

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

Ig μ 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 $\lambda 5$ and is incorporated, with the accessory chains Ig α and Ig β , 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 Ig κ locus (for review see references 3 and 4). The combined proliferative and antiapoptotic pre-BCR signals contribute to the expansion

of Ig μ -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 Ig μ , Ig β , $\lambda 5$, 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 Ig α and Ig β . First, in RAG-deficient mice, cross-linking of Ig β induces tyrosine phosphorylation of Ig α 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 Ig μ , Ig β cross-linking suppresses heavy chain rearrangement and activates light chain rearrangement (12). Third, in mice lacking the cytoplasmic

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

T. Tretter and A.E. Ross contributed equally to this work.

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domain of Ig β , development beyond the pro-B cell stage is dependent on the Ig α ITAM motifs (13). Fourth, pre-BCR cross-linking is associated with an increase in the amount of tyrosine-phosphorylated Ig β 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 Ig α and Ig β 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 Ig α and Ig β 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 \times SJL), bearing a cDNA encoding Blk(Y495F) under control of the H-2^k promoter and the Ig μ intronic enhancer, has been described (26). μ MT 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 Ig μ or in RAG-2 were generated by interbreeding Blk(Y495F) transgenic and μ MT 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 μ M β -mercaptoethanol, 10 mM Hepes, and 0.7 \times MEM nonessential amino acids (GIBCO BRL) at 37°C in 5% CO₂.

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 10⁵ cells per well. Thymidine incorporation was assayed after 3 d of culture after the addition of [³H]thymidine (1 μ Ci/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 10⁶/ml.

Flow Cytometric Analysis. Bone marrow cells or splenocytes from 3–5-wk-old mice were analyzed on a FACScanTM 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 _{κ} 2 region (29). Products were detected by hybridization to ³²P-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 μ 02, κ 03, or DFL16.1B (31). Products were detected by hybridization to ³²P-labeled probes specific for the germline C μ 0-JH3 region, the region 5' of DFL16.1, or the germline C κ 0-C κ 2 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 NaVO₃, 1 mM PMSF, and 10 μ g 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 μ g antibody was incubated overnight with cell lysate (5 \times 10⁷ 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 μ g 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 μ g 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 1. Phenotypic Analysis of B220⁺ Bone Marrow Populations in Nontransgenic and Blk(Y495F) Transgenic Mice

Genotype	No. mice	No. total BM × 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. Hierarchical 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 μ Mt/ μ MT 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 μ MT/ μ MT 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 supple-

ments 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

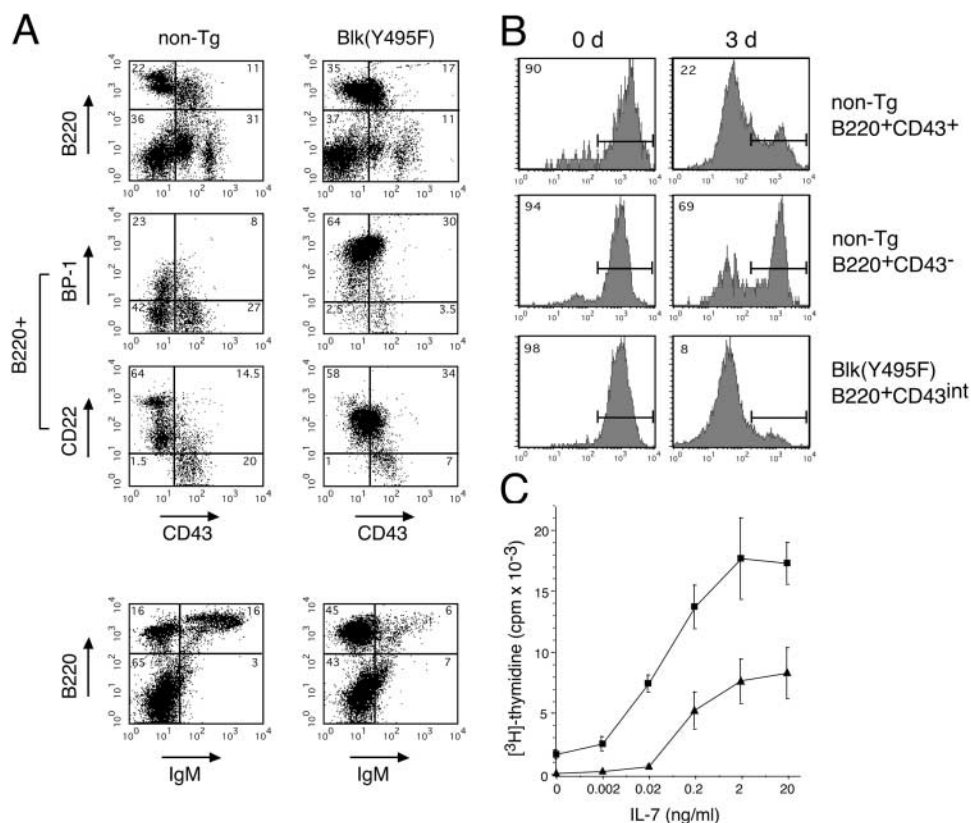


Figure 1. (A) Overrepresentation of B220⁺ CD43^{int} cells in bone marrow of Blk(Y495F) transgenic mice. Bone marrow cell suspensions from 3–4-wk-old transgenic or nontransgenic littermates were stained for B220 and additional surface markers as indicated. Plots of BP-1 or CD22 versus CD43 were gated 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 PKH26 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 [³H]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⁺

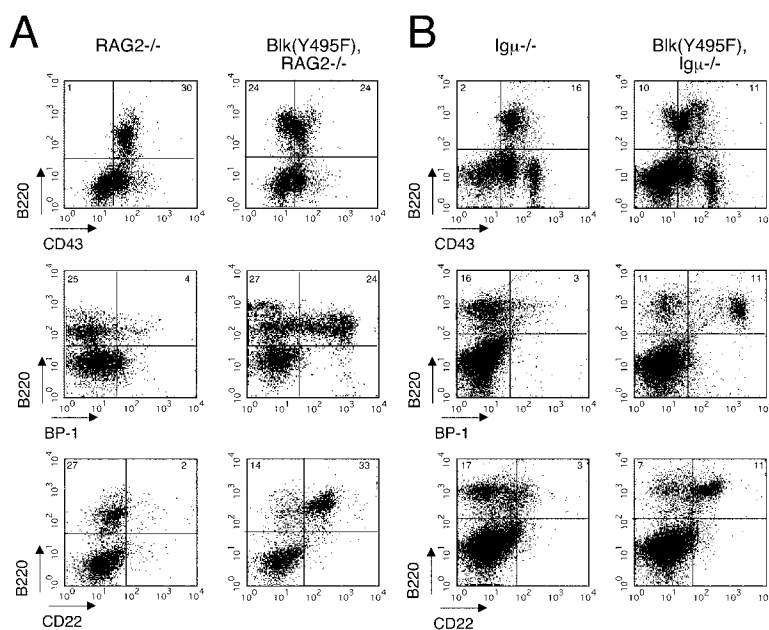


Figure 2. Expression of Blk(Y495F) circumvents developmental blocks in RAG-deficient or μ MT/ μ MT mice. (A) Bone marrow cell suspensions were prepared from 3–5-wk-old RAG2^{-/-} mice bearing the Blk(Y495F) transgene (right), or from age-matched, nontransgenic RAG2^{-/-} littermates (left). Cells were 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 μ MT/ μ MT mice bearing Blk(Y495F) transgene (right), or from age-matched, nontransgenic μ MT/ μ MT 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 μ MT/ μ MT 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 μ MT/ μ MT mice (27), in which the pre-BCR is not assembled because the μ transmembrane region is absent. In μ MT/ μ MT 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 μ MT/ μ MT 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 μ MT/ μ MT 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 μ MT/ μ MT 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 μ MT/ μ MT Mice. Light chain rearrangement is suppressed in homozygous μ MT mice and productive rearrangement of a μ MT allele fails to enforce allelic exclusion (35). To determine whether active Blk could drive devel-

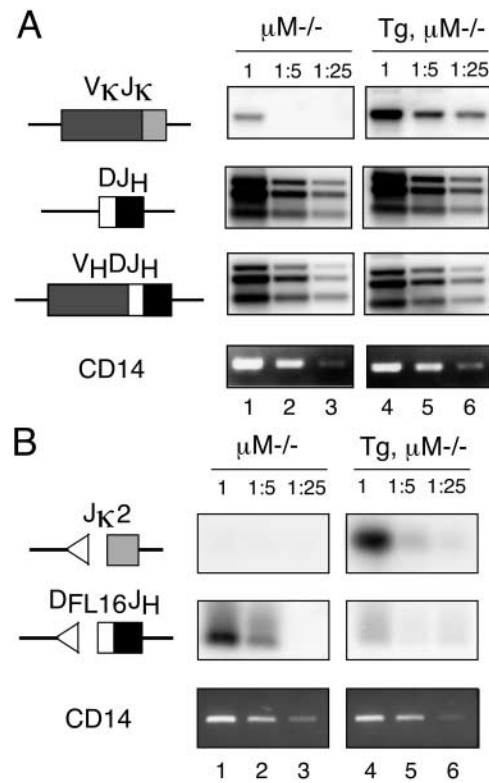


Figure 3. Activation of κ rearrangement and suppression of V_H to DJ_H rearrangement in μ MT/ μ MT mice expressing the Blk(Y495F) transgene. (A) Assay for completed rearrangements at $I\mu$ and κ loci. $V_{\kappa}J_{\kappa}$, DJ_H , or V_HDJ_H rearrangements were assayed by PCR in bone marrow cells from Blk(Y495F) transgenic (lanes 4–6) or nontransgenic (lanes 1–3) μ MT/ μ MT 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) μ MT/ μ MT mice by ligation-mediated PCR. Samples were diluted serially fivefold before amplification. Products were detected as in A.

opmental 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 μ MT/ μ MT mice and nontransgenic μ MT/ μ MT 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).

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,

V _H J558	Non-transgenic	J _H 3
TGT GCA AGA		GCC TGG TTT GCT TAC TGG
TGT GCA AGA	ACA ACT GGC TAC GGT AGT AGC TAC GAG AGG GT	C TGG TTT GCT TAC TGG
TGT GC	TCG GAA CTA CGG A	CT TAC TGG
TGT GCA AGA	C CTA CTA TAC	TGG TTT GCT TAC TGG
TGT GCA AGA	TCG AGA CAG CTC AGG TCA CCT	TTT GCT TAC TGG
TGT GCA AGA	TCG ATG GTT ACT CTC	G TTT GCT TAC TGG
TGT GCA A	GG ATG GCT GAT TAC GAG GAA	GCT TAC TGG
TGT GCA AGA	TCC CTA GCC CGG	GCT TAC TGG
TGT GCA AGA	AGG GTT TAC TAC GCC CCG	TTT GCT TAC TGG
TGT GCA AG	T CGG TAT GAT TAC GAC GGG	GCT TAC TGG
TGT GCA AGA	AAC CCC TCG GGG	GCT TAC TGG
TGT GCA AG	C GGT TAC TAC GGT AAT TCC	TTT GCT TAC TGG
TGT GCA AGA	GAT GAC TAT GAT TAC GAC G	GG TTT GCT TAC TGG
TGT GCA AGA	GGG GAC AAT AGT AAC CCG	GCC TGG TTT GCT TAC TGG
TGT GCA AGA	TTT ACT ACG GTT	CC TGG TTT GCT TAC TGG
TGT	CCA ACA ACT TGG GG	GG TTT GCT TAC TGG
TGT GCA AG	C AAA GGA GCA GCT AGG GGG GAC	TGG TTT GCT TAC TGG
TGT GC	T AGG TTT CCG ATC TCC TAT GGT AAC TT	G TTT GCT TAC TGG
TGT GCA AG	C TAC TTC	TTT GCT TAC TGG
TGT GCA AGA	TCG GGA TGG TTA CCG CCC G	GG TTT GCT TAC TGG
TGT GCA AG	G GAA GGG GCT CTA TGG TCC	TTT GCT TAC TGG
TGT GCA A	CT GGG AC	G TTT GCT TAC TGG
TGT GCA AGA	TCG ATA TAT GAT TAC GA	C TGG TTT GCT TAC TGG
TGT GCA AGA	GAT GGT AAC TAC AGG GGC TTG A	TT GCT TAC TGG
TGT GCA AGA	GGG GAG GGT AAC TAC G	GG TTT GCT TAC TGG
TGT GC	C CTT TAT TAC TAC GGT AGT AGC C	CC TGG TTT GCT TAC TGG
TGT GCA AGA	CCG TAT GGT AAC TAC GTA	GCC TGG TTT GCT TAC TGG
TGT GCA AGA	CCG ATT ACT ACG GTA GTG TGG	GG TTT GCT TAC TGG
TGT	ACT ACG GGG GAG	GCC TGG TTT GCT TAC TGG
TGT GC	C TCA TAC GGT AGT AGC TAC GTC GGT CCA	TTT GCT TAC TGG
TGT GC	T GGC CTT ATT ACT ACG GTA GAG GCC	TAC TGG
TGT GCA AGA	GAG GGG TTA CTA CCG GT	C TGG TTT GCT TAC TGG
TGT GCA A	TC TAC TAT GGT TAC GGA TTG GG	T GCT TAC TGG
TGT GCA AGA	TCT GAT GAT GGA GGG TAC	TTT GAC TAC TGG
TGT GCA A	AC GAT TAC TAC GGT AGA AGA GGG GAG	GCC TGG TTT GCT TAC TGG
TGT GCA AGA	GGG GAC TGG GC	G TTT GCT TAC TGG
TGT GCA AGA	TCC GCT CCA ACT GGG ACG ATT	GCC TGG TTT GCT TAC TGG
TGT GCA AG	G GGG GTA TTA CTA CCG TAG TAG C	CC TGG TTT GCT TAC TGG
TGT GCA AGA	CAG GGG GAC ACG TCA	GCC TGG TTT GCT TAC TGG
TGT GCA AGA	AAG TAT TAC TAC GGT AGT AGA GGG	GCC TGG TTT GCT TAC TGG
TGT GCA AGA	GGG GGG TAT GGT AAC G	GG TTT GCT TAC TGG
TGT GCA AG	C CAC TAC GGT AGT AGC CCC	CC TGG TTT GCT TAC TGG
TGT GCA AGA	GGG GGG GAA CGT TAC	GCC TGG TTT GCT TAC TGG

Nonproductive: 6/42 (14.3%)

V _H J558	Blk(Y495F)	J _H 3
TGT GCA AGA		GCC TGG TTT GCT TAC TGG
TGT GCA AG	G TGG GGG GTT ACG GTT	CC TTT GCT TAC TGG
TGT GCA AGA	AGG GAC GTT GGG GAC TAA C	CC TGG TTT GCT TAC TGG
TGT GCA	GGT TCC CTA CTA CCG TAG TAG CTA C	GG TTT GCT TAC TGG
TGT	ACT ACA TTT TAT GAT CTT TTA	GCT TAC TGG
TGT GCA AGA	CCT CTA TGA TGG TTA CCT C	TTT GCT TAC TGG
TGT GCA AGA	TGA TTA CCG GA	C TGG TTT GCT TAC TGG
TGT GCA AGA	TAG GGC CTC CTA CTA TAG TAA CTA CGT GGG	C TGG TTT GCT TAC TGG
TGT GCA A	AG GGC TTG GAT AGT AAC CC	G TTT GCT TAC TGG
TGT GCA AGA	TCG AGG CT	G TTT GCT TAC TGG
TGT GCA AGA	CCT ACG GTA GTA GCA ACC C	G TTT GCT TAC TGG
TGT GCA AGA	TCT CAG GCT ACG AG	GG TTT GCT TAC TGG
TGT	ACT ACG GGG GGA TTA CGA CCC CTC	TTT GCT TAC TGG
TGT GCA	TCT ATG ATG GTT ACT TTC T	TGG TTT GCT TAC TGG
TGT GCA	AGG GGG TAT GGT AAC CTC	TTT GCT TAC TGG
TGT GCA AGA	GAG GGG GTA TGG TAA CTA CGA A	GG TTT GCT TAC TGG
TGT GC	G GTC TAC TTT TCC CCT TAT CTA CTA TGA TTA CGA CCG CCTTTGG	TTT GCT TAC TGG
TGT GCA AGA	AGG GAC TAT AGT AAG G	GG TTT GCT TAC TGG
TGT GCA AGA	CBA GGC CCT TAT AAG GGG TTT	T GCT TAC TGG
TGT GCA AGA	GAT TAC TAC GGT AGT AGC TTC TCT	GCC TGG TTT GCT TAC TGG
TGT GC	T CTC TAT GGT TAC GAC GG	GG TTT GCT TAC TGG
TGT GCA	TAT GGC CCT	TTT GCT TAC TGG
TGT GCA AGA	TCG AGT TAC TAC GGT AGT AGC ACA	GCC TGG TTT GCT TAC TGG
TGT GCA AGA	GGG GGC TAA	C TGG
TGT GCA AGA	TCA GGC TAT AGT AAC TAC GGG	GG TTT GCT TAC TGG
TGT GCA A	AA CTG GGA CCG G	GG TTT GCT TAC TGG
TGT GCA A	TA TGG TAA CTA CG	T GCT TAC TGG
TGT GC	G AAA CTC CTC CFG GAC	TT GCT TAC TGG
TGT GCA AGA	AAA GGG TTC TAC TAT AGT AAC TGG	GCC TGG TTT GCT TAC TGG
TGT GCA	ATA AAT GAC GAC GCT CC	GCC TGG TTT GCT TAC TGG
TGT GCA AGA	GGC TAC TAT GGT TAC GGT CCC	TT GCT TAC TGG
TGT GCA AGA	CCT CTA CTA TGG TAA CCT GAC	GG TTT GCT TAC TGG
TGT GCG A	TC TGG ACT CCG CTA TGG TTA CGA CCG GGG A	TTT GCT TAC TGG
TGT GCA AGA	CCC TCT ACT ATA GTA ACT ACG A	G TTT GCT TAC TGG
TGT GCA AGA	CGC TAC GGT AGT AGC TAC CCT	TTT GCT TAC TGG
TGT GCA AG	G GGG GCT ATG ATT ACG ACT AG	GCC TGG TTT GCT TAC TGG
TGT GCT AG	C GGT ACT ACG GTA GTA GCT TGG A	TTT GCT TAC TGG
TGT GCA AGA	GGG AAC T	CT TAC TGG
TGT GCA AGA	TGG GTA GTA ACT ACG A	G TTT GCT TAC TGG
TGT	ACT ACT ATG GTA ACC TGA C	GG TTT GCT TAC TGG
TGT GCA AGA	TAA CCT ACT ATA GTA AAG	GG TTT GCT TAC TGG
TGT GC	T ACT ATA TC	CC TGG TTT GCT TAC TGG
TGT GCA AGA	TGA TGG GGA ATG GTT ACG GG	GCT TAC TGG
TGT GCA AGA	TAG GGA TTT ATT ACT ACG GTA GTA GCT ACT G	TGG TTT GCT TAC TGG
TGT GCA AGA	TCC CAC GCC CCG TCA GGC TAC GAA	TGG TTT GCT TAC TGG
TGT GCA AGA	TAT TAC TAC GGT AGT AGC TTT CC	G TTT GCT TAC TGG

Nonproductive: 30/45 (66.7%)

an indicator of ongoing V(D)J rearrangement, by ligation-mediated PCR. In the bone marrow of Blk(Y495F) transgenic μ MT/ μ MT mice, double strand DNA breaks at the J_k2 recombination signal, which indicate ongoing V_k to J_k rearrangement, were at least 25 times more abundant than in nontransgenic μ MT/ μ MT 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 μ MT/ μ MT animals than in nontransgenic μ MT/ μ MT 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_k to J_k rearrangement and suppress V_H to DJ_H rearrangement.

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. Non-productive rearrangements are shaded. The difference in abundance of nonproductively rearranged alleles from transgenic and nontransgenic mice was highly significant (P < 0.000001).

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 $BP-1^{high}$, 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 $\lambda 5$ (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 $Ig\beta$ (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.

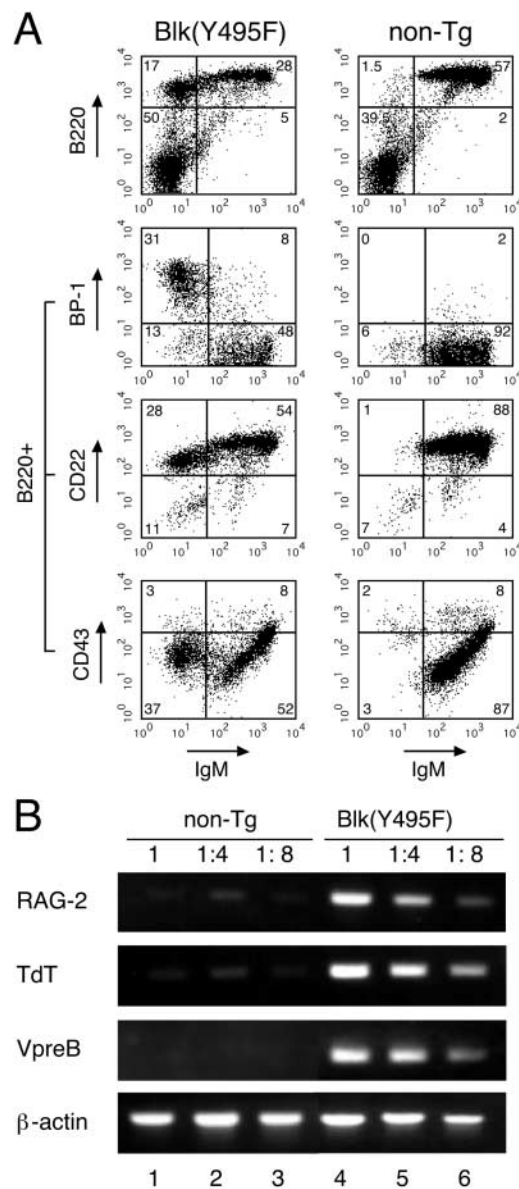


Figure 5. Appearance of cells bearing a B progenitor phenotype in spleens of *Blk(Y495F)* transgenic mice. (A) Flow cytometric analysis. Single cell suspensions from spleens of transgenic (left) or nontransgenic (right) littermates (3–5 wk old) were stained with antibodies to the indicated markers. Plots 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

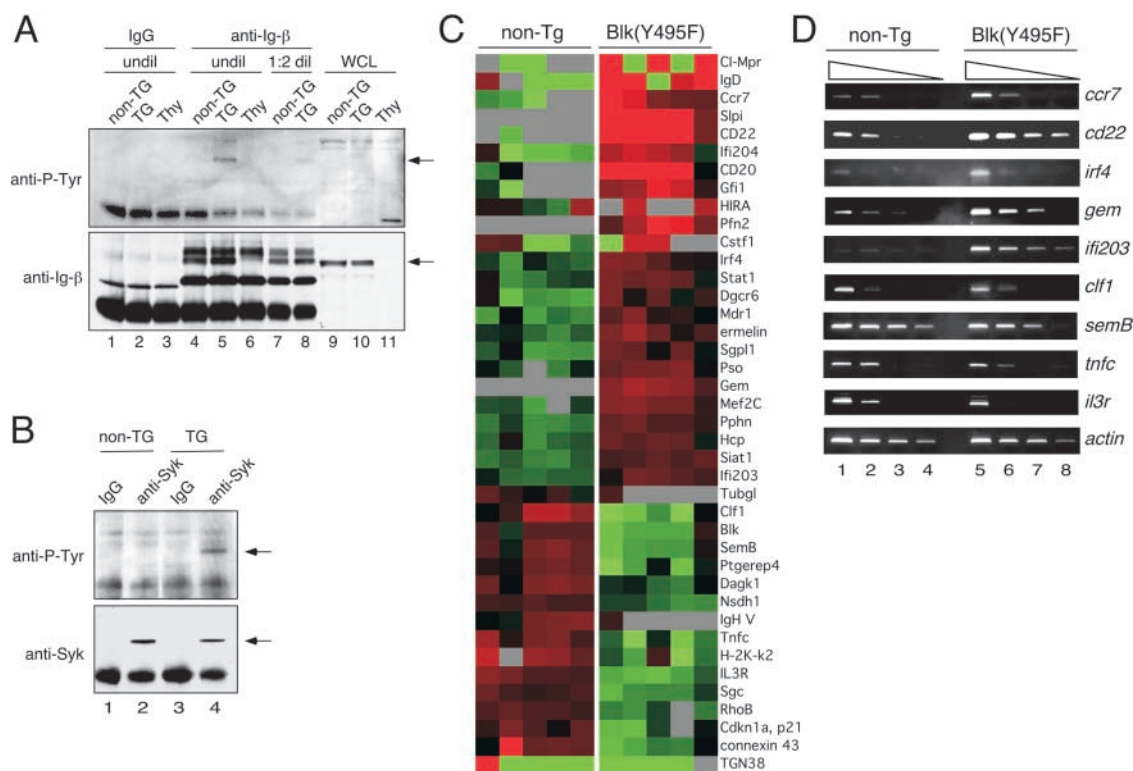


Figure 6. Targets of proximal and distal signaling in B cell progenitors from Blk(Y495F) transgenic mice. (A) Constitutive tyrosine phosphorylation of Ig β in transgenic B cell progenitors. Lysates were prepared from pro-B cells of nontransgenic (lanes 1, 4, and 7) or transgenic (lanes 2, 5, and 8) mice, and Ig β 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 Ig β . (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 (*hsp*; reference 42), CCR7 (*ccr7*; reference 43), and Ig δ , as well as *Irf-4* (*lsif*; *pip*), which stimulates germline *Ig* κ transcription (44), *Mef2C* (*mef2c*), which stimulates expression of J chain (45), and *CstF1* (*cstf1*), part of an RNA processing complex that generates *Ig* μ 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 (*ptgerea4*), 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 *D_{J_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 μ MT/ μ MT 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 Ig β (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 Ig β (11), but not in the presence of Blk(Y495F). Lastly, BP-1, which is induced upon cross-linking of Ig β 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 λ 5 (35, 53). Nonetheless, in

bone marrow B cell precursors from μ MT/ μ MT 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 Ig β 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 μ MT/ μ MT background are similar to those of Ig β 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 _{β} J _{β} 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 Ig β 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 PKC λ and activation of nuclear factor (NF)- κ B (25). In these animals the leakiness of the developmental block, as well as intact tyrosine phosphorylation of Ig α /Ig β 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- κ B 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- κ B was similarly active in nontransgenic and Blk(Y495F) transgenic pro-B cells (unpublished data), although it remains possible that differences in NF- κ B activity were masked by the conditions of ex vivo culture. Nonetheless,

differential NF- κ B 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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