Bruton’s tyrosine kinase is required for signaling the CD79b-mediated pro-B to pre-B cell transition

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Abstract

Formation of the pre-BCR complex is a critical check point during B cell development and induces the transition of pro-B to pre-B cells. CD79b (Igβ) is a signaling component in the pre-BCR complex, since differentiation to the pro-B phenotype is induced by cross-linking the CD79b expressed on developmentally arrested pro-B cells from recombination-activating gene (RAG)-2-deficient mice. Bruton’s tyrosine kinase (BTK) plays important roles in B cell development. However, its molecular mechanisms in early B cell development are not fully understood. To examine whether BTK functions in CD79b-mediated signaling for the pro-B/pre-B transition, we utilized RAG2/BTK double-knockout (DKO) mice. Pro-B cells from RAG2/BTK-DKO mice did not differentiate into pre-B cells following CD79b cross-linking, although tyrosine phosphorylation of cellular proteins including Erk1/2 and phospholipase Cγ2 was induced in the same manner as RAG2-KO mice. BTK is phosphorylated after cross-linking of CD79b on RAG2-deficient pro-B cells. These findings suggest that BTK-dependent pathways downstream of CD79b are critical for the pro-B/pre-B transition and BTK-independent signaling pathways are also activated via the pre-BCR complex.

Introduction

B cell development is a highly regulated process that includes the ordered rearrangement of Ig heavy and light chain genes (1). Formation and expression of a pre-BCR complex represents one requirement for B cell precursor maturation (2). The pre-BCR consists of a productive Ig heavy chain, Vpre-B, λ5, CD79a (Igα) and CD79b (Igβ) (3,4), and elicits signals for several critical events. These include the transition of pro-B cells to pre-B cells (5), selective expansion of pre-B cells producing a productive Ig heavy chain (6,7), allelic exclusion of Ig heavy chain genes (8) and the promotion of light chain gene rearrangements (9). Animals deficient for any component of the pre-BCR have a developmental block at the pro-B to pre-B transition (10–15). CD79b is believed to be a signal transmitting subunit of the pre-BCR since it carries an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain (16). In addition, we recently found that even before the expression of Ig heavy chain, small amounts of CD79b were expressed on the surface of pro-B cell lines and CD45R+ cells from recombination-activating gene (RAG)-2-deficient mice in a complex with calnexin (17).
Cross-linking of CD79b induced differentiation of the RAG2-deficient pro-B cells to small pre-B cells in vivo (17). This is analogous to the transition of CD4/CD8 double-negative thymocytes into CD4/CD8 double-positive thymocytes induced by anti-CD3 injection into Rag2 knockout (KO) mice (18). CD79b cross-linking induced tyrosine phosphorylation of Syk, phosphatidylinositol-3-kinase, Vav and SLP-76 proteins, and activation of Erk in RAG2-deficient pro-B cells in vitro (17), but it is not clear how these molecules transmit pro-B to pre-B differentiation signals.

**Methods**

**Mice**

BTK-KO mice (27) and RAG2-KO mice (32) were obtained from Dr F. W. Alt (Howard Hughes Medical Institute, The Children’s Hospital, Boston, MA), crossed and bred under specific pathogen-free conditions in the Animal Facility of the Institute of Medical Science, University of Tokyo. All mice used in this study were from a mixed background of C57BL/6 and 129/Sv.

**Antibodies**

Hamster anti-mouse CD79b mAb (HM79) (33) was purified from hybridoma culture supernatants (a gift from Dr T. Nakamura, Institute of Medical Science, University of Tokyo, Japan) using Protein G-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden). mAb RA3-6B2 (anti-CD45R), RM2-5 (anti-CD2), RC3/BP-1 (anti-Ly-51), S7 (anti-CD43), PC61 (anti-CD25) and 2C11 (anti-CD3) were purchased from PharMingen (San Diego, CA) in FITC-conjugated or biotinylated forms. Biotinylated Ack-2 (anti-c-kit) was kindly provided by Dr S.-I. Nishikawa (Graduate School of Medicine, Kyoto University, Japan). LM34 (anti-λ5) (4) was biotinylated by a standard method. 4G10 (anti-phosphotyrosine) and anti-phospholipase C (PLC)-γ2 antibody were purchased from Upstate Biotechnology (Lake Placid, NY). An anti-BTK antibody was raised by immunizing rabbits with a synthesized peptide corresponding to amino acids 176–193 of mouse BTK conjugated to keyhole limpet hemocyanin (KLH). Anti-BTK phosphopeptide-specific mAb were generated as described (34). Anti-Erk1/2 antibody and antibody that recognizes specifically Thr202/Tyr204-phosphorylated Erk1/2 were purchased from New England Biolabs (Beverly, MA).

**In vivo injection of antibody**

RAG2+/-BTK−/− or RAG2+/-BTK+/- mice (10–13 weeks old; designated as RAG2/BTK-DKO) were injected i.v. with 200 µg of anti-CD79b mAb. In some experiments, mice were injected with 700 µg of mAb. As a control, RAG2+/-BTK−/− or RAG2+/-BTK+/- mice (designated as RAG2-KO) were treated in parallel. After 4, 7, 11 or 14 days of treatment, mice were sacrificed for analysis.

**Flow cytometric analysis**

Bone marrow cells were collected from two femora of each mouse and suspended in 5% FCS/PBS. Cells were incubated with 10% heat-inactivated rat serum or 10 µg/ml of anti-Fcy III/I receptor mAb (2.4G2). Then cells were incubated with appropriately diluted FITC-conjugated or biotinylated mAb. For detection of biotinylated mAb, cells were subsequently incubated with phycoerythrin-conjugated streptavidin (A二人, Bayport, MN). Stained cells were evaluated by FACScan or FACSCalibur and the data were analyzed using CellQuest software (Becton Dickinson, Mountain View, CA). For detection of phosphorylated BTK, CD45R+ cells were sorted using an Auto-MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) after incubation with biotinylated anti-CD45R and streptavidin-coupled microbeads. Purified CD45R+ cells were then pretreated with 20 µg/ml of biotinylated anti-CD79b antibody for 5 min on ice. After addition of streptavidin (0.5 mg/ml), the cells were incubated at 37°C for 5 min. Cells were fixed by adding 1 ml of ice-cold fixative solution (PBS with 4% paraformaldehyde and 1 mM Na2VO4), and were permeabilized and stained with anti-BTK polyclonal antibody and anti-BTK223PY or anti-BTK551PY mAb as previously described (34).

**Immunoprecipitation and immunoblotting**

Purified CD45R+ cells were suspended in 0.1% BSA/RPMI 1640 at 2×10⁶ cells/100 µl and pretreated with 20 µg/ml of...
biotinylated anti-CD79b antibody for 5 min on ice. After addition of streptavidin (0.5 mg/ml), the cells were incubated at 37°C for various times. Stimulation was stopped by addition of cold inhibition buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl₂, 100 mM NaF, 1 mM Na₃VO₄, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor and 1 µg/ml α₅-antitrypsin). Then cells were pelleted and lysed with lysis buffer (inhibition buffer containing 1% Nonidet P-40). Cellular proteins in the lysate were separated by SDS–PAGE and then transferred to PVDF membranes (Millipore, Bedford, MA). Membranes were blotted with 4G10 mAb and horseradish peroxidase (HRP)-conjugated anti-mouse IgG. For the detection of tyrosine-phosphorylated BTK and PLC-γ2, lysates were immunoprecipitated with anti-BTK and anti-PLC-γ2 antibodies respectively, with separated SDS–PAGE, and transferred to PVDF membranes. The membranes were blotted with anti-BTK551PY, anti-BTK223PY or 4G10 mAb followed by HRP-conjugated anti-mouse IgG antibody. For the detection of phosphorylated Erk, lysates were applied to SDS–PAGE and immunoblotted with anti-phosphorylated-Erk antibody in combination with HRP-conjugated anti-rabbit IgG antibodies. All immunoblots were visualized by ECL reagent (Amersham Pharmacia Biotech, Uppsala, Sweden).

T cell reconstitution

For the T cell transfer experiment, lymph node cells were collected from 8-week-old C57BL/6 mice and 2×10⁷ cells were injected i.v. into RAG2-KO or RAG2/BTK-DKO mice. After 4 days of transfer, they were injected i.v. with 200 µg of anti-CD79b mAb. For the thymocyte transfer, 4×10⁷ thymocytes from 6-week-old C57BL/6 mice were injected i.v. and anti-CD79b mAb was injected after 3 weeks. For the thymus transfer (35), thymus lobes from 6-week-old C57BL/6 mice were implanted s.c. and anti-CD79b mAb was injected 5 weeks later. All mice were sacrificed 6 days after injection of anti-CD79b mAb and bone marrow cells were stained as above. Also, expression of CD3 on spleen cells or bone marrow cells was analyzed with flow cytometry following staining with anti-CD3 mAb.

Results

B lineage cells in the bone marrow of RAG2/BTK-DKO mice

We began with characterizing B lineage cells in bone marrow of RAG2/BTK-DKO mice. As shown in Fig. 1 (Untreated), proportions of CD45R⁺ cells and the expression patterns of CD25, CD2, BP-1, CD43 and λ₅ on CD45R⁺ cells were indistinguishable between RAG2/BTK-DKO and RAG2-KO mice. This implies that BTK is dispensable for development of pro-B cells, although BTK is known to be expressed from an early stage (36). This is consistent with a previous report that BTK-deficient mice have normal numbers of pro-B cells (27).

RAG2/BTK-DKO mice are unresponsive to anti-CD79b treatment

We next treated RAG2-KO and RAG2/BTK-DKO mice by i.v. injection with 200 µg of HM79 anti-CD79b mAb to determine if the pro-B cells in these animals could differentiate in response to CD79b cross-linking. Seven days after antibody treatment, the expression of CD25, BP-1 and CD2 on CD45R⁺ cells was up-regulated, and expression of CD43 and λ₅ was down-regulated in RAG2-KO mice (Fig. 1A and B, upper panels) as we previously reported (17). In addition, the proportion of small CD45R⁺ cells with a FCS value <70 was significantly increased (29–54%) after treatment (Fig. 1B). These changes have been used to characterize events associated with the pro-B to pre-B transition (37,38). In contrast to RAG2-KO mice, their responses were significantly reduced in RAG2/BTK-DKO mice (Fig. 1A and B, lower panels). Most dramatically, the increase in CD25⁺ cells almost completely disappeared (Fig. 1A). CD79b stimulation also induced an increase in absolute numbers of CD45R⁺ cells in bone marrow of RAG2-KO mice. However, in RAG2/BTK-DKO mice, the number of CD45R⁺ bone marrow cells did not change after CD79b stimulation (Fig. 1C). To test the possibility that stronger CD79b stimulation is required for RAG2/BTK-DKO pro-B cells to differentiate into pre-B cells, we also tried 3.5 times higher doses (700 µg/mouse i.v.) of anti-CD79b mAb (data not shown), but RAG2/BTK-DKO pro-B cells did not differentiate. We examined mice 4, 7, 11 and 14 days after anti-CD79b injection, but found no CD25⁺CD45R⁺ cells in RAG2/BTK-DKO mice (Fig. 2). These results demonstrate that RAG2/BTK-DKO mice have impaired differentiation responses to anti-CD79b.

The unresponsiveness of RAG2/BTK-DKO mice to anti-CD79b treatment is due to a defect in the CD79b signaling

Although the surface markers on pro-B cells were comparable between untreated RAG2/BTK-DKO and RAG2-KO mice (Fig. 1), there was a possibility that RAG2/BTK-DKO mice might have a deficiency of CD79b-expressing cells. As CD79b is too low to detect quantitatively on pro-B cells (17), we examined tyrosine phosphorylation of intracellular proteins after CD79b cross-linking in vitro. As previously described (17), rapid tyrosine phosphorylation of intracellular proteins was induced in RAG2-KO bone marrow cells by anti-CD79b. Similar levels of tyrosine phosphorylation of intracellular proteins were induced in RAG2/BTK-DKO bone marrow cells (Fig. 3). Therefore the defect in CD79b-induced differentiation of RAG2/BTK-DKO pro-B cells was not due to the absence of responsive cells or lower expression of CD79b, but resulted from a defect in the CD79b-mediated signal transduction pathway. Furthermore, the gross activity of tyrosine kinases or the time course of activation induced by CD79b cross-linking was not affected by the absence of BTK kinase.

Intracellular signaling through CD79b in the RAG2/BTK-DKO pro-B cells

Next, we evaluated particular intracellular signaling events after CD79b cross-linking of pro-B cells. First, we examined whether BTK became phosphorylated on tyrosine by cross-linking CD79b on RAG2-KO pro-B cells. For this purpose, we utilized phosphopeptide-specific anti-BTK mAb. 223PY and 551PY antibodies specifically recognize BTK protein only when its tyrosine residues at position 223 (autophosphorylation site) and position 551 (transphosphorylation site) are phosphorylated (34,39). Purified CD45R⁺ cells were stimu-
Bruton's tyrosine kinase in the CD79b signaling

Fig. 1. Differentiation of pro-B cells from RAG2-KO mice (upper panels) or RAG2/BTK-DKO mice (lower panels) induced by anti-CD79b. Anti-CD79b mAb was injected i.v. and bone marrow cells were analyzed 7 days later. (A) These representative two-color fluorescence plots show expression of CD45R and CD25 on cells from untreated (left panels) or anti-CD79b-treated mice (right panels). (B) Single-parameter histograms show expression of CD2, BP-1, CD43, \( \lambda 5 \) and cell size (FSC) of CD45R/H11001 lymphocytes from untreated (open histograms) or anti-CD79b-treated mice (filled histograms). The data shown are representative of seven independent experiments. (C) The absolute numbers of CD45R\(^+\) cells in two femora of individual RAG2-KO (open circles) and RAG2/BTK-DKO (closed circles) mice were calculated and shown.

Fig. 2. Time course of appearance of CD25\(^+\) B lineage cells after anti-CD79b antibody treatment. Bone marrow cells were analyzed 4, 7, 11 and 14 days after anti-CD79b injection. Dots represent percentages of CD25\(^+\) cells among the CD45R\(^+\) B lineage cells from individual RAG2-KO (open circles) or RAG2/BTK-DKO mice (closed circles).

Fig. 3. Tyrosine phosphorylation of intracellular proteins induced by cross-linking of CD79b. Bone marrow cells from RAG2-KO (left four lanes) or RAG2/BTK-DKO (right four lanes) mice were stimulated with anti-CD79b mAb for the indicated times. The whole cell lysates of stimulated cells were subjected to Western blotting with an anti-phosphotyrosine mAb (4G10).
Bruton’s tyrosine kinase in the CD79b signaling

Fig. 5. (A) Phosphorylation of intracellular signaling molecules. Cells were stimulated with anti-CD79b for the indicated times. PLC-γ2 was immunoprecipitated and subjected to immunoblotting with 4G10 anti-phosphotyrosine mAb (upper panel) or with anti-PLC-γ2 (lower panel). (B) Total cell lysates were also subjected to immunoblotting with phospho-specific anti-Erk1/2 antibody.

Fig. 4. Tyrosine phosphorylation of BTK in RAG2-KO pro-B cells. Bone marrow suspensions enriched for CD45R+B cells were stimulated for 5 min with anti-CD79b. (A) After cell fixation and permeabilization, cells were incubated with anti-BTK antibody and mAb specific to Tyr551-phosphorylated BTK (upper panel) or Tyr223-phosphorylated BTK (lower panel) followed by incubation with appropriate secondary reagents. Results of FACScan analysis are shown as two-parameter plots of total BTK and phosphorylated BTK. Percentages of phosphorylated BTK in the lymphocyte gate are shown. (B) Stimulated cells were lysed and immunoprecipitated with anti-BTK antibody. Immunoprecipitants were subjected to immunoblotting with anti-PY551 (upper) or anti-PY223 (middle) BTK mAb. The total amount of BTK in each sample is shown by immunoblotting with anti-BTK antibody (bottom).

After the stimulation with CD79b, the cross-linking, phospho-Tyr223-BTK+ population increased from 5.6 to 24.7%. In the same stimulation, the phospho-Tyr551-BTK+ population increased from 10.7 to 68.6%. These changes are comparable to the BTK phosphorylation induced by IgM cross-linking of Ramos human B cell line (34). Immunoblotting with the same mAb detected phosphorylated BTK (77 kDa), confirming the specificity of the mAb (Fig. 4B). This result shows that BTK undergoes tyrosine phosphorylation after cross-linking CD79b and that BTK is directly activated downstream of CD79b signaling.

PLC-γ2 and Erk1/2 are phosphorylated in the RAG2/BTK-DKO pro-B cells upon cross-linking of CD79b

Recently, BTK was shown to be upstream of PLC-γ2 and Erk, and a regulator of Ca2+ influx in DT40 cells, a chicken B lymphoma line (40,41). PLC-γ2 became tyrosine phosphorylated by CD79b stimulation in RAG2-KO pro-B cells. Comparable amounts of PLC-γ2 phosphorylation were also detected in RAG2/BTK-DKO cells (Fig. 5A). We could not observe co-immunoprecipitation of BTK with PLC-γ2 in RAG2-KO cells (data not shown). Next, we examined the activation of Erk1/2 by phosphorylation. In contrast to BTK-deficient DT40 cells which have partial abrogation of Erk1/2 phosphorylation after BCR ligation (41), Erk1/2 were activated by CD79b cross-linking in RAG2/BTK-DKO cells as well as RAG2-KO cells (Fig. 5B). Equal amounts of total Erk1/2 protein were verified by reprobing the membranes with anti-Erk1/2 antibody (Fig. 5B, lower panel).
Table 1. Anti-CD79b antibody treatment of the T cell-reconstituted RAG2-KO or RAG2/BTK-DKO animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mouse</th>
<th>Anti-CD79b injection</th>
<th>CD45R&lt;sup&gt;+&lt;/sup&gt;CD25&lt;sup&gt;+&lt;/sup&gt; in bone marrow (%)</th>
<th>CD3&lt;sup&gt;+&lt;/sup&gt; in bone marrow (%)</th>
<th>CD3&lt;sup&gt;+&lt;/sup&gt; in spleen (%)</th>
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<tr>
<td>Thymocyte transfer&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>RAG2/BTK</td>
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<td></td>
<td>+</td>
<td>RAG2/BTK</td>
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<td>7.0</td>
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<td>0.4</td>
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<td>T cell transfer&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup>Thymocytes, <sup>b</sup>lymph node cells or <sup>c</sup>thyms lobes were transferred to RAG2-KO or RAG2/BTK-DKO mice. Expression of CD25 on bone marrow CD45<sup>+</sup> cells and CD3 on bone marrow cells or spleen cells after 6 days from the injection of anti-CD79b mAb is shown.

T cell reconstitution in RAG2/BTK-DKO mice does not restore the unresponsiveness of their pro-B cells to the anti-CD79b treatment

Both nude and XID mice have normal numbers of pre-B cells. However, XID/nude doubly mutant mice show severe defects in B cell development at the pre-B stage indicating that B cell development is largely dependent on T cells in the absence of BTK (42). Since RAG2-KO mice (and RAG2/BTK-DKO mice) have no T cells, this might influence B cell development in the RAG2/BTK-DKO mice. We transferred peripheral T cells from lymph nodes of the wild-type mice, or thymus grafts from the wild-type mice, into RAG2/BTK-DKO mice and examined whether CD79b cross-linking induces pro-B/pre-B transition in the presence of T cells. After the transfer of T cells or thymus graft, both RAG2-KO and RAG2/BTK-DKO mice showed CD3<sup>+</sup> cells in their bone marrow or spleens indicating T cells are reconstituted in these animals. However, pro-B cells from RAG2/BTK-DKO mice did not show any anti-CD79b induced up-regulation of CD25 expression (Table 1).

Discussion

BTK is a tyrosine kinase required for B cell development. However, appropriate models were not available for investigating its importance at early stages of B cell development. We devised a unique differentiation system utilizing RAG/BTK-DKO mice and found that BTK functions downstream of CD79b to induce the transition from the pro-B to the pre-B stage. Anti-CD79b-induced differentiation of pro-B cells was greatly impaired in the absence of BTK, indicating that BTK is essential. This result is particularly impressive since XID and BTK-KO mice have normal numbers of pre-B cells (27).

The disadvantage of BTK malfunction in pro-B/pre-B transition has been demonstrated by Kerner et al. using chimeric mice in which BTK-deficient progenitors show poor expansion at the pro-B stage (30). In the mice heterozygous for the lacZ-replaced btk locus, BTK-deficient progenitors were affected only after the immature B cell stage (31). Our results demonstrated the importance of BTK in the transition from the pro-B to the pre-B stage and are consistent with the observation by Kerner et al. (30).

Several explanations can be proposed to account for the discrepancy between these results. First, CD79b cross-linking on the RAG2-deficient pro-B cells utilized in this study was done in the absence of Ig heavy chains. Cross-linking of CD79b in the absence of Ig heavy chains might have a limited capacity to produce complete pre-BCR signaling. Alternatively, pre-BCR signaling transmitted with Ig heavy chain that cannot associate with CD79b (43). This indicates that there could be CD79b-independent signals mediated through a pre-BCR complex. CD79b cross-linking may deliver a BTK-dependent signal, whereas additional Ig heavy chain-dependent signals appear to compensate for a loss of function of BTK in mice.

Second, we must also consider the differentiation stages represented by the stimulated cells. Pre-BCR complex is normally formed and expressed on large pre-B cells. In contrast, we stimulated CD79b in a complex with calnexin on cells at the pro-B stage. This difference in maturation of the target cells might affect the extent of BTK dependency.

Third, BTK might also transmit negative signals in early B lineage development. In several in vitro studies, XID B lineage cells have had an advantage over wild-type cells. One is the
more rapid establishment of long-term bone marrow cultures from XID mice (44) and another is the high frequency of colony-forming units responsive to IL-7 (CFU-IL-7) in XID bone marrow cells (45). We also observed better expansion and production of cytoplasmic µ- pre-B cells in stroma/serum-free cultures initiated with lineage marker (Lin) c-kit- bone marrow cells from XID mice as compared to controls (T. Kouro and Paul W. Kincade, unpublished observation). Thus, BTK can be inhibitory in the formation of pre-B cells in some circumstances. The normal pre-B cell numbers found in BTK-deficient mice may reflect the sum of multiple signals.

Finally, development of BTK-deficient B cell progenitors depends on T cells in some conditions, as demonstrated by the early arrest of B cell development in nude, XID mice (42). Although there appear to exist strain difference in the T cell dependency of B cell progenitors (46), lack of mature T cells in RAG2-KO mice might explain the unresponsiveness of pre-B cells from RAG2/BTK-DKO mice. However, this possibility was clearly ruled out by transferring peripheral T cells or thymocytes into RAG2/BTK-DKO mice.

By taking advantage of phosphopeptide-specific anti-BTK mAb, we showed tyrosine phosphorylation of BTK after cross-linking of CD79b. This is the first report showing that BTK is directly downstream of mouse pre-BCR. Phosphorylation of Tyr551 of BTK causes an increase in kinase activity of BTK and autophosphorylation of Tyr223. Phosphorylation of Tyr223 is located in the SH3 domain of BTK. Phosphorylation of the CD79b mAb treatment has been shown to be a specific effect of anti-CD79b mAb but not to be a non-specific effect of hamster mAb by using control antibody (17). Pre-BCR may have different or redundant signaling pathways leading to the activation of PLC-γ2 and Erk1/2, although both pre-BCR and BCR share CD79b as a signal transmitting molecule. Alternatively, activation mechanisms of PLC-γ2 and Erk1/2 could differ between murine and the chicken DT40 cell line. Tyrosine phosphorylation of PLC-γ2 induced by FceRI was indistinguishable between wild-type and BTK-deficient mast cells, although degranulation from BTK-deficient mast cells was severely reduced (50). Such a BTK-dependent, PLC-γ2-independent pathway might be important also to the CD79b-induced differentiation signal of pro-B/pre-B transition. Recently, human pre-BCR was shown to be located in membrane domains rich in glycosphingolipids and cholesterol called ‘lipid rafts’. Ligation of pre-BCR results in recruitment of several signaling molecules including BTK and PLC-γ2 to form a signaling complex within the raft, which is indispensable for calcium flux (51). Although we have been unable to detect increases in cytoplasmic Ca^{2+} in RAG2-deficient pro-B cells stimulated with anti-CD79b mAb (17), it is still possible that the absence of BTK causes defective recruitment of signaling molecules to the lipid raft and results in impaired differentiation signal transmission.

Our results, using RAG2/BTK-DKO mice and anti-CD79b administration, demonstrated the involvement of BTK in a signaling pathway downstream of CD79b in the pre-BCR complex. This showed that the pro-B/pre-B transition is a critical target for BTK malfunction even in mice. Moreover, our system provides a potential way to identify target molecule(s) of BTK and gene(s) required for the pro-B/pre-B transition. Further experiments using this model should clarify the pathogenesis of XID and XLA as well as the molecular basis of B cell development.

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BTK</td>
<td>Bruton's tyrosine kinase</td>
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<tr>
<td>DKO</td>
<td>double knockout</td>
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<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
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<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
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<tr>
<td>PLC</td>
<td>phospholipase C</td>
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<tr>
<td>RAG</td>
<td>recombination-activating gene</td>
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<tr>
<td>TH</td>
<td>Tec homology</td>
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<tr>
<td>XID</td>
<td>X-linked immunodeficiency</td>
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<tr>
<td>XLA</td>
<td>X-linked agammaglobulinemia</td>
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