Mimicry of Pre–B Cell Receptor Signaling by Activation of the Tyrosine Kinase Blk

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Abstract

During B lymphoid ontogeny, assembly of the pre–B cell receptor (BCR) is a principal developmental checkpoint at which several Src-related kinases may play redundant roles. Here the Src-related kinase Blk is shown to effect functions associated with the pre-BCR. B lymphoid expression of an active Blk mutant caused proliferation of B progenitor cells and enhanced responsiveness of these cells to interleukin 7. In mice lacking a functional pre-BCR, active Blk supported maturation beyond the pro–B cell stage, suppressed V_H to DJ_H rearrangement, relieved selection for productive heavy chain rearrangement, and stimulated κ rearrangement. These alterations were accompanied by tyrosine phosphorylation of immunoglobulin β and Syk, as well as changes in gene expression consistent with developmental maturation. Thus, sustained activation of Blk induces responses normally associated with the pre-BCR.

Key words: B cell development • signal transduction • allelic exclusion • V(D)J recombination • Src kinases

Introduction

Igμ heavy chain genes are assembled from discrete segments by V(D)J recombination, a process initiated by RAG-1 and RAG-2 (1). The joining of coding segments is random with respect to reading frame, and most primary products of V(D)J recombination are nonproductive. Heavy chain gene assembly begins in pro–B cells with the formation of DJ_H joints on both alleles. V_H to DJ_H joining is then activated sequentially at the two alleles. Productive assembly of a heavy chain gene and expression of an intact μ chain marks the transition from the pro–B to the pre–B cell stage of development (2).

At this developmental checkpoint, the μ chain associates with the chaperones VpreB and λ5 and is incorporated, with the accessory chains Igα and Igβ, into the pre–B cell receptor (BCR). The pre-BCR signals several cellular responses, including: (a) cessation of further V_H to DJ_H joining, (b) increased sensitivity to IL-7, (c) cell proliferation, (d) suppression of apoptosis, (e) developmental progression, and (f) activation of rearrangement at the Igκ locus (for review see references 3 and 4). The combined proliferative and antiapoptotic pre-BCR signals contribute to the expansion of Igμ-expressing clones. Increased responsiveness to IL-7, by allowing proliferation and survival at diminished cytokine concentrations, may function in the positive selection of cells that have undergone productive heavy chain rearrangement and developmental progression to light chain rearrangement (4, 5).

Although the molecular details of signaling through the pre-BCR are poorly understood, genetic approaches have identified several critical components of the signaling machinery. In mice lacking Igμ, Igβ, λ5, the tyrosine kinase Syk, or the docking protein BLNK, B cell development beyond the pre–B cell stage is impaired, as evidenced by a marked reduction in the number of peripheral B cells and an increase in the proportion of $B220^+$ $CD43^+$ progenitor B cells in the bone marrow relative to the more mature $B220^+$ $CD43^-$ cells (6–10).

The intracellular signaling events after appearance of the pre-BCR are not clearly defined, but several lines of evidence indicate a role for phosphorylation of Igα and Igβ. First, in RAG-deficient mice, cross-linking of Igβ induces tyrosine phosphorylation of Igα and Syk, as well as differentiation of pro–B cells to small pre–B cells (11). Second, in mice lacking the membrane-bound form of Igμ, Igβ cross-linking suppresses heavy chain rearrangement and activates light chain rearrangement (12). Third, in mice lacking the cytoplasmic

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Abbreviations used in this paper: BCR, B cell receptor; NF, nuclear factor.

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domain of Igβ, development beyond the pro–B cell stage is dependent on the Igα ITAM motifs (13). Fourth, pre-BCR cross-linking is associated with an increase in the amount of tyrosine-phosphorylated Igβ that is associated with the pre-BCR in lipid rafts (14).

The Src-related tyrosine kinases Blk, Lyn, and Fyn associate with the BCR, and current models of BCR or pre-BCR signaling propose that one or more of these Src family kinases, possibly in combination with Syk, participate in phosphorylation of the Igα and Igβ ITAM motifs (for review see reference 15). Blk and Lyn prefer similar consensus substrate sequences, distinct from that of Src and resembling sites in the Igα and Igβ ITAM motifs (16). Blk, Lyn, and Fyn play functionally redundant roles in supporting the pro–B to pre–B cell transition. Single or pairwise deficiencies of these or other Src-like kinases have little or no effect on early B cell development (17–24). In contrast, the pro–B to pre–B cell transition is attenuated in triply mutant $Blk^{-/-}$ $Lyn^{-/-}$ $Fyn^{-/-}$ mice (25). Thus, any one of these three kinases is essential for effective pre-BCR signaling.

Although loss of function mutations have revealed a redundant requirement for Blk, Lyn, or Fyn in B cell development, gain of function mutations, by revealing the consequences of kinase activation, would be expected to provide additional mechanistic insight. To this end we asked whether a constitutively active form of Blk, the only member of the group expressed preferentially in B cells, could provide some or all of the functions associated with the pre-BCR. Our results indicate that in B cell progenitors, active Blk mimics several consequences of pre-BCR signaling.

Materials and Methods

Mice. The transgenic line Blk(Y495F)-15 (B6 × SJL), bearing a cDNA encoding Blk(Y495F) under control of the H-2^k promoter and the Igμ intronic enhancer, has been described (26). μMT mice (C57BL/6 background; reference 27) were obtained from The Jackson Laboratory. RAG-2–deficient mice (129S6 background; reference 28) were obtained from Taconic Laboratories. Blk(Y495F) transgenic mice deficient in membrane-bound Igμ or in RAG-2 were generated by interbreeding Blk(Y495F) transgenic and μMT or RAG2-deficient mice, respectively.

Cell Culture. Cells were maintained in RPMI 1640 supplemented with 10% FCS, 50 u/ml penicillin/streptomycin, 1 mM sodium pyruvate (Sigma-Aldrich), 2 mM L-glutamine (GIBCO BRL), 50 μM β-mercaptoethanol, 10 mM Hepes, and 0.7 × MEM nonessential amino acids (GIBCO BRL) at 37°C in 5% CO_2.

For thymidine uptake assays, bone marrow cell suspensions from 3–4-wk-old mice were cultured with 10 ng/ml rIL-7 for 5 d, by which time the nonadherent cell population contained >95% $B220^+$ cells, >80% of which were also $CD43^+$. Triplicate samples of $B220^+$ $CD43^+$ cells were separated from dead cells by ficoll centrifugation, washed three times with PBS, and incubated with rIL-7 or media alone in 96-well plates at 10^5 cells per well. Thymidine incorporation was assayed after 3 d of culture after the addition of [^{3}H]thymidine (1 μCi/well) 16 h before assay.

For isolation of RNA or protein, $B220^+$ $CD43^+$ cells were cultured as described above, but for 10 d in the presence of 10 ng/ml IL-7 added every 3 d of culture. Cell concentration was maintained below 10^6/ml.

Flow Cytometric Analysis. Bone marrow cells or splenocytes from 3–5-wk-old mice were analyzed on a FACScan™ instrument (Becton Dickinson). The following mAbs were obtained from BD Biosciences: FITC-conjugated anti-CD43 (S7 clone), anti–c-kit (CD117), anti-CD24 (HSA), anti–BP-1, anti-CD22, and anti-CD2; PE-conjugated anti-CD43, anti–BP-1, anti-CD22, anti-CD25, and anti-B220; and Cy3-conjugated anti-B220. FITC-conjugated anti-IgM and PE-conjugated anti-IgD were obtained from Southern Biotechnology Associates, Inc. Cells stained with PKH26 (Sigma-Aldrich) or annexin V (BD Biosciences) were counterstained with anti–B220-Cy3 and anti–CD43 (S7 clone)-FITC or anti–BP-1 (BD Biosciences).

V(D)J Recombination Assays. DNA from $B220^+$ $CD43^-$ or unsorted bone marrow cells was assayed for rearrangement by PCR as previously described, using primers specific for the V_H VJ558 family, for the DFL16 and DSP2 families, for V_κ segments, for the J_H3 region, and for the $J_\kappa2$ region (29). Products were detected by hybridization to ^{32}P-labeled probes (30). The ligation-mediated PCR assay for signal end breaks was performed using the linker-specific primer BW-1 and one of the locus-specific primers μ02, κ03, or DFL16.1B (31). Products were detected by hybridization to ^{32}P-labeled probes specific for the germline Cμ0-JH3 region, the region 5′ of DFL16.1, or the germline Cκ0-Cκ2 region.

Analysis of Protein Tyrosine Phosphorylation. Cells were lysed in a buffer containing 50 mM TrisCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1% deoxycholic acid, 0.1% SDS, 1 mM $NaVO_3$, 1 mM PMSF, and 10 μg each leupeptin, aprotinin, and pepstatin. Antibodies against Syk (Santa Cruz Biotechnology, Inc.) or CD79b (Southern Biotechnology Associates, Inc.) were affixed to protein A/G agarose. 10 μg antibody was incubated overnight with cell lysate (5×10^7 cell equivalents) at 4°C. Beads were collected by centrifugation and washed in lysis buffer. Immunoprecipitates were fractionated by SDS-PAGE and phosphotyrosine was detected by immunoblotting with antibody 4G10 (Upstate Biotechnology).

RNA Isolation and Analysis. Total RNA was extracted from cell suspensions using the TRIzol reagent (Invitrogen). 1 μg total polyadenylated mRNA, isolated by adsorption to oligo-dT–coated beads (Oligotex; QIAGEN), was used as a template for synthesis of double stranded cDNA using reverse transcriptase (Superscript II; Invitrogen) and a T7-$(dT)_{24}$ primer. Biotin-labeled cRNA probes for array hybridization were transcribed from cDNA templates using T7 RNA polymerase (Enzo Biochem).

For RT-PCR, 1 μg total RNA was synthesized using reverse transcriptase (Superscript II; Invitrogen) and random hexameric primers. Reverse transcripts were amplified by PCR. Sequences of oligonucleotide primers are provided in Table S4, available at http://www.jem.org/cgi/content/full/jem.20030729/DC1.

Oligonucleotide Array Hybridization and Data Analysis. Biotin-labeled cRNA probes were hybridized to oligonucleotide microarrays (mouse U74Av2; Affymetrix, Inc.) containing 12,488 probe sets. Transcripts that were scored by Affymetrix Microarray Suite as present on at least one array were analyzed using GeneSpring 4.0 (Silicon Genetics). The signal intensity of each probe set was normalized to the median value of all intensities measured in the corresponding array, and then further normalized to the median of all array-normalized intensities determined for that gene over all hybridizations. Three sets of genes were selected for further study. A gene was included in the first set if (a) its normalized expression in Blk(Y495F) transgenic samples deviated from expression in nontransgenic controls by at least twofold and (b) its expression deviated from that of the control samples with a significance cutoff of $P < 0.01$ (Welch's approximate *t* test). A gene

Table I. *Phenotypic Analysis of B220$^+$ Bone Marrow Populations in Nontransgenic and Blk(Y495F) Transgenic Mice*

Genotype	No. mice	No. total BM $\times 10^{-6}$	Percent B220$^+$	Percent B220$^+$ CD43$^+$ CD22$^{lo/-}$	Percent B220$^+$ CD43^{int} CD22^{int}	Percent B220$^+$ CD43$^-$ IgM$^-$ CD22^{lo}	Percent B220$^+$ IgM$^+$ CD22^{hi}
Nontransgenic	3	22.4 ± 1.2	37.7 ± 2.9	9.2 ± 0.5	<4.5 ± 1.5	12.5 ± 1.7	12.4 ± 0.6
Blk(Y495F)	3	23.4 ± 0.4	45.9 ± 2.6	3.9 ± 0.2	34.1 ± 1.6	4.3 ± 0.3	4.9 ± 0.5

B220$^+$ lymphocyte populations were identified by flow cytometry as described in Materials and Methods and Fig. 1 A. Bone marrow samples were collected from 3–5-wk-old mice in littermate groups. Percentages are defined according to a lymphocyte gate in which 12,000 events were acquired for each mouse. Total cellularity is derived from the number of gated events. Mean values ± SEM are given.

was included in the second set if it was (a) absent from all control arrays but present in all transgenic arrays or (b) present in all control arrays but absent from all transgenic arrays. A gene was included in the third set if its normalized expression differed by more than fivefold between transgenic and nontransgenic samples. Hierarchal clustering was performed using the standard correlation coefficient as a distance metric.

Online Supplemental Material. Fig. S1 supplements Fig. 2 and shows the effect of Blk (Y495F) on expression of the B cell developmental markers CD24, CD25, CD2, and c-kit in RAG-deficient or μMt/μMT mice, as assessed by fluorescence cytometry. Table S1 shows the distribution of B220 CD43$^+$ and B220 CD43$^-$ cells in the bone marrow of RAG-deficient or μMT/μMT mice expressing the Blk (Y495F) transgene. Table S2 shows the distribution of B220 IgM$^+$ and B20 IgM$^-$ B cells in the spleens of Blk (Y495F) transgenic mice. Table S3 supplements Fig. 6 C and assigns the differently expressed genes of known function to functional categories. Table S4 depicts the primer pairs used for RT-PCR, whose results are shown in Fig. 6 D. Fig. S1 and Tables S1–S4 are available at http://www.jem.org/cgi/content/full/jem.20030729/DC1.

Results

Expansion of B Cell Progenitors in Bone Marrow of Blk(Y495F) Transgenic Mice. We first considered whether active Blk might deliver proliferative signals independent of pre-BCR expression using a line of transgenic mice, Blk(Y495F)-15, in which a constitutively active Blk mutant is expressed specifically in the B lymphoid lineage (26). Bone marrow B lymphoid progenitors from 3–5-wk-old

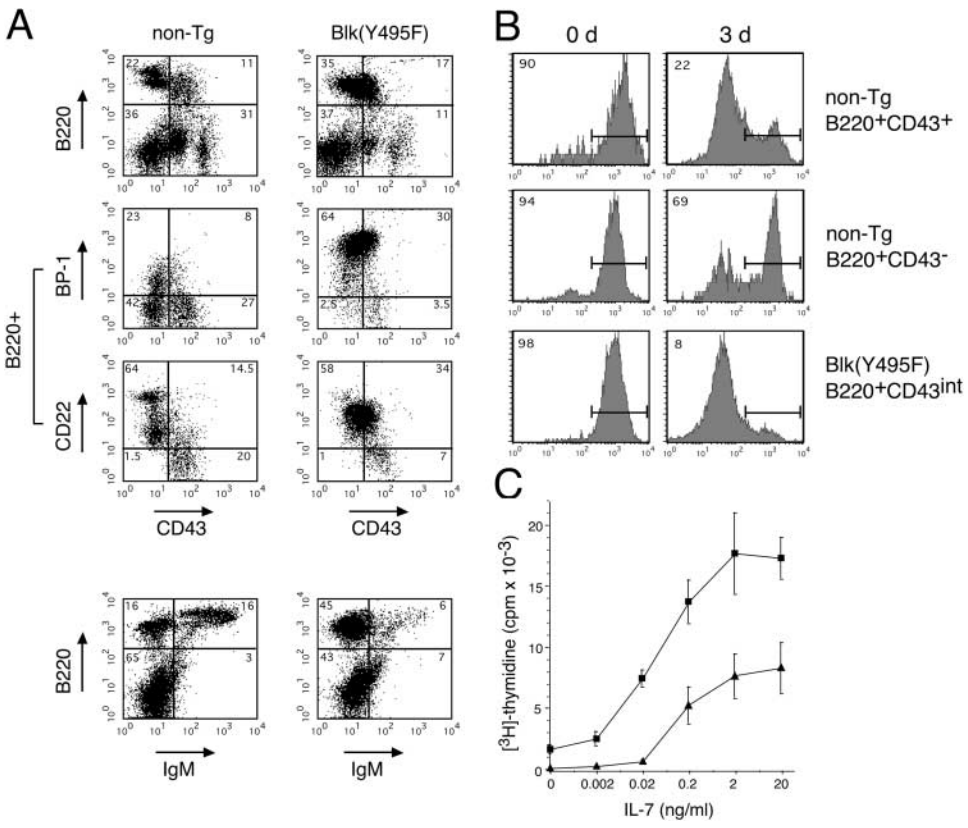

Figure 1. (A) Overrepresentation of B220$^+$ CD43^{int} cells in bone marrow of Blk(Y495F) transgenic mice. Bone marrow cell suspensions from 3–4-wk-old transgenic or nontransgenic littermates were stained for B220 and additional surface markers as indicated. Plots of BP-1 or CD22 versus CD43 were gated on B220$^+$ cells. Numbers indicate percentages of cells in the corresponding quadrants. (B) Hyperproliferation of Blk(Y495F) transgenic B cell progenitors. Bone marrow cells from transgenic or nontransgenic littermates were labeled with PKH26 and maintained in the presence of 20 ng/ml IL-7. At 3 d, cells were counterstained for B220, CD43, and BP-1. Top and middle panels show PKH-26 fluorescence gated on nontransgenic B220$^+$ CD43$^+$ or B220$^+$ CD43$^-$ populations. Bottom panel shows PKH26 fluorescence gated on the transgenic B220$^+$ CD43^{int} population. (C) Hyperresponsiveness of Blk(Y495F) transgenic B cell progenitors to IL-7. Proliferation of B cell progenitors from nontransgenic (▲) or transgenic (■) littermates in response to IL-7 was assayed by [^{3}H]thymidine incorporation (mean ± SEM of three independent trials) as described in Materials and Methods.

transgenic mice and nontransgenic littermates were examined. Blk(Y495F) transgenic and nontransgenic mice showed similar bone marrow cellularity. Transgenic mice, however, exhibited a slight increase in the percentage of $B220^+$ bone marrow cells (Table I).

Phenotypic analysis revealed an expanded subset of $B220^+$ bone marrow cells with a CD43-intermediate ($CD43^{int}$) phenotype, reminiscent of cells at the pro–B to pre–B cell transition (Fig. 1 A and Table I). The $CD43^{int}$ population was homogeneously BP-1^{high} and $CD22^{int}$ and comprised 74.1 ± 6.2% of the $B220^+$ bone marrow compartment. In contrast, $B220^+$ $CD43^+$ and $B220^+$ $CD43^-$ cells comprised 24.5 ± 1.7% and 75.5 ± 9.5%, respectively, of the $B220^+$ bone marrow compartment in nontransgenic mice. The transgenic animals also exhibited decreases in pro–B cells ($B220^+$ $CD43^+$ $CD22^{lo/-}$) and late pre–B cells ($B220^+$ $CD43^-$ $CD22^{lo}$ IgM^-; reference 32). In addition, the $B220^+$ IgM^+ population was relatively diminished in transgenic mice. With respect to their $CD22^{int}$ phenotype, these cells resemble a B progenitor population that accumulates in RAG-deficient mice bearing a μ heavy chain transgene and is expanded upon coexpression of Bcl-2 (33).

The distribution of DJ_H rearrangements from bone marrow cells was consistent with a polyclonal expansion of B cell progenitors in Blk(Y495F) transgenic mice (not depicted). The polyclonality of the B cell expansion in the 3–5-wk-old transgenic mice stands in contrast to the clonality of the $B220^+$ $CD43^+$ tumors that arise in these animals after a protracted latency period of 6–12 mo (26).

Increased IL-7 Responsiveness of B Cell Progenitors from Blk(Y495F) Transgenic Mice. The accumulation of $B220^+$ $CD43^{int}$ cells in the bone marrow suggested that Blk(Y495F) had stimulated cellular proliferation. To examine this, bone marrow cells were loaded ex vivo with PKH-26 and cultured with IL-7 at 20 ng/ml in the presence of autologous stromal cells. After 3 d, the PKH-26 signal remained undiluted in 22 and 69% of the nontransgenic $B220^+$ $CD43^+$ and $B220^+$ $CD43^-$ populations, respectively (Fig. 1 B). In contrast, only 8% of viable $B220^+$ $CD43^{int}$ cells from Blk(Y495F) mice had not undergone cell division by 3 d, as evidenced by PKH-26 fluorescence (Fig. 1 B). Gating of transgenic samples on $B220^+$ $CD43^+$ and $B220^+$ $CD43^-$ populations yielded similar results, as expected because the majority of $B220^+$ cells in transgenic bone marrow are homogeneously $B220^+$ $CD43^{int}$ (unpublished data).

To determine whether the Blk(Y495F) transgene could confer hypersensitivity to IL-7, we cultured bone marrow from transgenic or nontransgenic mice for 5 d under conditions that favor outgrowth of $B220^+$ $CD43^+$ cells. Nonadherent cells were stimulated with increasing amounts of IL-7 in the absence of stromal cells. Thymidine incorporation was measured after 3 d of restimulation (Fig. 1 C). The maximal proliferative response of cells from Blk(Y495F) transgenic mice was more than twice that of cells from nontransgenic animals (17,457 ± 1,501 cpm vs. 8,434 ± 2,079 cpm) and we observed a shift in IL-7 sensitivity. Cells from transgenic mice responded to IL-7 at concentrations as low as 20 pg/ml, whereas cells from nontransgenic animals required a 10-fold higher concentration for a similar response. In this respect, pro–B cells from Blk(Y495F) transgenic mice resembled μ heavy chain transgenic pro–B cells, which also exhibit a lower threshold for responsiveness to IL-7 (5).

We asked whether differences in the frequency of apoptosis might contribute to the outgrowth of B cell progenitors in the transgenic animals. In freshly isolated B lymphoid progenitors from bone marrow of Blk(Y495F) transgenic mice, we observed slight decreases in the apoptotic fraction, as defined by annexin V staining, relative to wild-type. This held whether we gated on $B220^+$ $CD43^+$

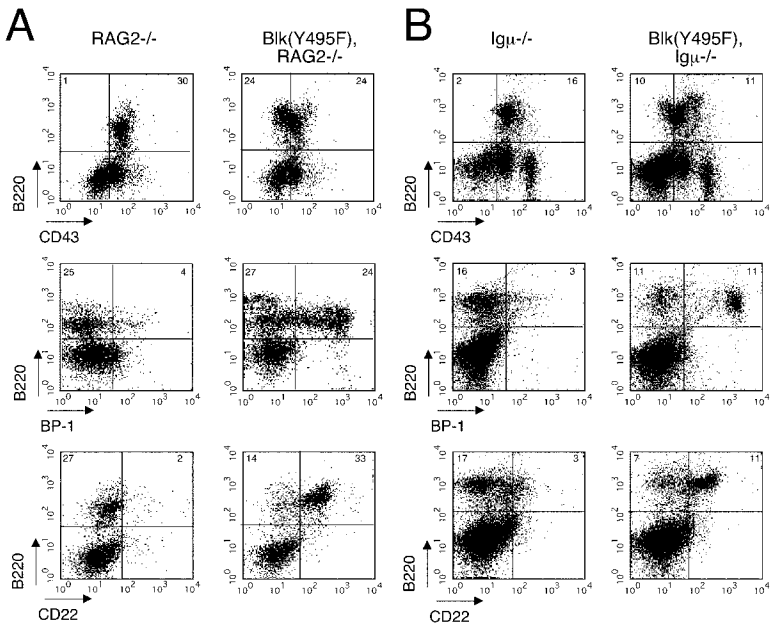

Figure 2. Expression of Blk(Y495F) circumvents developmental blocks in RAG-deficient or μMT/μMT mice. (A) Bone marrow cell suspensions were prepared from 3–5-wk-old RAG-$2^{-/-}$ mice bearing the Blk(Y495F) transgene (right), or from age-matched, nontransgenic RAG-$2^{-/-}$ littermates (left). Cells were stained with an anti-B220 antibody and counterstained with antibodies for additional surface markers as indicated. Numbers indicate percentages of cells in the corresponding quadrants. (B) Analysis, as in A, of bone marrow cells from 3–5-wk-old μMT/μMT mice bearing Blk(Y495F) transgene (right), or from age-matched, nontransgenic μMT/μMT littermates (left).

(16.1 ± 1.5 in transgenic vs. 22.8 ± 2.7 in wild-type) or $B220^+$ $CD43^-$ (15.8 ± 1.6 in transgenic vs. 18.1 ± 1.4 in wild-type) cells. These observations suggest that the accumulation of B cell progenitors expressing Blk(Y495F) results primarily from increased proliferation.

Constitutively Active Blk Overcomes Developmental Blocks in RAG-2$^{-/-}$ and μMT/μMT Mice. In mice lacking RAG-2, B lymphoid development is arrested at the $CD43^+$ pro–B cell stage (Fig. 2 A; reference 28). This block can be overcome by introduction of a μ transgene (34). To test the ability of the Blk(Y495F) mutant to bypass the requirement for μ heavy chain in signaling the pro–B to pre–B cell transition, Blk(Y495F) transgenic animals were crossed with RAG-2–deficient mice. A $B220^+$ $CD43^{int}$ population emerged in the bone marrow of RAG-2–deficient mice bearing the Blk(Y495F) transgene, indicating developmental progression beyond the $CD43^+$ pro–B cell stage (Fig. 2 A and Fig. S1 and Table S1, which are available at http://www.jem.org/cgi/content/full/jem.20030729/DC1). The appearance of the differentiation markers BP-1 and CD22, increased expression of CD24 (HSA) and CD2, and decreased expression of c-kit were all consistent with this interpretation (Fig. 2 A and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20030729/DC1). Therefore, these cells resembled the expanded $CD43^{int}$ population we observed in Blk(Y495F) RAG-$2^{+/+}$ mice.

We then asked whether Blk(Y495F) could support progression to light chain gene rearrangement in the absence of the pre-BCR. To do so, we crossed transgenic animals with μMT/μMT mice (27), in which the pre-BCR is not assembled because the μ transmembrane region is absent. In μMT/μMT mice B lymphoid development is blocked at the pro–B cell stage, although the V(D)J recombination machinery is intact (Fig. 2 B; references 27 and 35). Expression of Blk(Y495F) on the μMT/μMT background supported developmental progression beyond the block imposed by the absence of functional Ig heavy chain, as evidenced by decreased expression of CD43 and increased expression of BP-1 and CD22 (Fig. 2 B and Table S1, available at http://www.jem.org/cgi/content/full/jem.20030729/DC1). Thus, the more mature B progenitor population that emerged when μMT/μMT mice were supplemented with the Blk(Y495F) transgene was similar to the one that accumulated when the transgene was expressed on a wild-type or RAG-deficient background. Moreover, as we observed on the RAG-deficient background, the late pre–B cell marker CD25 (36) was not expressed in the $B220^+$ $CD43^{int}$ population from Blk(Y495F) transgenic μMT/μMT mice (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20030729/DC1), despite the ability of B progenitors from these animals to advance to κ light chain rearrangement (see below).

Initiation of κ Rearrangement in Blk(Y495F) Transgenic μMT/μMT Mice. Light chain rearrangement is suppressed in homozygous μMT mice and productive rearrangement of a μMT allele fails to enforce allelic exclusion (35). To determine whether active Blk could drive developmental progression to κ rearrangement in the absence of a pre-BCR, V_HDJ_H, DJ_H, and $V_\kappa J_\kappa$ rearrangements were assayed in the bone marrow of Blk(Y495F) transgenic μMT/μMT mice and nontransgenic μMT/μMT littermates at 4 wk of age (Fig. 3 A). In Blk(Y495F) transgenic mice, a striking increase in completed $V_\kappa J_\kappa$ rearrangement was observed (Fig. 3 A, top).

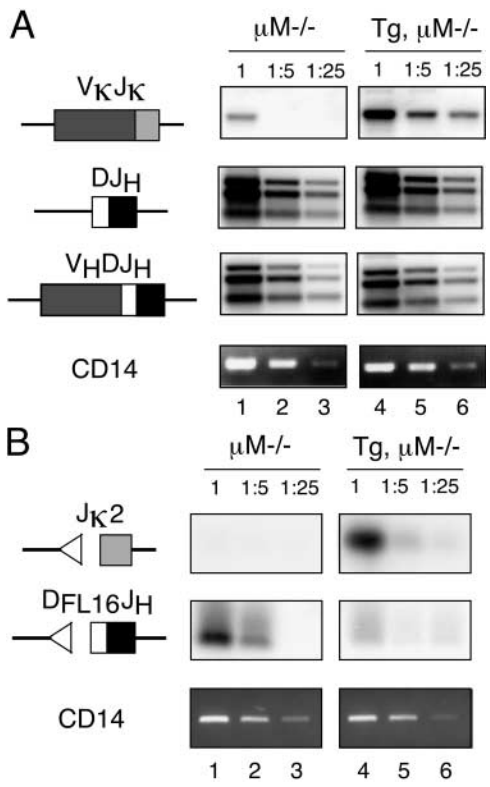

Figure 3. Activation of κ rearrangement and suppression of V_H to DJ_H rearrangement in μMT/μMT mice expressing the Blk(Y495F) transgene. (A) Assay for completed rearrangements at Igμ and κ loci. $V_\kappa J_\kappa$, DJ_H, or V_HDJ_H rearrangements were assayed by PCR in bone marrow cells from Blk(Y495F) transgenic (lanes 4–6) or nontransgenic (lanes 1–3) μMT/μMT mice. Samples were diluted serially fivefold before amplification. Products were separated by gel electrophoresis and detected by hybridization to radiolabeled, locus-specific probes. Amplification of a nonrearranging locus (CD14, bottom) was performed as a control. (B) Assay for DNA cleavage at V(D)J recombination signal sequences. Double strand DNA breaks at the $J_\kappa 2$ recombination signal sequence (top) or the 5′ recombination signal sequence of DFL16.1 (middle) were detected in bone marrow DNA from nontransgenic (lanes 1–3) or transgenic (lanes 4–6) μMT/μMT mice by ligation-mediated PCR. Samples were diluted serially fivefold before amplification. Products were detected as in A.

Detection of completed rearrangements, however, is not a direct indicator of recombinase activity at a given locus because differences in the frequency of rearrangement can be masked by differential outgrowth and survival of cells after completion of recombination. Indeed, when we examined completed V_HDJ_H and DJ_H rearrangements we observed no significant difference between transgenic and wild-type bone marrow (Fig. 3 A, second and third panels from top). To evaluate recombinase activity at κ and heavy chain loci directly, we assayed signal end recombination intermediates,

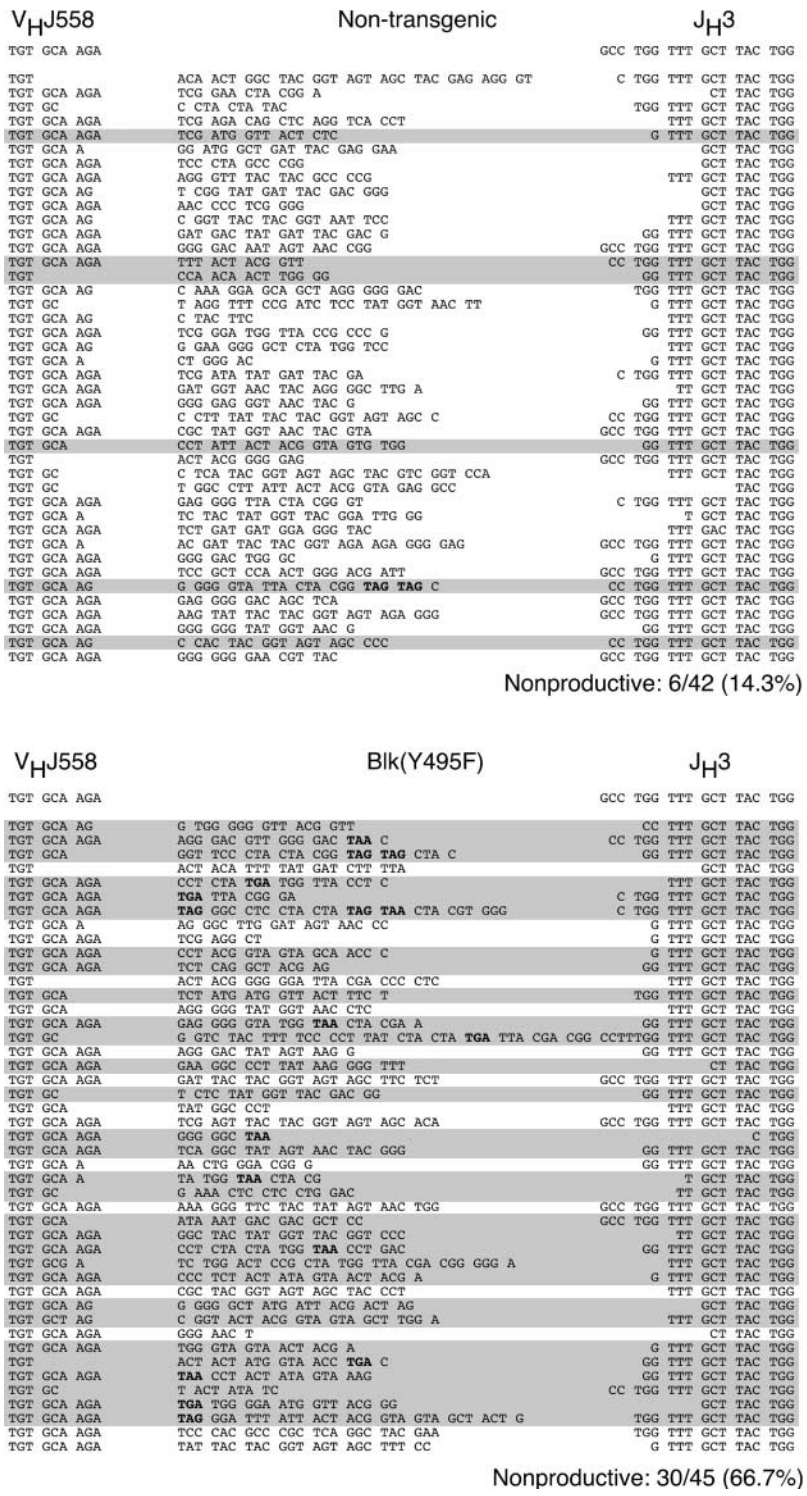

Figure 4. Blk(Y495F) relieves selection for productive heavy chain gene rearrangement. Nucleotide sequences of V_HDJ_H junctions from nontransgenic or Blk(Y495F) transgenic littermates are shown. Genomic DNA was purified from sorted $B220^+$ $CD43^-$ bone marrow cells of 3-wk-old nontransgenic or Blk(Y495F) transgenic mice. V_HJ558-D-J_H3 junctions were amplified by PCR. Nucleotide sequences of individual, cloned junctions are displayed. The 3′ and 5′ ends of germline V_HJ558 and J_H3 segments, respectively, are shown at the top. For each entry below, the 3′ end of sequence derived from V_HJ558 and the 5′ end of sequence derived from J_H3 are separated by sequence derived from the D segment and any N or P nucleotide additions. Nonproductive rearrangements are shaded. The difference in abundance of nonproductively rearranged alleles from transgenic and nontransgenic mice was highly significant ($P < 0.000001$).

an indicator of ongoing V(D)J rearrangement, by ligation-mediated PCR. In the bone marrow of Blk(Y495F) transgenic μMT/μMT mice, double strand DNA breaks at the $J_κ2$ recombination signal, which indicate ongoing $V_κ$ to $J_κ$ rearrangement, were at least 25 times more abundant than in nontransgenic μMT/μMT littermates (Fig. 3 B, middle). In contrast, recombination signal ends associated with DFL16, which indicate continuing V_H to DJ_H rearrangement, were at least five times less abundant in transgenic μMT/μMT animals than in nontransgenic μMT/μMT littermates (Fig. 3 B, top). Taken together, then, these observations provide direct evidence that the Blk(Y495F) transgene mimics the ability of the pre-BCR to activate $V_κ$ to $J_κ$ rearrangement and suppress V_H to DJ_H rearrangement.

Relief of Selection for Functional Rearrangement at the Heavy Chain Locus in Blk(Y495F) Transgenic Mice. B cell progenitors that do not assemble a functional heavy chain gene are eliminated by apoptosis. We wished to test whether the Blk(Y495F) transgene relieves selection for functional heavy chain gene rearrangement at this checkpoint. V_HDJ_H joints were amplified by PCR from sorted $B220^+$ $CD43^-$ bone marrow cells of three 3–4-wk-old Blk(Y495F) transgenic mice and three nontransgenic littermates (Fig. 4). Of 42 V_HJ558-D-J_H3 rearrangements obtained from nontransgenic mice, 6 (14.3%) were found to be nonproductive, whereas of 45 V_HJ558-D-J_H3 rearrangements isolated from Blk(Y495F) transgenic mice, 30 (66.7%) were nonproductive. The increased representation of nonproductive rearrangements in transgenic mice is highly significant ($P < 0.000001$) and approximates the level expected for random rearrangement in the absence of selection. Similar results were obtained when rearrangements were amplified from unsorted bone marrow cells (unpublished data). These results are consistent with the interpretation that constitutively active Blk relieves the selection for functional heavy chain rearrangement at the pro-B to pre-B transition by enabling cellular survival in the absence of the pre-BCR.

Peripheral Accumulation of IgM^- B Lymphoid Progenitors in Blk(Y495F) Transgenic Mice. B cell progenitors can accumulate in secondary lymphoid tissues under conditions of stress or polyclonal activation (37, 38), as well as in RAG- or heavy chain–deficient mice expressing activated Ras in the B lymphoid compartment (39, 40). This also occurred when Blk(Y495F) was expressed in the B lineage. Although overall splenic structure and cellularity were not significantly affected by the transgene, a substantial decrease in the percentage of $B220^+$ cells expressing IgM was seen in spleens of 3–5-wk-old transgenic animals relative to nontransgenic littermates (16.9 ± 2.4 in transgenic animals, 51.6 ± 1.7 in nontransgenic controls; Fig. 5 A and Table S2, available at http://www.jem.org/cgi/content/full/jem.20030729/DC1). Most remaining B lymphoid cells were $B220^{lo}$, $CD43^{int}$, $CD22^{int}$, and $BP\text{-}1^{high}$, suggesting that they were derived from the B progenitor population that accumulates in bone marrow. Consistent with this interpretation, splenic RNA from transgenic mice contained transcripts corresponding to *RAG-2*, *TdT*, *VpreB* (Fig. 5 B), and *λ5* (not depicted), whose expression is characteristic of lymphoid progenitors. The B progenitors found in the spleens of young transgenic mice were polyclonal (unpublished data), in contrast to the clonal tumors that arise in these mice after 6–12 mo. These observations suggest that in Blk(Y495F) transgenic mice, B cell progenitors emigrate to peripheral lymphoid organs in the absence of BCR expression.

Consequences of Blk Activation. The ability of Blk(Y495F) to mimic multiple pre-BCR signals suggested a point of action near proximal targets of pre-BCR signaling. Accordingly, basal tyrosine phosphorylation of Igβ (Fig. 6 A) and Syk (Fig. 6 B) were substantially increased in pro–B cells from transgenic mice relative to pro–B cells from nontransgenic animals.

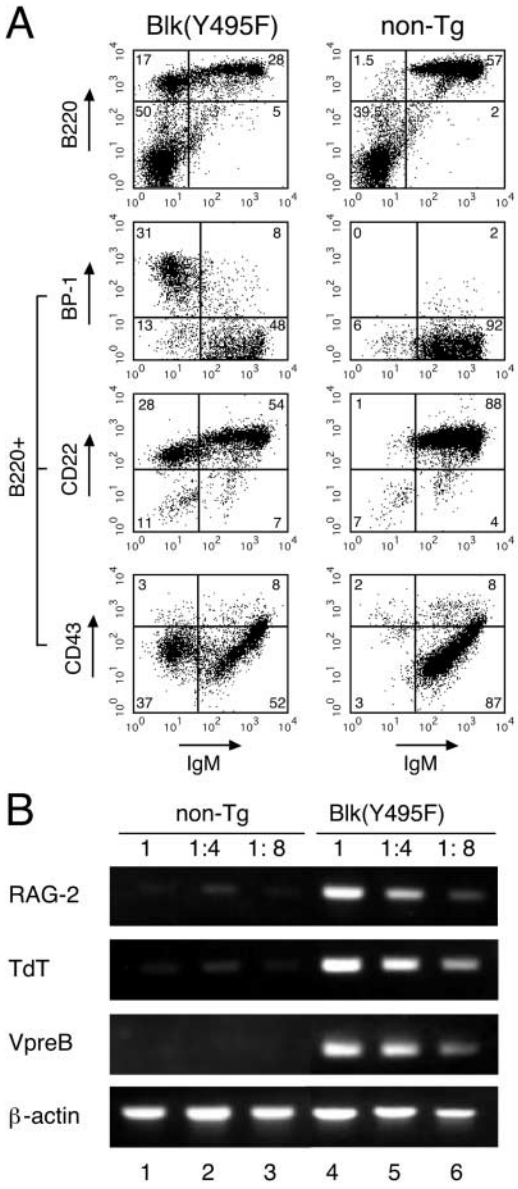

Figure 5. Appearance of cells bearing a B progenitor phenotype in spleens of Blk(Y495F) transgenic mice. (A) Flow cytometric analysis. Single cell suspensions from spleens of transgenic (left) or nontransgenic (right) littermates (3–5 wk old) were stained with antibodies to the indicated markers. Plots of BP-1, CD22, or CD43 versus IgM (bottom three pairs) are gated on $B220^+$ cells. Numbers indicate the percentage of cells in the corresponding quadrant. (B) Peripheral expression of immature B cell markers in Blk(Y495F) transgenic mice. RNA was prepared from spleens of nontransgenic (lanes 1–3) or transgenic (lanes 4–6) littermates and transcripts encoding RAG-2, terminal nucleotidyl transferase (TdT), VpreB, or actin were detected by RT-PCR. Products were diluted as indicated above, fractionated by gel electrophoresis, and detected by staining with ethidium bromide.

The availability of matched, pro–B cell populations allowed us to examine the effects of Blk activation on gene expression in B lymphoid progenitors. Bone marrow cells from transgenic and nontransgenic animals were maintained with IL-7 for 10 d to obtain $B220^+$ $CD43^+$ cells (>98% purity), from which RNA was isolated. Preparations from three

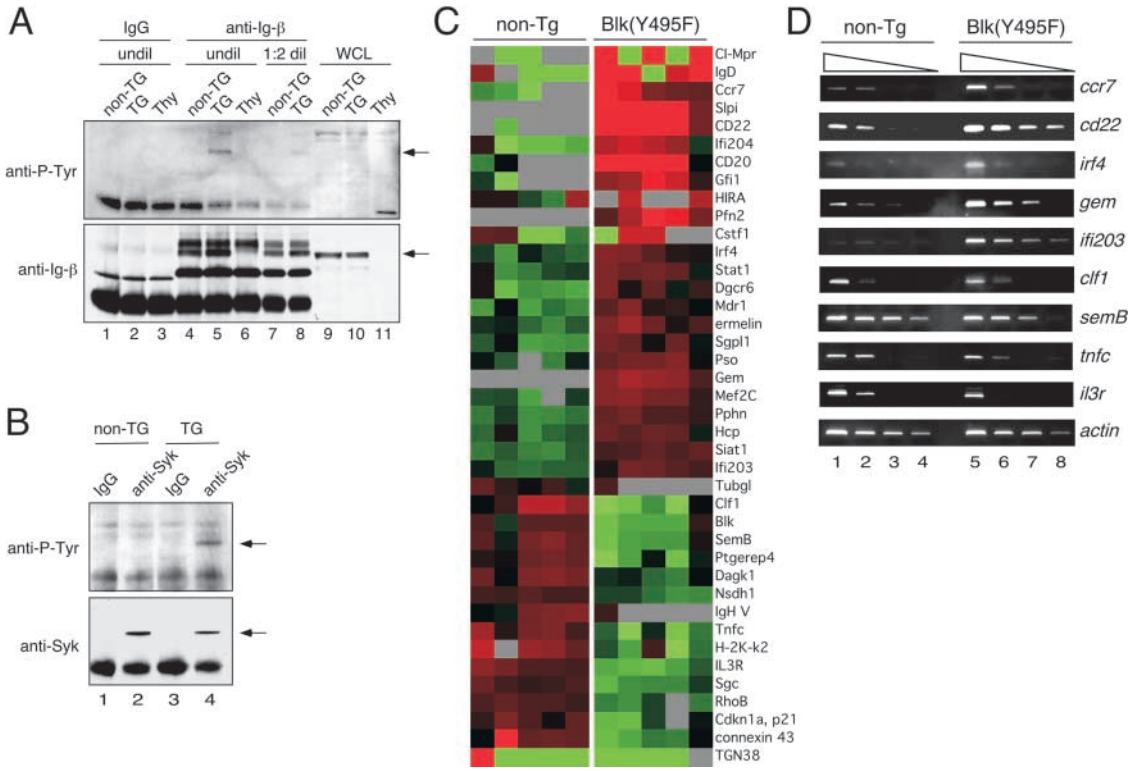

Figure 6. Targets of proximal and distal signaling in B cell progenitors from Blk(Y495F) transgenic mice. (A) Constitutive tyrosine phosphorylation of Igβ in transgenic B cell progenitors. Lysates were prepared from pro–B cells of nontransgenic (lanes 1, 4, and 7) or transgenic (lanes 2, 5, and 8) mice, and Igβ was immunoprecipitated (lanes 4–8). Control immunoprecipitations were performed from a thymocyte lysate (lanes 3 and 6) or with nonimmune IgG (lanes 1–3). Undiluted (lanes 1–6) and twofold diluted (lanes 7 and 8) immunoprecipitates were fractionated by electrophoresis alongside whole cell lysates (lanes 9–11). Phosphotyrosine (top) and Ig-β (bottom) were detected by sequential immunoblotting. Arrows mark the position of Igβ. (B) Constitutive tyrosine phosphorylation of Syk in transgenic B cell progenitors. Lysates were prepared from nontransgenic (lanes 1 and 2) or transgenic (lanes 3 and 4) pro–B cells as in A. Syk was immunoprecipitated (lanes 2 and 4) and control immunoprecipitations were performed with nonimmune IgG (lanes 1 and 3). Phosphotyrosine (top) and Syk (bottom) were detected by sequential immunoblotting. (C) Differential gene expression in pro–B cells from transgenic and nontransgenic mice. 35 genes of known function that were differentially expressed in nontransgenic (left) and transgenic (right) pro–B cells are shown. Each column corresponds to one microarray. Red represents expression above and green represents expression below the median value. Black represents expression at the median and gray represents no detectable expression. (D) Confirmation of differential expression. Total RNA from nontransgenic (lanes 1–4) or transgenic (lanes 5–8) pro–B cells was reverse transcribed, diluted serially fourfold, and used as a template for amplification of the transcripts indicated at right. Products were fractionated by gel electrophoresis and detected with ethidium bromide.

mice of each group were pooled and probes from pooled RNA samples were hybridized to arrays representing 12,488 markers. Five replicate hybridizations were performed.

Of the genes assayed, 51 were scored as differentially expressed and of these, the 35 genes of known or inferred function (Fig. 6 C) were assigned to 9 categories (Table S3, available at http://www.jem.org/cgi/content/full/jem.20030729/DC1) as defined by the Gene Ontology Consortium (www.geneontology.org). More than one third (13/35) of these genes encode markers or regulators of B lymphoid development. Those up-regulated in transgenic pro–B cells include CD22, CD20, Siat1 (*siat1*; reference 41), SHP-1 (*hcp*; reference 42), CCR7 (*ccr7*; reference 43), and Igδ, as well as Irf-4 (*lsirf*; *pip*), which stimulates germline *Igκ* transcription (44), Mef2C (*mef2c*), which stimulates expression of J chain (45), and CstF1 (*cstf1*), part of an RNA processing complex that generates Igμ secretory transcripts (46). Consistent with the ability of Blk(Y495F) to suppress heavy chain rearrangement, V_H transcripts (*IgH V*) were diminished in transgenic $B220^+$ $CD43^+$ cells. Down-regulation of transcripts for the prostaglandin E2 receptor (*ptgerep4*), a positive regulator of apoptosis in B lymphoid cells (47), and the IL-3 receptor (*il3r*) are also in agreement with the maturation-promoting effects of active Blk. The results obtained by microarray were confirmed by RT-PCR for five markers that were overexpressed (*ccr7*, *CD22*, *irf4*, *gem*, and *ifi203*) and four markers that were underexpressed (*clf1*, *semB*, *tnfc*, and *il3r*) in transgenic cells (Fig. 6 D).

Discussion

We have shown that a constitutively active form of Blk, expressed in the B lineage at a level comparable to that of the endogenous protein, effects a suite of responses normally stimulated by productive rearrangement and expression of the μ heavy chain, including: (a) increased proliferation of $CD43^{int}$ B progenitor cells, (b) enhanced responsiveness of these cells to IL-7, (c) maturation as reflected by changes in phenotype, (d) suppression of V_H to DJ_H rearrangement, and (e) initiation of κ rearrangement.

IL-7 may limit expansion of B cell progenitors in the bone marrow. Exogenous IL-7 provokes an overexpansion of B cell precursors (48), whereas in IL-7–deficient mice the transition from the pro-B to pre-B stage is partially impaired (49). Assembly of a pre-BCR is associated with increased responsiveness to IL-7 (50), perhaps reflecting convergence of pre-BCR and IL-7 signals at the level of MAP kinase activation (5). A similar increase in IL-7 sensitivity was seen in progenitor B cells from Blk(Y495F) transgenic mice. Increased responsiveness of pre-BCR–expressing cells to IL-7 would present a selective growth advantage when the availability of IL-7 is reduced, as may occur in some stromal microenvironments (4). The increase in IL-7 sensitivity conferred by Blk(Y495F) may contribute to the overrepresentation of B cell progenitors and the apparent lack of selection for μ heavy chain expression observed in Blk(Y495F) transgenic mice. As BP-1 is induced by IL-7 (51), increased sensitivity to this lymphokine may in part explain the BP-1^{high} phenotype of $B220^+$ $CD43^{int}$ transgenic B progenitors.

Expression of active Blk in the B lineage of μMT/μMT or RAG-$2^{-/-}$ mice advances development past the blocks induced by these deficiencies. This is evident from decreased expression of CD43 and c-kit, increased expression of CD24, and appearance of BP-1, CD2, and CD22. This action of Blk resembles the effects of transgenic μ heavy chain (33, 52) or cross-linking of Igβ (11), both of which support differentiation of RAG-deficient pro–B cells to pre–B cells, with concomitant down-regulation of c-kit and CD43 and increased expression of CD24 and CD2.

In several ways, however, Blk(Y495F) and μ heavy chain transgenes differ in the extent to which they support B cell development. In the RAG-deficient setting, μ heavy chain drives the emergence of $B220^+$ $CD43^-$ cells (33, 34, 52), whereas active Blk supports accumulation of a $B220^+$ $CD43^{int}$ population. Moreover, CD25, a marker characteristic of pre–B II cells (36), is acquired by RAG-deficient B progenitors upon the introduction of μ heavy chain (33) or cross-linking of Igβ (11), but not in the presence of Blk(Y495F). Lastly, BP-1, which is induced upon cross-linking of Igβ on RAG-deficient pro–B cells (11), is expressed in the predominant B progenitor population in Blk(Y495F) transgenic RAG-$2^{-/-}$ mice but not in the progenitors that accumulate in μ transgenic $RAG^{-/-}$ animals (11).

Thus, the predominant B progenitor phenotype in Blk(Y495F) transgenic RAG-deficient animals is phenotypically identical to the B progenitor population that is expanded in recombination-competent, Blk(Y495F) transgenic mice, but less mature than the most advanced progenitors observed in μ transgenic RAG-deficient mice. This distinction suggests that the pre-BCR delivers additional signals, perhaps supplied by activation of other BCR-associated kinases, which effect further developmental progression.

The pre-BCR stimulates κ gene rearrangement and suppresses heavy chain rearrangement. A functional pre-BCR, however, is not essential for activation of light chain rearrangement, which occurs at a low level in the absence of membrane-bound μ chain or λ5 (35, 53). Nonetheless, in bone marrow B cell precursors from μMT/μMT mice, Ig light chain gene rearrangement is attenuated and the incidence of specific DNA cleavage at the κ locus is greatly reduced (27, 35). Cross-linking Igβ reverses this attenuation and suppresses ongoing V(D)J rearrangement at the heavy chain locus (12).

With respect to V(D)J recombination, the effects of Blk(Y495F) in a μMT/μMT background are similar to those of Igβ cross-linking. In bone marrow B lineage cells, κ rearrangement is stimulated, whereas the yield of V_H to DJ_H recombination intermediates is reduced. The effects of the Blk(Y495F) transgene on heavy and light chain rearrangement are likely not related to increased IL-7 sensitivity, which would have been expected to promote V_H to DJ_H recombination and suppress κ rearrangement (54). In the T lineage, Lck can supply functions associated with the pre-TCR, including suppression of V_β to $D_\beta J_\beta$ rearrangement and promotion of TCR-α rearrangement (55). An active Ras transgene promotes TCR-α rearrangement but fails to stimulate allelic exclusion at the TCR-β locus (56), suggesting that the ability of Lck to enforce allelic exclusion at the TCR-β locus is not exerted through Ras. The ability of activated Ras to induce κ rearrangement in J_H-deficient mice (39) raises the possibility that Ras mediates the stimulatory effect of Blk on κ rearrangement.

Expression of Blk(Y495F) was associated with constitutive tyrosine phosphorylation of Igβ and Syk, suggesting that the most proximal sequels of pre-BCR signaling are mimicked by Blk activation. A comparison of gene expression in Blk(Y495F) transgenic and nontransgenic B cell progenitors was used to identify direct or indirect targets of pre-BCR signaling. Fewer than 1% of expressed markers exhibited significant differences in levels of expression. Of the 21 annotated genes whose expression increased in transgenic cells, markers associated with development beyond the pro-B stage were disproportionately represented (>41%), validating the expression screen and providing further evidence that active Blk promotes developmental progression.

A recent report demonstrates that mice triply deficient in Blk, Lyn, and Fyn suffer an attenuation of the pro–B to pre–B cell transition, accompanied by deficiencies in tyrosine phosphorylation of PKCλ and activation of nuclear factor (NF)-κB (25). In these animals the leakiness of the developmental block, as well as intact tyrosine phosphorylation of Igα/Igβ and Syk, may reflect the action of residual tyrosine kinases such as Hck, Fgr, and Lck. In this light, our studies of Blk(Y495F) transgenic animals are consistent with and complementary to those obtained with the triple mutant mice.

Impaired activation of the p50-p65 NF-κB heterodimer by the pre-BCR (25) seems unlikely to account for the developmental defect seen in animals lacking Blk, Lyn, and Fyn because B cell development is unimpaired in mice deficient in p50 or p65 (RelA; references 57 and 58). NF-κB was similarly active in nontransgenic and Blk(Y495F) transgenic pro–B cells (unpublished data), although it remains possible that differences in NF-κB activity were masked by the conditions of ex vivo culture. Nonetheless,

differential NF-κB activity is not essential for maintaining the differences in proliferation and developmental maturity that we observed between transgenic and nontransgenic cell populations.

Despite their partial redundancy in supporting the pro-B to pre-B transition, the functions of Blk may differ in detail from those of other Src-related kinases expressed in B lymphoid cells. For example, a constitutively active form of Lyn, unlike the Blk(Y495F) mutant, affects neither the proliferation of B progenitors nor their responsiveness to IL-7 (18). Although such differences are consistent with nonequivalent roles for Blk and other Src-related kinases in early B lymphoid development, the ability of Blk, Lyn, or Fyn to sustain B cell development in the absence of the other two kinases suggests considerable functional overlap.

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