Activated Ras Signals Developmental Progression of Recombinase-activating Gene (RAG)-deficient Pro-B Lymphocytes

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Summary

To elucidate the intracellular pathways that mediate early B cell development, we directed expression of activated Ras to the B cell lineage in the context of the recombination-activating gene 1 (RAG1)-deficient background (referred to as Ras–RAG). Similar to the effects of an immunoglobulin (Ig) \( \mu \) heavy chain (HC) transgene, activated Ras caused progression of RAG1–deficient progenitor (pro)-B cells to cells that shared many characteristics with precursor (pre)-B cells, including downregulation of surface CD43 expression plus expression of \( L5, \) RAG2, and germline \( \kappa \) locus transcripts. However, these Ras–RAG pre-B cells also upregulated surface markers characteristic of more mature B cell stages and populated peripheral lymphoid tissues, with an overall phenotype reminiscent of B lineage cells generated in a RAG-deficient background as a result of expression of an Ig \( \mu \) HC together with a Bcl-2 transgene. Taken together, these findings suggest that activated Ras signaling in pro-B cells induces developmental progression by activating both differentiation and survival signals.

Key words: B cell development • pre-B cell receptor • signal transduction • Ras • recombinase-activating gene 2–deficient blastocyst complementation

B lymphocyte development proceeds through a series of stages defined by the expression of surface markers and by the status of Ig gene rearrangement (1). In this developmental program, upon productive rearrangement and expression of Ig \( \mu \) heavy chain (HC) genes, B220–CD43+ pro-B cells progress to B220–CD43– pre-B cells. This transition requires expression of the pre-B cell receptor (pre-BCR) complex consisting of \( \mu \) HC associated with the invariant surrogate light chain proteins \( \lambda 5 \) and \( \lambda \) preB, most likely on the cell surface (2). Consistent with this notion, targeted germline deletion of the \( \mu \) membrane exon arrested murine B cell development at the pro-B cell stage (3). Moreover, germline inactivation of mice in either the recombinase-activating gene (RAG)1 or RAG2 genes, which encode components of the V(D)J recombinase required for initiation of antigen receptor gene rearrangement, again resulted in a block in B lymphocyte development at the pro-B cell stage (4, 5). However, expression of a rearranged \( \mu \) HC transgene in the RAG-deficient background partially rescued this developmental block in the B lineage, leading to the generation of B220–CD43– pre-B cells and demonstrating that \( \mu \) chain expression was sufficient to drive this developmental transition (6, 7).

Because the pre-BCR, like the mature BCR, has no known intrinsic enzymatic functions, it must rely upon associated proteins to provide a functional linkage with intracellular signaling pathways. The mature and pre-BCR-associated Ig\( \alpha \) and Ig\( \beta \) contain immunoreceptor tyrosine-based activation motifs (ITAMs), which are targets for phosphorylation by tyrosine kinases (8); these proteins are required for normal B cell development (9, 10). Furthermore, the importance of an ITAM-associated tyrosine kinase activity during early B lymphopoiesis was demonstrated in mice deficient in the syk tyrosine kinase, in which an incomplete block in development was observed at the B220–CD43+ pro-B cell stage (11, 12). Although several downstream signaling pathways can be induced in B cell lines (13), the identity of the targets downstream of the

1Abbreviations used in this paper: BCR, B cell receptor; ES, embryonic stem; DP, double positive (CD4+CD8+); HC, immunoglobulin heavy chain; RAG, recombinase-activating gene; RT, reverse transcriptase.
nonreceptor tyrosine kinases that are activated by the pre-B cell complex has remained unclear. In this context, the R as family of GTPases (14) represents an attractive candidate. In numerous vertebrate systems, Ras proteins have been implicated in linking tyrosine kinase-mediated signal transduction to downstream effectors (15). In the T cell lineage, constitutive expression of activated Ras as in a RAG-deficient background has been shown to drive the expansion and differentiation of double negative thymocytes to the CD4+ CD8+ (double positive, or DP) stage (16). Moreover, Ras-dependent signaling after cross-linking of the mature BCR has also been observed in lymphocyte cell lines (17, 18). We hypothesized that if activation of endogenous Ras represents a necessary event in pre-B cell signaling, then introduction of constitutively activated Ras into RAG-deficient pro-B cells could mimic signaling by the pre-B cell and result in developmental progression.

**Materials and Methods**

**DNA Constructs.** The plasmid pEμ was constructed through ligation of a 1,042-bp fragment containing the Ig Hc enhancer (Eμ) linked to a variable region promoter (19) into the Smal site of Bluescript II SK. A BamHI/PstI fragment containing two exons of the human β-globin gene was introduced at the KpnI site of Bluescript II SK to provide splice sites and a polyadenylation signal. pEμ was digested with Sall, treated with Klenow fragment and alkaline phosphate, and ligated to the c-Ha-ras V12 cDNA (20) to complete pEμRasV12.

Embryonic Stem Cell Transfection and RAG2-deficient Chimera Generation. Cotransfection of RAG1−/− (16) CCE embryonic stem (ES) cells was carried out with 10 μg NotI-linearized pEμRasV12 together with 1 μg linearized PGK-puro (a gift of P.W. Laird, University of Southern California School of Medicine, Los Angeles, CA). DNA was added to 107 ES cells, and transfection was via electroporation at 300 V, 70 μF twice. Cells were selected in 0.5 mg/ml puromycin (Sigma Chemical Co.). Drug-resistant ES colonies were picked and subcloned for injection into RAG2-deficient blastocysts as previously described (21).

A subset of RAG2-deficient chimeras were maintained in a pathogen-free environment, and analyzed at 4–6 wk of age. FACS analysis of stained samples was performed on a Becton Dickinson FACScan®, and sorted of B220− Ly 9.1− B lineage cells was carried out on an Ortho Cytofluorograf II or Becton Dickinson FACScan®. Dot plots were generated using Cell Quest software (Becton Dickinson).

**Western Blot Analysis.** After red blood cell lysis in ammonium chloride, single-cell splenocyte or lymph node suspensions were treated with RIPA lysis solution (0.15 mM NaCl, 0.05 mM Tris-HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) at 106 cells per ml, and postnuclear supernatants were prepared following standard procedures. Proteins were resolved using SDS/10% PAGE (loading 2 × 106 cells equivalents of lysate per lane), transferred to Immobilon-P membranes (Millipore), and probed with an anti-Ha-ras monoclonal antibody (clone F235; Calbiochem), followed by a horseradish peroxidase-linked F(ab')2 sheep anti-mouse Ig (Boehringer Mannheim). For detection we used the ECL system (Amersham), and Ponceau-S staining was employed to verify equivalent protein loading.

**Reverse Transcription PCR Analysis.** RNA was isolated from 106 sorted B220−Ly 9.1− cells derived from RAG1−/− chimeras or wild-type lymph node and spleen, together with sorted B220−CD43− IgM− wild-type pre-B cells, and B220−CD43+ RAG2-deficient pro-B cells using the TRIZOL reagent (GIBCO BRL) and the manufacturer's protocol. 2 μg of RNA was used for first-strand cDNA synthesis using SuperScript II reverse transcriptase (RT) (GIBCO BRL), following conditions recommended by the manufacturer. 1% of each cDNA synthesis reaction was used for PCR amplification, together with two serial fivefold dilutions. 5- and 25-fold cDNA dilution samples were mixed with cDNA derived from J1 ES cells to equalize template amount in reactions amplifying lymphoid-specific λ5 and RAG2; because Bcl-2 and Bcl-xL were both expressed in ES cDNA (data not shown), ES cell cDNA was not diluted into these reactions. The ES cell cDNA dilution was determined through amplification of β-actin. 25 μl reactions contained: 1× PCR buffer, 200 μM dNTPs, 2 μM of both sense and antisense oligonucleotide primers, and 1 U Taq polymerase (Qiagen). Primers for λ5, RAG2, Bcl-2, and β-actin were as previously described (23), except that the 5′ λ5 oligonucleotide primer was 5′ CTGAGGGTCATTAGAGCTCAGA 3′. Primers for Bcl-xL were as previously described (24). All primers contained sequences spanning at least two exons, allowing clear distinction between RAG2 and genomic DNA signals. PCR amplification conditions were as previously described (23). Expected sizes of amplified products were: λ5, 337 bp; RAG2, 515 bp; Bcl-2, 315 bp, Bcl-xL, 557 bp; β-actin, 623 bp. For analysis of germline k transcripts, a primer 5′ of Jκ1 (5′CCACGCATGCTTGAGAGGGT3′) and a 3′ primer within the coding sequence of Jκ2 were used (25). RNA samples were treated with 5 U of RNAase-free DNase (Boehringer Mannheim) in 1× RT buffer for 30 min at 37°C. The DNase was inactivated at 75°C for 10 min, followed by reverse transcription as above. Since germline k transcripts could not be distinguished from contaminating genomic DNA samples, transcript not treated with RT were subjected to PCR analysis. Conditions for amplification were 95°C for 2 min, then 24 cycles at 95°C for 30 s, 60°C for 1 min, and 72°C for 1.5 min. PCR products were resolved on 1.5% agarose gels, blotted onto Zetaprobe GT (Biorad), and probed with 32P-labeled cDNAs for λ5 (6), with cloned PCR fragments for RAG2, β-actin, Bcl-2, and Bcl-xL, or with a HindIII fragment containing the germline Jκ region (26).

**Results**

To direct expression of activated Ras as to B lineage cells, we used an expression construct containing a c-Ha-ras12 cDNA and Ig HC regulatory sequences (Fig. 1 A). This expression construct was transduced into RAG1-deficient ES cells (16), and the resulting ES clones were tested for their ability to generate B lineage cells in the RAG2-deficient blastocyst complementation assay (21). Flow cytometry analysis of bone marrow cells from RAG chimera revealed low numbers of IgM− B220−CD43− B lineage cells (which are absent in RAG-deficient mice); however, these cells were also found in the spleen and lymph nodes in numbers approaching those of normal, Ig-positive B cells.
in wild-type mice (Fig. 2 and data not shown). To verify that B220+ cells were derived from ES cells, the clonotypic marker Ly 9.1 was used (data not shown). Furthermore, expression of the Ha-ras protein in these chimeric mice was confirmed by Western analysis of spleen and lymph node cell lysates using an Ha-ras-specific monoclonal antibody (expression of endogenous Ras in lymphocytes is limited to N-ras and K-ras reference 27) (Fig. 1 B). Therefore, activated Ras as expression results in the generation of B lineage cell populations that substantially populate the peripheral lymphoid tissues of RAG1-deficient mice.

To further delineate the stage of maturation of B lineage cells in RAG mice, we assayed for expression of various genes used to define stages of B cell differentiation. RT-PCR assays demonstrated that populations of splenic or lymph node RAG B lineage cells expressed substantial levels of λ5 and RAG2 transcripts, which are normally transcribed in the pre-B and pre-B cells but generally are absent during later stages of development (23, 28). On the basis of semiquantitative RT-PCR analyses, we determined that RAG cell populations expressed λ5 and RAG2 at levels comparable to those in purified wild-type pre-B cells (Fig. 3). In normal mice, productive rearrangement and expression of μH C genes in developing B cell progenitors leads to the transcriptional activation and rearrangement of κ light chain genes (29–33). To study if signaling by activated Ras could mimic the induction of κ germline transcription normally induced by expression of H C in pro-B cells, we determined the levels of germline κ transcripts in RAG B lineage cells. We observed that such transcripts were present in RAG B lineage cells at levels similar to those in wild-type pre-B cells (Fig. 4). These results suggest that activated Ras as signaling in RAG-deficient B lineage cells promotes transcriptional activation of the κ light chain gene locus.

Given the large number of peripheral B lineage cells in RAG mice, we further assayed for staining of more mature B cell surface markers. On the basis of these assays, we also found that the RAG B lineage cells in both the bone marrow and periphery expressed surface antigens usually associated with later stages of B cell development, such as the low affinity IgE Fc receptor CD23 (34), the BCR coreceptor CD22 (35), and complement receptor CD21/CD35 (36) (Fig. 2 and data not shown). Thus, our data suggest that expression of activated Ras as results in the development of RAG-deficient B lineage cells that retain major properties of pre-B lymphocytes while also expressing cell surface markers usually found only in mature B cell stages. These RAG B lineage cells are distinct from those generated in the RAG-deficient background via expression of an Igμ H C transgene, which only promotes differentiation to cells that show pre-B cell characteristics and remain primarily in the bone marrow (6, 7), but are similar in patterns of gene expression and tissue distribution to those observed in the RAG-deficient mouse.
Bcl-2 and Bcl-xL in Ras–RAG B cells were more comparable levels of Bcl-2 expression (Fig. 5).

The similarity of the R as-R AG phenotype to that promoted by μ-HC plus Bcl-2 transgenes suggested to us that activated R as may signal both differentiative and cell survival processes. During normal B cell development, the anti-apoptotic gene Bcl-2 is expressed at the pro-B stage, but is downregulated in pre-B cells and later upregulated in mature B lymphocytes (23, 38). In contrast, the cell survival gene Bcl-xL displays a reciprocal pattern of expression, with high levels in pre-B cells that are downregulated in mature B cells (39). To assay for Bcl-2 and Bcl-xL expression in Ras-RAG peripheral B lineage cells, we used RT-PCR analysis and determined that the expression levels of Bcl-2 and Bcl-xL in R as-R AG B cells were more comparable with those in wild-type mature B cells with substantial levels of Bcl-2 expression (Fig. 5).

Discussion

Several studies have implicated R as as an intermediate in signal transduction downstream of the BCR (17, 18). Our studies of R as-R AG mice demonstrate that activated R as can induce differentiation of pro-B cells in the absence of μ-HC. B lineage cells that develop in R as-R AG mice acquired characteristics shared with normal pre-B cells, including expression of RAG and λ5, and the induction of germline κ light chain locus transcripts. However, these cells also expressed surface markers characteristic of more mature stages of B lymphopoiesis. Thus, we believe it is most likely that R as-R AG B cells have progressed in their differentiation beyond the pre-B cell stage, at least to the pre-B-B cell junction. It also remains possible, given their peripheral location, that R as-R AG B cells may be developmentally similar to the recently described subset of germinal center B cells that reinitiate light chain gene rearrangement as a result of antigenic challenge (40-42). Although such cells, unlike R as-R AG B cells, lack CD23 expression (43), both cell types demonstrate the concurrent expression of λ5 and RAG genes with mature B cell surface markers. The accumulation of the R as-R AG B lineage cells in the periphery could be due to several potential effects of activated R as expression, including acquisition of surface markers necessary for transit from the bone marrow or prolonged survival allowing exit from the marrow and accumulation in the periphery.

Previous work has demonstrated that the introduction of a rearranged μ-transgene into RAG-deficient pro-B cells induced their differentiation to pre-B cells (6, 7). Although such cells remained predominantly within the bone marrow and did not express surface markers characteristic of more mature B cells, the expression of a Bcl-2 transgene in the B lineage of μ-R AG mice resulted in the appearance of cells in the marrow and periphery with a phenotype that resembles B lineage cells found in R as-R AG mice (22, 37). These findings suggested that survival signals provided by Bcl-2 may advance B lymphopoiesis beyond the stage achieved by μ-HC alone. In this regard, we found that R as-R AG cells expressed significantly higher levels of endogenous Bcl-2 than normal pre-B cells; in fact, the observed Bcl-2 expression levels approached those in mature B cells. At present, we do not know whether this upregulation of Bcl-2 expression in R as-R AG cells is directly in-
duced by activated R as, or, alternatively, occurs as a result of developmental progression to a more mature stage. Nevertheless, the phenotypic similarities between μ H C/Bcl-2/ R AG and R as-R AG B cells suggest that introduction of activated R as may induce and/or enable both differentiation and survival signals in R AG-deficient and, presumably, normal progenitor B lineage cells.

Our finding that B cells in R as-R AG mice develop to a stage beyond that of B cells in μ-R AG mice indicates that signaling events triggered by constitutively activated R as may surpass or differ from those initiated upon H C-mediated activation of endogenous R as. In this context, in other experimental systems the effects of activated R as on cultured cells varied depending on the level and duration of R as expression (44). It is also possible that the expression of activated R as in B lineage cells mimics signaling from other surface receptors, in addition to the pre-BCR, which normally trigger endogenous R as. Numerous R as effector pathways have been identified to date, including a well-characterized mitogen-activated protein kinase cascade and a growing number of stress-activated protein kinase cascades (45). R as has also been shown to induce phosphatidylinositol-3 kinase (46, 47), as well as the Rho family of GTPases which regulate the actin cytoskeleton (48). It remains to be established which of these (or other) R as effector pathways are involved in mediating the developmental progression of pro-B cells. Selective engagement of R as effectors using activated mutant alleles may facilitate further elucidation of these issues.

Recent data demonstrate that R as signaling is used during several stages of B and T lymphocyte development. For example, a dominant negative R as transgene was shown to cause an incomplete block in B cell development at the earliest known B cell precursor stage before B220<sup>+</sup>CD43<sup>+</sup> pro-B cells (49). In T lineage cells, several studies with dominant negative alleles implicated the R as/Raf/mitogen-activated protein kinase pathway in the development of CD4<sup>+</sup>CD8<sup>+</sup> (DP) thymocytes and mature T cells (50-52). Notably, a complete reconstitution of DP thymocytes was induced by activated R as in R AG-deficient mice; however, in these mice no developmental progression beyond the DP stage was observed, and no T cells were detected in the peripheral lymphoid organs (16). These results suggested that additional signals are required for the T cell positive selection process that normally results from signaling events accompanying ligation of the T cell receptor with self-MHC ligands of specific avidity (53). Such a requirement for additional signaling events, independent of R as, in the development of T cells beyond the DP stage suggests that an important distinction may exist in the signals required to effect further development of precursor B versus precursor T lineage cells.

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