Cutting Edge: A/WySnJ Transitional B Cells Overexpress the Chromosome 15 Proapoptotic Blk Gene and Succumb to Premature Apoptosis

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Cutting Edge: A/WySnJ Transitional B Cells
Overexpress the Chromosome 15 Proapoptotic Blk Gene and Succumb to Premature Apoptosis

Ian J. Amanna, Karen Clise-Dwyer, Faye E. Nashold, Kathleen A. Hoag, and Colleen E. Hayes

Better knowledge of the factors that regulate peripheral B lymphocyte homeostasis is needed to address the human hypogammaglobulinemia diseases. A defect in the Bcmd gene shortens the B cell life span and causes B cell deficiency in A/WySnJ mice. Previous genetic mapping placed Bcmd near Srebf2 on chromosome 15. Inspection of the human chromosome 22 syntenic region identified the proapoptotic Bik gene as a candidate. Two mapping methods placed the homologous mouse gene, Blk, near Srebf2. The Bik genomic structure was highly homologous to Bik. Sequence analysis ruled out coding region mutations, but Bik transcripts were overly abundant in sorted A/WySnJ T1 B cells. Moreover, enriched transitional B cells showed a cell-autonomous defect leading to excessive apoptosis. Thus, Bcmd may be a direct mutation in Blk, or in a gene involved in Blk regulation, such that excess expression pushes the A/WySnJ transitional B cells past the apoptosis checkpoint to cell death. The Journal of Immunology, 2001, 167: 6069–6072.

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nowledge of the factors that regulate peripheral B lymphocyte homeostasis is incomplete. Perturbations of B cell homeostasis can cause hypogammaglobulinemia or B cell lymphoma (1). Our discovery of a heritable B cell deficiency in A/WySnJ mice has afforded us the opportunity to investigate B cell homeostasis (2). The A/WySnJ mice have 90% fewer peripheral B cells than normal and fail to make significant Ig memory responses (3). The deficiency is controlled by a single, autosomal, incompletely dominant gene, B cell maturation defect (Bcmd),3 that does not alter lymphopoiesis (3) but leads to premature B cell apoptosis at the transitional type-1 (T1) to transitional type-2 (T2) stage of peripheral B cell development (4, 5). These features make the A/WySnJ mouse a valuable genetic model for defining regulatory processes in peripheral B cell development.

Our previous genetic analyses placed Bcmd on chromosome (Chr) 15, between D15 Mit259 and D15 Mit118, an empirical distance of ~0.8 centimorgans (cM) (6, 7). The human Chr 22 syntenic region (8) contains the Bcl-2-interacting killer (Bik) gene that encodes a death-inducing BCL-2 protein family member (9, 10). In human B104 B lymphoma cells, surface IgM ligation increased Bik transcription and triggered apoptosis (11). These two reports suggested that a Bik homolog involved in excessive negative selection might be a Bcmd candidate gene. Investigating this hypothesis, we mapped the putative Bik homolog, Bik-like killer (Blk), to Chr 15, demonstrated its overexpression in A/WySnJ transitional B cells, and showed that these transitional B cells have a cell-autonomous defect leading to excessive apoptosis. These results are discussed in the context of a second suggested Bcmd candidate gene, provisionally designated Baffr (12).

Materials and Methods
Mice

The production and simple sequence-length polymorphism (SSLP) genotyping of (A/WySnJ × CAST/Ei)F1 × A/WySnJ mice for Chr 15 markers was described (7). The mice were housed in our pathogen-free mouse colony; protocols were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin (Madison, WI).

Nucleic acid isolation and PCR

The DNA isolation, PCR, and electrophoresis were described (6). The expressed region of Bik was cloned and sequenced, and transcript abundance studies were performed with the PCR primers 5’-AGGAGCTGTTGCAGAAGCAC-3’ and 5’-GGGGGACAGTCAGAAACAC-3’. The PCR primers for the intronic, Blk-associated SSLP were 5’TGTAGGACTTTCACAGT-3’ and 5’-CCATTGGAAACAGGACACAC-3’. The Blk-Q1 transcript abundance studies were performed with the PCR primers 5’-TTTTCTGATTTACGCCG-3’ and 5’-GGGAGGTAGAGGTAGTG-3’. The Bax and Bcl-2 specific primers were described (13).

RH mapping

The DNA from each T31 radiation hybrid (RH) cell line (Research Genetics, Huntsville, AL) and controls was amplified with Bik-specific PCR primers and analyzed for the unique 305-bp mouse gene product three times.

B cell enrichment, analysis, and culture

Flow-sorted splenic T1 B cells were obtained from adult mice 13 days after 500-rad irradiation (14). Splenocytes were stained following RBC lysis with biotin-goat Ab to mouse μ (Fab; Jackson ImmunoResearch Laboratories, West Grove, PA), and PE Ab to CD23 (clone B3B4), FITC Ab to CD21 (clone 7G6) and Cy5PE-streptavidin, all from BD PharMingen (San Francisco, CA).
Enriched transitional B cells were obtained from the spleens of 1-wk old mice as described (15). A/J B cells, labeled with CellTracker Orange and A/WySnJ B cells labeled with CellTracker Green (Molecular Probes) were mixed in equal proportions and cultured. After culture, cells were analyzed for CellTracker dyes and propidium iodide (PI) exclusion. Some cell cultures also included splenic adherent cells (SAC). Splenocytes (10^7 cells in 1.0 ml/well; 48-well plates) were cultured overnight and nonadherent cells were removed before the purified B cells were added.

For apoptosis assays, RBC-depleted splenocytes (viability >95%) from neonatal mice were cultured in triplicate (2 x 10^6 cells in 1.0 ml/well; 48-well plates) in IMDM supplemented with 5% FBS, 50 μg/ml 2-ME, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Samples were analyzed by flow cytometry for apoptotic B cells after B220 staining, ethanol fixation, and PI staining.

Data analysis

The interval mapping data were analyzed with Map Manager QT software (Whitehead Center for Genome Research) (16) (http://www-genome.wi.mit.edu). The map positions in centimorgans were obtained from the 2000 Chromosome Committee Reports, Mouse Genome Database, Mouse Genome Informatics, The Jackson Laboratory (Bar Harbor, ME) (17) (http://www.informatics.jax.org/ccr/).

Results

RH mapping of the Blk gene

To find the Chr 15 syntenic region, we searched the annotated human Chr 22 sequence (http://www.sanger.ac.uk/HGP/Chr22) for loci mapped in the human and the mouse (8). Sreb2 has been mapped in both species (8, 17) and is very close to D15 Mit118, the closest known SSLP to Bcmd (6). Near Sreb2 on human Chr 22 is the Blk locus (8, 9). The mouse Blk gene is believed to be the Blk homolog, because mouse BLK protein is 43% homologous to human BLK protein (18), but Blk has not been mapped.

We mapped Blk using the T31 RH mapping panel (Research Genetics). The mouse Blk gene retention pattern was analyzed by PCR and entered into a server at the Whitehead Center for Genome Research (Cambridge, MA) (http://www-genome.wi.mit.edu). The RHMAPPER software (Whitehead Center for Genome Research) determined that the Blk gene cosegregated with the Chr 15 markers D15 Mit118 and D15 Mit33 at 48.6 cM on the Mouse Genome Database map (Fig. 1A). This position is very near to D15 Mit118, within the interval defined by our most current analysis (7).

Structure of the Blk gene and identification of a Blk gene SSLP

To confirm the RH mapping data, we sought a Blk-associated polymorphism between strains A/WySnJ and CAST/Ei that could be used to genotype our panel of Chr 15 recombinant (A/WySnJ × CAST/Ei)F1 × A/WySnJ mice (7). To this end we examined a Blk intron, anticipating polymorphisms between A/WySnJ and CAST/Ei due to less sequence conservation. To determine the genomic organization of Blk, basic local alignment search tool searches were done by comparing the Blk mRNA sequence to the publicly available High Throughput Genomic Sequence database (www.ncbi.nlm.nih.gov/genome/seq/MmHome.html). This search yielded a Chr 15 mouse genomic clone (National Center for Biotechnology Information LocusID AL583887) encompassing the Blk coding region. Comparing the mRNA sequence (18) to the genomic sequence, and following the gt/ag rule for intron/exon boundaries, yielded the gene structure (Fig. 1, B and C). The mouse Blk gene structure was similar to the human Blk gene, with five exons and four introns of similar size and functional organization. Inspection revealed a putative SSLP in intron 2. When genomic A/WySnJ and CAST/Ei DNA were amplified with PCR primer pairs designed to bracket this repeat, the two strains yielded distinct band sizes (CAST/Ei = 150 bp and A/WySnJ = 180 bp).

SSLP mapping of the Blk gene using Chr 15 recombinant strains

For fine resolution mapping of Bcmd, we assembled a panel of 17 (A/WySnJ × CAST/Ei)F1 × A/WySnJ N2 progeny with recombinations in the interval between D15 Mit144 and D15 Mit118, and measured splenic B cell phenotypes (7). The DNA from this recombinant panel was examined for the Blk-associated intronic SSLP. The genotyping results placed the Blk gene within the gray area shown in Fig. 2, consistent with the RH mapping data.

Overexpression of the Blk gene in A/WySnJ transitional B cells

We next sought evidence for a Blk coding sequence mutation. The cDNA from A/J and A/WySnJ B cells was PCR-amplified, cloned, and sequenced (data not shown). There were no sequence differences between these strains and the published Blk coding sequence (18). Therefore, we investigated the level of Blk transcript expression in sorted T1 B lymphocytes through the use of RT-PCR. Serially diluted cDNA was examined for expression of Blk and a
second proapoptotic gene, Bax, as well as the two antiapoptotic genes that interact with \( \text{Blk} \), Bcl-2, and Bcl-x\(_L\). 

**Discussion**

The evidence presented in this work establishes that the \( \text{Blk} \) gene fits the \( \text{Bcmd} \) candidate gene profile by placement, expression polymorphism, and function. Two methods mapped \( \text{Blk} \) within the region with the highest probability of including \( \text{Bcmd} \). Moreover, the A/WySnJ T1 B cells overexpressed \( \text{Blk} \), and showed a cell-autonomous defect leading to premature apoptosis, consistent with increased expression of a proapoptotic gene. The molecular basis for this expression polymorphism is not yet known.

The \( \text{Blk} \) gene encodes a death-inducing member of the BCL-2 protein family (10). The BCL-2 protein family members share one or more of the BCL-2 homology (BH) domains, BH1, BH2, BH3,
and BH4 (20). The Bcl-2 prosurvival gene was discovered at the characteristic chromosomal translocation breakpoint t(14;18) of follicular B cell lymphoma (21). The Bax pro-death gene was discovered due to interactions between Bcl-2-associated X protein (BAX) and BCL-2 (22). The BCL-2:BAX ratio determined a cell’s susceptibility to apoptosis, leading to the concept that the ratio of proapoptotic and antiapoptotic BCL-2 family proteins serves as a checkpoint or rheostat regulating apoptosis (20).

Bik was discovered due to interactions between BIK and prosurvival BCL-2 family members (10). Bik promoted cell death in transient transfection assays, and Bcl-2 and Bcl-xL suppressed this function. Thus, Bik is a proapoptotic protein and lacks all BH domains except BH3. The BH3-only proteins (BIK, BAD, BID, and HRK) are particularly potent death agonists (20). The mouse homolog of Bik, Blk, was discovered by searching GenBank for sequences encoding conserved BH3 domains (18). The BLK BH3 domain interacts directly with BCL-XL protein (18). In transfec-tion experiments, Bik was a more potent death effector than Bik or Bax, and the apoptotic cell percentage doubled with each doubling of the Blk to Bcl-xL ratio, indicating that this ratio is a very sensitive apoptosis rheostat (18). In the present experiments, Bik was overexpressed 3-fold and Bcl-xL was overexpressed 2-fold, so the Bik:Bcl-xL ratio was higher in the A/WySnJ T1 B cells than in the control cells.

Little is known about regulation of Bik transcription (18). Jiang and Clark (11) showed that surface IgM but not surface IgD ligation initiated Bik transcription and apoptosis in B104 B lymphoma cells, a model for B cell Ag receptor (BCR)-mediated negative selection. We reported that excessive apoptosis occurs at the T1→T2 transition of B cell maturation in A/WySnJ mice (5). The T1 B cells are IgM+IgD− and retain their sensitivity to negative selection via a BCR-mediated apoptotic signal, whereas the T2 B cells are IgM+IgD+ and apparently insensitive to negative selection (23). Thus, our evidence is consistent with a model wherein the A/WySnJ B cells have difficulty making the T1→T2 transition because they express inappropriately high levels of a BCR-induced death gene, and excessive apoptosis is the result.

Intriguingly, Thompson et al. (12) very recently identified a new receptor for B cell activating factor belonging to the TNF family (BAFF), and showed that the gene encoding this receptor, provisionally termed Baffr, maps to human Chr 22q13.1, which is syntenic to the mouse Chr 15 Bcmd region. Also, they showed by Southern blotting that A/WySnJ and A/J genomic DNA are polymorphic at this locus, and that the A/WySnJ Baffr transcript was shortened and exon 3 was not intact. The exact nature of the Baffr mutation in A/WySnJ is not known, and whether the A/WySnJ BAFF receptor (BAFF-R) retains any signaling function is also unclear. Nevertheless, their studies together with ours and those of Jiang and Clark (11) suggest a very interesting model, wherein membrane IgM and BAFF-R may compete for control of Bik transcription and thus the maturation or apoptosis fate of the transitional B cells. The IgM may enhance Bik transcription and BAFF-R may inhibit it. The outcome of the competition might determine peripheral B cell homeostasis. The A/WySnJ T1 B cells may be unable to inhibit Bik transcription due to a BAFF-R defect, explaining their premature apoptosis phenotype. It is also interesting to note that Baffr and Bik are only ~1.3 Mb apart in the human genome, raising the possibility that chromatin remodeling during peripheral B cell development may lead to coordinated expression of these two genes, which may play critical roles in the life or death fate of the transitional B cell. Additional experiments will no doubt test these hypotheses and provide a detailed model of this most interesting B cell developmental transition.

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