absence of Btk. To elucidate the function of different potential functional domains of Btk in vivo, complementation studies with mutated *Btk* transgenes can be performed. Crossing Btk⁻ mice with knockout mice deficient for other candidate PTKs (e.g., Itk and Tec) or for particular cytokines or their receptors may provide insights into the molecular basis of potential compensatory mechanisms. Such mice ultimately may prove useful as a gene therapy model for XLA because they lack Btk as opposed to *Xid* mice, where presence of a mutated protein might complicate interpretation of results perhaps by acting as a dominant negative.

### Experimental Procedures

#### Construction of Btk Targeting Vectors

There are four ATG in-frame translation initiation sites located in exon 2, 4, 6, and 7 of the *Btk* gene. In vitro translation has shown that all these methionines can be used as start sites. To inactivate the PH domain specifically, exons 2 and 3 were replaced with the *neo^r* gene. If, in vivo, only the first initiation codon is used, this mutation will result in complete inactivation of *Btk*. To construct the *Btk*–PH domain targeting vector, a *Btk* genomic clone containing 15.0 kb DNA fragment containing the 5′ end of the *Btk* gene was isolated from a 129Sv DNA library using a genomic fragment, as probe, isolated by PCR amplification by Atk-1 and Atk-2 primers as described earlier (Sideras et al., 1995). This clone containing most of the PH domain was used to make

the targeting construct to replace coding exon 2 and 3 with pGK–*neo*. A thymidine kinase (pGK–*tk*) gene was subcloned into SalI–XhoI sites of a modified SKII pBluescript from which HindIII site of the polylinker was destroyed (tkpBS). A 5.5 kb NotI–BamHI fragment providing the 5′ flank of the construct, was subcloned into NotI–BamHI of this tkpBS. A 4.15 kb BamHI–EcoRI fragment, containing the 3′ flank of the construct, was isolated by BamHI and partial EcoRI digestion and inserted between the 5′ flank and pGK–*tk*. Finally, pGK–*neo* was inserted as a HindIII–BamHI fragment in between the two flanking sequences to generate the final targeting construct (Figure 1A). The construct was linearized with NotI prior to transfection into CCE ES cells.

To construct the kinase domain targeting vector, an 8.0 kb BamHI fragment containing the entire kinase domain was subcloned into the above described tkpBS. Two HindIII fragments (1.2 and 1.0 kb) containing exons 12 and 13 were deleted and replaced by pGK–*neo* to give the targeting construct (Figure 1A, right). The construct was linearized with NotI prior to transfection into J1 ES cells.

### Generation of *Btk* Mutant ES Cell Lines and Mice

For the construction of *Btk*–PH$^-$ ES cell lines, approximately 2 × $10^7$ CCE embryonic cells were transfected with 20 μg linearized construct by two pulses at 300 V and 70 μF in phosphate-buffered saline (PBS). Selection for growth in the presence of G418 (GIBCO) and ganciclovir (Sigma) was done as previously described (Shinkai et al., 1992). Screening for mutant ES cell clones was done by Southern blotting of HindIII digested DNA from approximately 750 clones and hybridization to a 700 bp PvuII fragment located outside the construct. The endogenous fragment is 12 kb and the targeted fragment is 9.0 kb. Three mutant CCE clones were obtained, subcloned, and used for generation of chimeric mice.

For the construction of *Btk–kin*$^-$ ES cell lines, approximately 2 × $10^7$ J1 embryonic cells were transfected with 25 μg linearized construct by a single pulse at 400 V and 25 μF in PBS. Selection for growth in the presence of G418 (GIBCO) and gancyclovir (Sigma) was as previously described (Shinkai et al., 1992). Screening for mutant ES cell clones was done by Southern blotting of EcoRI digested DNA from approximately 200 clones and hybridization to a 700 bp BamHI–HindIII fragment located outside the construct. Four mutant J1 clones were obtained and subcloned and two of the clones were used for injection into blastocysts from C57BL/6 for germline transmission. Therefore, the mice (*Btk–kin*$^-$/GL) analyzed have a mixed genetic background comprised of both 129Sv and C57BL/6.

To obtain the mice chimeric for the *RAG2*$^{-/-}$ locus and a mutant *Btk* allele, two of the independent *Btk*–PH$^-$ (CCE cells) and *Btk–kin*$^-$ (J1 cells) subclones were injected into blastocysts from *RAG2*-deficient mice (which are of the mixed genetic background: 129Sv/C57.BL/6) and transplanted into foster mothers (B6/CBA) as described (Chen et al., 1993). Thus, the CCE–*Btk*–PH$^-$/*RAG2*$^{-/-}$ and J1–*Btk–kin*$^-$/*RAG2* chimeric mice have a mixed genetic background of 129Sv/C57.BL/6.

### Mice

Normal littermates (*Btk*-XY sib of a X$^M$ Y mouse) and mutant X$^M$ Y, were bred at Children's Hospital, Boston, and Umeå University, Umeå, Sweden. CBA/NcaHN (X$^{xid}$Y) from Jackson Laboratories, CBA.*xid*/HHW (Wortis et al 1982) and C57BL/6.*Xid* males obtained from J. Kenney at National Cancer Institute were compared.

### Flow Cytometry Analysis

Single cell suspensions from BM, spleen, thymus, lymph node, and peritoneal exudates from normal, *Xid*, *RAG2*$^{-/-}$, *Btk*–PH$^-$/*RAG2*$^{-/-}$, and *Btk–kin*$^-$/*RAG2*$^{-/-}$ chimeras were prepared and stained by standard methods (Parks et al., 1986), using specific antibodies to lymphocyte cell surface markers. Cells in the lymphocyte gate by light scatter were analyzed at Howard Hughes Medical Institute/Children's Hospital on a FACScan (Becton Dickinson, Mountain View, California) using LYSYS software. For experiments performed with chimeric mice, the antibodies purchased from Pharmingen (San Diego, California) were the following: fluorescein isothiocyanate(FITC)–conjugated RA 3-6B2 (B220/CD45R), CD43(S7), AMS 9.1 (IgD$^a$), (IgM$^a$), phycoerythrin(PE)-conjugated DS-1 (IgM$^a$), (B220/CD45R; Figure 3A), CD5.

FACS analysis of 9- to 13-week-old *Btk–kin*$^{-/Y}$ (germline mutant mice), their normal littermates (*Btk–kin*$^{+/Y}$) and *Xid* (CBA/NcaHN) mice were performed using the methods and reagents described previously (Parks et al., 1989; Kantor et al., 1992). Monoclonal antibodies conjugates with fluorophores, as previously used (Wells et al., 1994), were purified, prepared, and used according to standard procedures (Hardy, 1986; Kantor et al., 1992). In brief, single cell suspensions were prepared in deficient RPMI 1640 and EDTA (1 mM) was added to the medium for staining in microtiter plates. BM cells were stained with CD43/S7(PE), B220/6B2(APC), and IgM/331 (FITC), or IgM/331(TR-AV). All experiments were done using 1.0 mg/ml propidium iodide to identify dead cells. Spleen cells were stained with IgD/1126(TR-AV), CD4/GK1.5(PE), CD8/53-5(PE), CD5/53-7(APC), IgM/331(FITC), and B220/6B2(APC). Peritoneal exudates were stained with IgM/331(FITC), IgD/11–26(TR-AV), Mac1/M1/70(APC), and CD5/53–7(APC). Cells were analyzed at the shared FACS facility at Stanford University on "Flasher," an extensively modified dual laser (488 and 595 nm excitation) FACS II (Parks et al., 1986) (Becton Dickinson, Mountain View, California) interfaced with a VAX 6300 computer (Digital Equipment, Maynard, Massachusetts) running FACS/Desk software (Stanford University, Stanford, California) (Parks et al., 1989; Moore and Kautz, 1986). The probability contour plots contain an equal number of cells between each pair of contour levels. The number of lines in each region of the map is essentially proportional to the number of cells in that region. All plots presented here have 5% probability contours.

### B Cell Proliferation Assays

To purify splenic B cells, single cell suspensions were treated with anti-Thy1 and guinea pig complement (Hawrylowicz et al., 1984), and enriched for live cells by lympholyte M. Adherent cells were depleted by incubating on a tissue culture plate (Nunc) at 37°C. B cell preparations were typically 85% pure for *Xid* (CBA/CaHN–*Xid*/J) (Jackson Laboratories), *Btk*–PH$^-$, and *Btk–kin*$^-$, and 95% B220$^+$ for normal mice as assayed by FACS analysis. Cells were cultured at $10^5$/100 ml in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 50 nM 2-ME, and 2.0 μg/ml goat anti-mouse IgM (Southern Biotechnology Associates), 20 ng/ml anti-IgD–dextran (Mandler et al., 1993), 2.0 μg/ml anti-CD40 (Serotech), and several concentrations of LPS as indicated in the legend to Figure 6. For optimal B cell proliferation, conditioned supernatants from IL-4-transfected cells (Karasuyama and Melchers, 1988; Luzkar et al., 1988) was added, where indicated. DNA synthesis was assayed by pulsing the cultures with 1.0 μCi [$^3$H]thymidine/well at 60 hr and incubated for additional 6–8 hr, harvested, and counted on a scintillation counter. All assays were done in triplicates.

### Immunizations

To measure the thymus-independent immune responses, *Btk*–PH$^-$/*RAG2*$^{-/-}$ and *Btk*–Kin$^-$/*RAG2*$^{-/-}$ chimeric or *Btk*–Kin$^-$/GL mice were immunized with 10 μg TNP–Ficoll (gift of Dr. J. Inman, National Institutes of Health, Bethesda, Maryland) in PBS was injected intraperitoneally. The serum was analyzed at day 7 for TNP-specific total immunoglobulin antibodies by ELISA using TNP–Ficoll-coated plates. To measure the thymus-dependent immune responses, chimeric or germline mutant mice were immunized with 100 μg TNP–KLH precipitated with alum and injected intraperitoneally together with $10^9$ Bordetella pertussis (gift of Dr. A. Abbas). A booster dose of 50 μg of antigenin PBS was given at day 14. For the analysis of secondary immune responses, the serum was analyzed for isotype-specific anti-KLH or anti-TNP antibodies by ELISA after day 7 of booster dose. The plates were coated with TNP–OVA, or KLH (100 μg/ml).

### ELISA Assays

Immunol 1 plates (Dynatech) were coated with 5 μg/ml of isotype-specific rabbit or goat anti-mouse antibodies. Diluted serum samples were incubated in the plates and revealed by alkaline phosphatase–labeled secondary antibody. The antibodies were purchased from Southern Biotechnology Associates Incorporated, Birmingham, Alabama.

### DNA Probes, Southern, and Northern Blotting Analysis

For Southern blot analysis, DNA was prepared, restriction digested, electrophoresed, blotted on to zeta probe membranes using standard methods. The probe for detection of *Btk*–PH knockout allele was a 700 bp PvuII fragment from the 5′-end of the *Btk* gene containing exon

4 (82 bp) and sequences from intron V (Sideras et al., 1995). The probe for detection of *Btk–kin* knockout allele was a 700 bp HindIII–BamHI fragment from the 3′-end of the *Btk* gene containing sequences from intron 18 (Sideras et al., 1995).

For Northern blot analysis, 15 μg of cytoplasmic RNA was electrophoresed in a 1.2% formaldehyde agarose gel, transferred to ζ probe membranes using standard methods. The probe used for the detection of 5′-end sequences in *Btk* transcript was a 1.5 kb EcoRI fragment from the 5′-end of the *Btk* cDNA containing the coding sequences up to the kinase domain. The 3′-end sequences in the *Btk* transcript were detected with a 1.4 kb EcoRI fragment from the 3′-end of the *Btk* cDNA that contains the coding sequences of the kinase domain and the 3′ UTR. (Sideras et al., 1995) and *neo*′ gene sequences were detected with a 600 bp PstI–PstI fragment of the *neo*′ gene.

### Generation of A-MuLV-Transformed Cell Lines

A-MuLV-transformed pre-B cell lines were established as described by Rosenberg and Baltimore (1976). For the generation of *Btk–kin*$^-$ A-MuLV-transformants, BM cells from *Btk–kin*$^-$/GL mice were infected with A-MuLV and plated in soft agar; individual colonies were picked, transferred to liquid medium, and expanded. *Btk*–PH$^-$ cell lines were derived from BM of chimeric mice made by injection of *Btk*–PH$^-$ ES cells into *RAG2*$^{-/-}$ blastocysts. Chimeric mice were identified by screening peripheral blood samples by flow cytometry for the presence of mature T and B cells, indicating ES cell contribution to the lymphocyte compartment. BM cells from chimeric mice were subsequently infected with A-MuLV and plated as above. Individual colonies were expanded in 24-well plates, screened by PCR for the presence of the *RAG2*$^{+/+}$ genotype, indicating ES derivation, and selected cell lines were expanded. The *Btk*–PH$^-$ or *Btk*–Kin$^-$ genotypes of all cell lines were confirmed by Southern blotting analysis (data not shown).

### Protein Analysis

Rabbit polyclonal anti-Btk antibodies used in immunoprecipitation and Western blot analysis were specific for the unique N-terminal region of Btk and for the last 15 aa (645–659) of the C terminus (a gift of Dr. O. Witte) of Btk, respectively. Antibodies were immunoaffinity purified. Immunoprecipitation was performed on Abelson-transformed *Btk–kin*$^-$ A-MuLV and *RAG2*$^{-/-}$ A-MuLV transformants (as a positive control) and T cell line D010 (as negative control). Cells ($10 \times 10^6$) for positive and negative control cell lines and $30 \times 10^6$ cells for *Btk–kin*$^-$ cell line were labeled with 1.0 μCi, $10 \times 10^6$ cells of [$^{35}$S]methionine and cysteine for 15 min in RPMI medium lacking methionine and cysteine. After labeling, cells were solubilized in lysis buffer containing 1% Nonidet P-40, Tris–HCl (pH 7.5), 150 mM NaCl, and protease inhibitors, aprotinine, leupeptin, ABSF, 1 mM PMSF, and incubated with 10 μg antibodies for 4 hr at 4°C. The immune complexes were recovered by protein A–Sepharose and analyzed on SDS–PAGE and autoradiography. For Western blot analysis, lysates of $3–5 \times 10^6$ cells were denatured and separated on SDS–PAGE and transferred to immobilon (Millipore) membrane. The filter was blocked by 10% milk in TBST for 4 hr and incubated with 1:2000 diluted anti-N-terminal or anti-C-terminal antibodies for 2 hr. The antibody-reactive proteins were visualized by horseradish peroxidase–conjugated anti-rabbit antibodies and enhanced chemiluminescence light-emitting detection system (Amersham).

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