

# Control of B cell development by Ras-mediated activation of Raf

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**Cell fate commitment in a variety of lineages requires signals conveyed via p21<sup>ras</sup>. To examine the role of p21<sup>ras</sup> in the development of B lymphocytes, we generated transgenic mice expressing a dominant-negative form of Ras in B lymphocyte progenitors, using a novel transcriptional element consisting of the E $\mu$  enhancer and the *lck* proximal promoter. Expression of dominant-negative Ras arrests B cell development at a very early stage, prior to formation of the pre-B cell receptor. Furthermore, an activated form of Raf expressed in the same experimental system could both drive the maturation of normal pro-B cells and rescue development of progenitors expressing dominant-negative Ras. Hence p21<sup>ras</sup> normally regulates early development of B lymphocytes by a mechanism that involves activation of the serine/threonine kinase Raf. *Keywords:* B cell development/Raf/Ras/transgenic mice**

## Introduction

*Ras* genes encode a family of structurally related GTP-binding proteins which regulate differentiation in a number of different systems (reviewed in Eisenmann and Kim, 1994; Wassarman *et al.*, 1995). Each of the three mammalian *ras* genes (*H-ras*, *K-ras* and *N-ras*) encodes a membrane-associated 21 kDa protein that acts as a molecular switch to convey extracellularly derived signals into the cell interior. Ras proteins normally exist in an equilibrium between active (GTP-bound) and inactive (GDP-bound) states. In numerous instances where receptor-type protein kinases have been studied, receptor activation leads to tyrosine phosphorylation of the receptor itself, recruitment of highly conserved SH2 domain-containing adaptor proteins [e.g. Grb-2 (Schlessinger, 1994)], and subsequent binding of the guanine-nucleotide releasing protein Son of Sevenless (Sos). Formation of this complex stimulates an exchange of GDP for GTP on p21<sup>ras</sup> which, in its GTP-bound form, stimulates a variety of downstream effector enzymes including the serine/threonine kinase c-Raf-1 (Moodie *et al.*, 1993; Vojtek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993; Marais *et al.*, 1995), phosphatidylinositol-3 kinase (PI3 kinase) (Rodriquez-Viciana *et al.*, 1994), other low-molecular-weight GTPases

(Hofer *et al.*, 1994; Prendergast *et al.*, 1995; Qiu *et al.*, 1995), and possibly the Ras-GAP protein (which also serves as a negative regulator of Ras itself) (Adari *et al.*, 1988; Cales *et al.*, 1988).

While Ras proteins are ubiquitously expressed, tissue and developmental stage-specific differences in the expression of the three different Ras isoforms encourage the view that each Ras protein may subserve a specialized function (Furth *et al.*, 1987; Leon *et al.*, 1987). Hematopoietic cells contain both the N- and K-*ras* isoforms, and where Ras mutations occur in hematopoietic malignancies, N-*ras* is most frequently affected (Guerrero *et al.*, 1984, 1985; Lemoine, 1990). Surprisingly, mice made deficient in N-*ras* expression are phenotypically normal, presumably due to compensatory activation of other *ras* gene products or their downstream targets (Umanoff *et al.*, 1995).

To explore the importance of *ras*-mediated pathways in T lymphopoiesis, we previously expressed a dominant-negative human H-*ras* mutant, H-*ras*N17, as a tissue-specific transgene, thereby avoiding problems of gene redundancy or lethality associated with disruption of ubiquitously expressed genes (Swan *et al.*, 1995; Perlmutter and Alberola-Ila, 1996). Transgenic mice which express this dominant-negative form of Ras under control of the thymocyte-specific *lck* proximal promoter manifest a profound defect in positive selection, the late-stage cell fate commitment event wherein CD4<sup>+</sup>CD8<sup>+</sup> (double-positive) cells mature to become immunocompetent CD4<sup>+</sup>CD8<sup>-</sup> or CD8<sup>+</sup>CD4<sup>-</sup> (single-positive) T cells (Swan *et al.*, 1995). Similarly, expression of dominant-negative Raf (O'Shea *et al.*, 1996), or a kinase-inactive form of MAP kinase kinase (dMek) (Alberola-Ila *et al.*, 1995, 1996), again using the *lck* proximal promoter, yields mice with an identical phenotype, indicating that this step in thymocyte development depends at least in part on activation of Ras, which in turn stimulates Raf, and thereby activates MEK (Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Seger *et al.*, 1994). Ras also regulates late-stage maturation of monocytes, as demonstrated by experiments employing a myeloid-specific promoter element derived from *c-fms* to drive expression of either the catalytic domain of the negative regulator Ras-Gap, or a dominant-negative form of Ets-2 (Jin *et al.*, 1995), a transcription factor which has been shown to participate in developmental events controlled by p21<sup>ras</sup> in other systems (Langer *et al.*, 1992; Wasyluk *et al.*, 1993; Bradford *et al.*, 1995). These studies in T cells and monocytes together suggested that p21<sup>ras</sup>, perhaps by stimulating the Raf-induced serine/threonine kinase cascade, might act to regulate B cell maturation as well.

Early B lineage cells can be identified in the bone marrow by virtue of their expression of B220 (CD45R) in the absence of detectable surface or cytoplasmic IgM (Li *et al.*, 1996). These cells subsequently mature according to

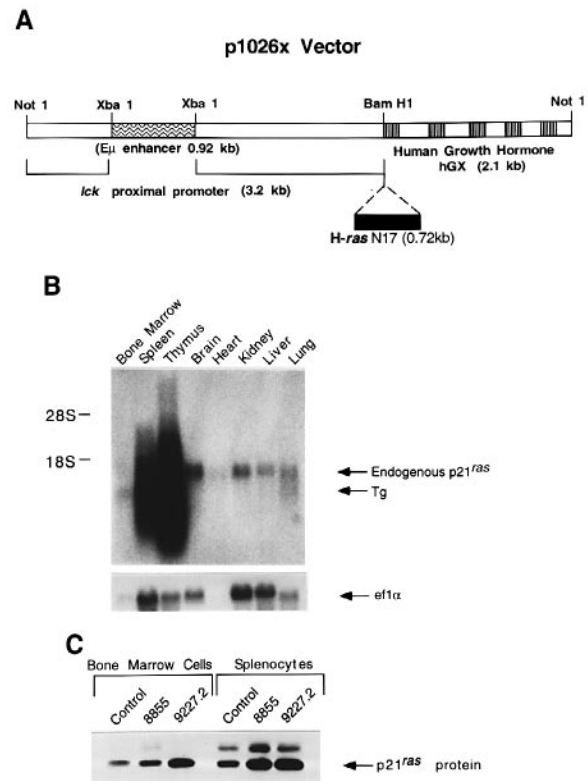
a developmental program that mimics in most respects the process that governs thymocyte maturation. In particular, B220<sup>+</sup>IgM<sup>-</sup> pro-B cells sustain V-D-J rearrangements of their heavy chain genes leading to expression of a cytoplasmic  $\mu$  heavy chain. The  $\mu$  heavy chain then appears at the cell surface, along with the Ig $\alpha$  and Ig $\beta$  chains, and a 'surrogate' light chain composed of the VpreB and  $\lambda 5$  polypeptides, as a component of the pre-B cell receptor. Thereafter, signals derived from the pre-B cell receptor stimulate light chain gene rearrangement and further maturation, giving rise to immature B220<sup>lo</sup>IgM<sup>+</sup> B cells. It is these cells that initially populate peripheral lymphoid tissues, where development continues (for a review see Melchers *et al.*, 1994). Although these stages of B cell maturation can be resolved phenotypically, little is known about the signaling pathways that govern B cell development.

To elucidate control points in B lymphopoiesis, we have initiated studies aimed at enumerating signal transduction pathways that regulate B cell maturation. Here we report that p21<sup>ras</sup> serves as a pivotal regulator of signals governing the transition from B220<sup>+</sup> pre-pro-B cells to the more mature pro-B cell stage. In addition, we show that expression of an activated form of Raf both stimulates this critical transition in wild-type mice, and rescues B cell development in transgenic mice which co-express dominant-negative Ras.

## Results

### Generation of transgenic mice expressing a dominant-negative form of p21<sup>ras</sup> in B lymphocytes

To examine the role of p21<sup>ras</sup> in the development of B lymphocytes, we generated transgenic mice in which a dominant-negative form of p21<sup>ras</sup> (H-rasN17) was expressed throughout B lymphopoiesis. We chose to express H-rasN17 rather than other dominant inhibitors of Ras because the asparagine-for-serine mutation at position 17 has been shown to potently inhibit normal Ras function in other systems (Szeberenyi *et al.*, 1990; de Vries-Smits *et al.*, 1992; Medema *et al.*, 1993; Schweighoffer *et al.*, 1993), and because we have previously shown that H-rasN17 functions effectively in the lymphocytes of transgenic mice to inhibit MAP and S6 kinase activation (Swan *et al.*, 1995). All evidence supports the view that H-rasN17 competes directly for the Sos guanine nucleotide exchange factor, thus inhibiting activation of all Ras isoforms (Chen *et al.*, 1994; Alberola-Ila *et al.*, 1995). We employed a novel, compound transcriptional regulatory element in which the immunoglobulin intronic heavy chain enhancer (E $\mu$ ) and the *lck* proximal promoter are juxtaposed. This vector makes use of the well-characterized *lck* proximal promoter, which directs high-level expression primarily in immature T lineage cells (Wildin *et al.*, 1991), and the intronic enhancer from the immunoglobulin heavy chain locus (Alexander *et al.*, 1987), which was expected to permit expression in B lineage cells as well. It has been shown previously that the immunoglobulin enhancer is active in the earliest committed B lymphocyte progenitor (pre-pro-B cells), when D<sub>H</sub> to J<sub>H</sub> rearrangement first takes place (Li *et al.*, 1996). Hence we hoped that this compound promoter/



**Fig. 1.** Schematic representation of the *lckEμH-rasN17* transgene and total Ras expression in lymphoid tissues. (A) The p1026x vector includes a 3.2 kb fragment of the mouse *lck* gene that contains the proximal promoter to +37 with respect to the transcription start site (open box), a 0.92 kb fragment of the intronic heavy chain enhancer (wavy lines) and a 2.1 kb mutated (non-translatable) version of the human growth hormone gene (vertically striped and stippled segments represent exons and introns respectively). The H-rasN17 cDNA (black box) was inserted into the *Bam*HI site. (B) Expression of transgene-derived and endogenous p21<sup>H-rasN17</sup> mRNA. Shown is a representative tissue RNA blot from a 16-week-old *lckEμH-rasN17* transgenic mouse from the A9227.2 line. 15  $\mu$ g of total RNA from each tissue was separated by agarose electrophoresis, transferred to nylon and hybridized with a 720 bp <sup>32</sup>P-labeled H-rasN17 cDNA fragment as described in experimental procedures. Transgene-derived p21<sup>H-rasN17</sup> RNA includes multiple hybridizing bands, the products of alternative splicing of hGH exons. The position of the endogenous *ras* transcript and of the predominant transgene transcript are noted at the right of the figure. The blot was stripped and reprobbed with a <sup>32</sup>P-labeled efl $\alpha$  cDNA probe as a loading control (see Materials and methods). (C) Expression of p21<sup>ras</sup> protein in wild-type and transgenic lymphoid tissue. Shown is a representative immunoblot performed using 20  $\mu$ g of total cellular protein from bone marrow cells and splenocytes comparing control (non-transgenic littermate) and two independent 12-week-old *lckEμH-rasN17* transgenic (A8855 line and A9227.2 line) mice. Protein-containing samples were separated using 12% SDS-PAGE, transferred to nitrocellulose and visualized using an anti-pan-Ras specific antibody. The slower-migrating band in spleen samples represents an unknown cross-reacting species.

enhancer combination would function throughout B lymphopoiesis.

Figure 1A provides a schematic diagram of the E $\mu$  enhancer/*lck* promoter vector, p1026X, containing a *Bam*HI cloning site within a modified (non-translatable) human growth hormone gene cassette (see Materials and methods). A 7 kb *Not*I fragment containing the *lckEμH-rasN17* expression construct was injected into (C57BL/6/J $\times$ DBA2) F2 mouse zygote pronuclei using standard techniques, and 17 founder mice were generated. Eleven

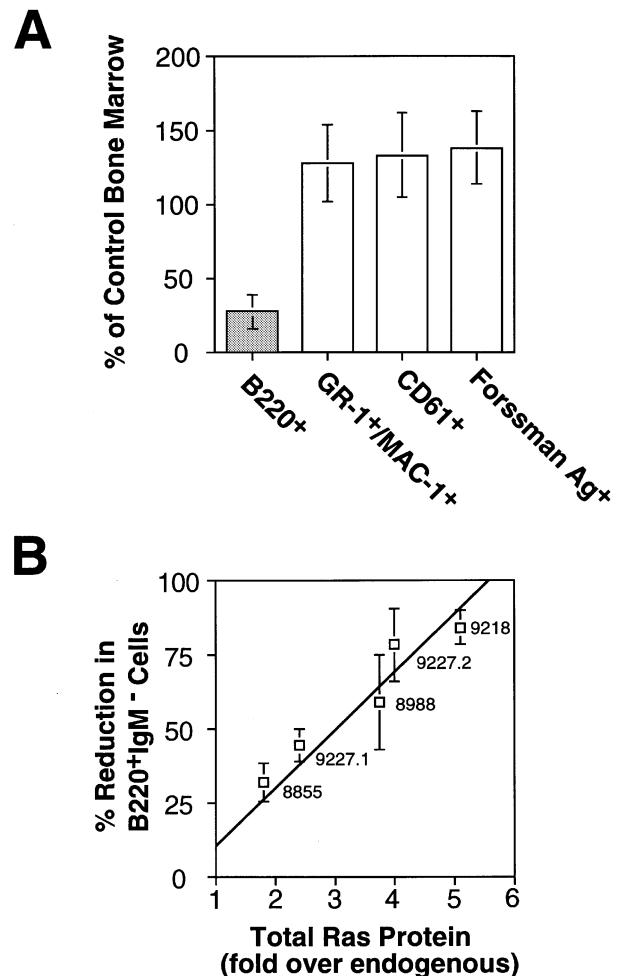
of these founders were examined directly, and six transgenic lines were generated by backcrossing to C57BL/6 mice.

Normal lymphocytes express little detectable p21<sup>ras</sup> in either bone marrow, spleen or thymus, as determined using immunoblotting techniques. However, representative animals from each *lckEμH-rasN17* transgenic line expressed 2- to 30-fold more mRNA (Figure 1B), and immunoreactive p21<sup>ras</sup> protein (Figure 1C) in these tissues. Since there is a paucity of B cells present in bone marrow from transgenic versus control mice (as described below), transgene expression in B cell progenitors is very high on a per-cell basis in *lckEμH-rasN17* transgenic mice. Thus, enrichment for B cell progenitors in bone marrow cells from *lckEμH-rasN17* transgenic mice by depletion of myeloid, T lymphoid and erythroid lineage cells using lineage-specific antibodies and magnetic beads results in a proportional increase in Ras protein detected by Western blot (data not shown). As expected, transgene expression was restricted to lymphoid cells (Figure 1B). Importantly, the *lck* proximal promoter by itself never directs expression of transgenes at appreciable levels in immature B lineage cells (J.Alberola-Ila and R.M. Perlmutter, unpublished data). Hence, introduction of the heavy chain intronic enhancer had the desired effect. The p1026x vector behaves reliably: all of more than 45 transgenic founders generated using this construct manifested significant transgene expression (data not shown). Finally as discussed below, the p1026x vector directs transgene expression very early in B lymphopoiesis.

#### Transgenic mice expressing p21<sup>H-rasN17</sup> manifest a profound block in B cell development

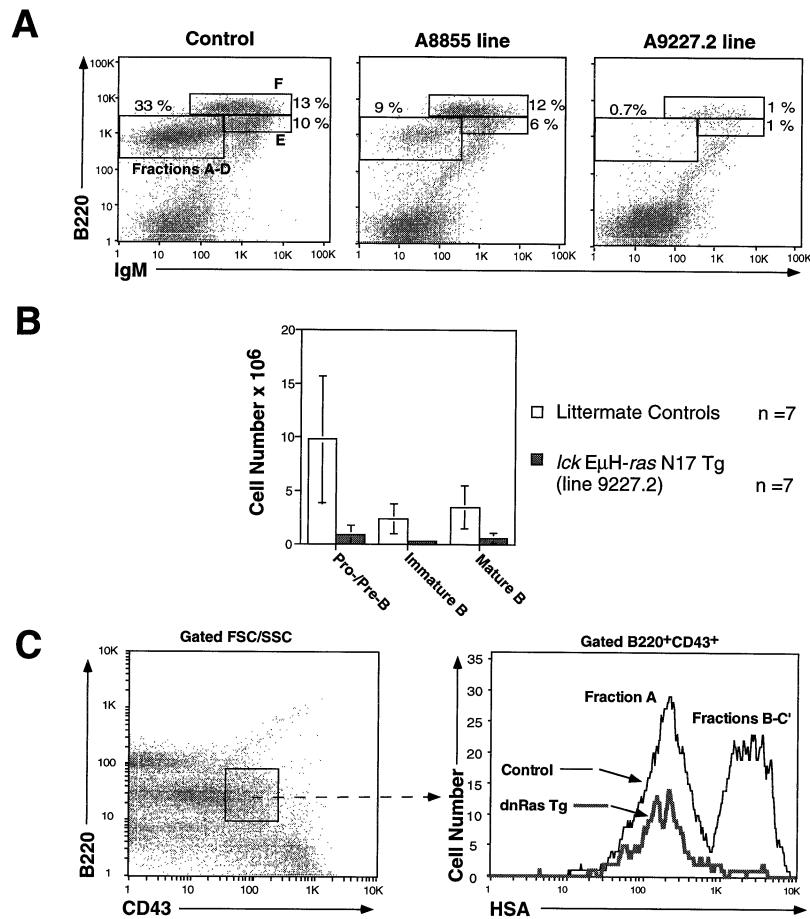
To determine whether p21<sup>ras</sup> function regulates the development of B lymphocytes, total bone marrow cells from transgenic mice expressing *lckEμH-rasN17* were analyzed by flow cytometry for expression of surface markers which distinguish different hematopoietic cell lineages. Although the cellularity of the bone marrow was normal, a significant decrease in the percentage of total bone marrow cells that express the B cell lineage-specific marker B220 (CD45R) relative to control animals was observed, while the representation of non-lymphoid cells within the bone marrow was proportionately increased (Figure 2A). Thus expression of the *lckEμH-rasN17* transgene did not inhibit the generation of GR1/MAC1-, CD61- and Forssman antigen-bearing cells, representing myeloid, megakaryocytic and erythroid progenitors, respectively. Transgenic mice expressing *lckEμH-rasN17* also had normal hematocrits and complete blood counts, normal proportions of circulating white blood cells and normal numbers of myeloid progenitors as judged by CFU-GM assays performed using bone marrow cells (data not shown).

The decrease in B cell progenitors in the bone marrow was dependent on transgene dose, as determined by comparing total Ras protein expression with the percent reduction in B220<sup>+</sup>IgM<sup>-</sup> B cell progenitors in five different lines of *lckEμH-rasN17* transgenic mice (Figure 2B). While the measurement of total Ras protein does not discriminate between transgene-derived and endogenous Ras, RNA analysis confirmed that the observed increase in Ras protein levels correlated directly with the abundance



**Fig. 2.** The development of B lineage cells is selectively perturbed in *lckEμH-rasN17* transgenic mice. **(A)**  $1 \times 10^6$  total bone marrow cells from 8- to 12-week-old *lckEμH-rasN17* transgenic (9227.2 line) and littermate control mice were stained with combinations of phycoerythrin (PE)-conjugated anti-CD45R/B220 and fluorescein isothiocyanate (FITC)-conjugated anti-IgM, which identify mature and immature B cells; PE-conjugated anti-GR1 and FITC-conjugated anti-MAC1, which together identify mature and immature myeloid cells; and PE-conjugated anti-CD61 and biotin-conjugated anti-Forssman antigen followed by streptavidin-tricolor, which identify megakaryocytes and erythroblasts respectively. The percentage of total bone marrow cells from *lckEμH-rasN17* transgenic mice which express the B cell-specific marker B220 is decreased, while the percentage of total bone marrow cells which express the myeloid-specific markers MAC1 and GR1, megakaryocyte marker CD61 and erythroblast marker Forssman antigen is proportionately increased, relative to littermate control mice. Each box represents the mean  $\pm$  SD for six animals analyzed in two separate experiments. **(B)** *lckEμH-rasN17* transgenic mice have a dose-dependent reduction in B lymphocyte progenitors. Total bone marrow cells from 6- to 8-week-old animals were stained with PE-conjugated anti-B220 and FITC-conjugated anti-IgM. 20  $\mu$ g of whole cell lysates from total bone marrow cells were probed by immunoblot for total Ras protein expression as described in Materials and methods. The relative amount of Ras protein expressed was assessed by densitometry. Shown are means  $\pm$  SD for three to five animals of the 9227.1, 8855, 8988 9218 and 9227.2 lines of *lckEμH-rasN17* transgenic mice.

of transgene-derived transcripts (Figure 1B). Collectively, these results demonstrate that the *lckEμH-rasN17* transgene selectively perturbs the development of B lymphocytes in the bone marrow, leaving other non-lymphoid lineages intact.



**Fig. 3.** Dose-dependent reduction of Pro-B/Pre-B, immature B and mature recirculating B lymphocytes in *lckEμH-rasN17* transgenic mice. (A) Shown are representative two-dimensional flow cytometric histograms of bone marrow cells from two lines of 12-week-old *lckEμH-rasN17* transgenic mice and control mice. Bone marrow cells were stained with PE-conjugated anti-CD45R/B220 and FITC-conjugated anti-IgM. The results are presented as a two-dimensional dot plot in which each dot represents an individual cell from a pre-defined lymphocyte forward/side-scatter gate. The percentage of cells in each box is indicated. A8855 is a low-expressing *lckEμH-rasN17* transgenic line while A9227.2 is a high-expressing *lckEμH-rasN17* transgenic line (see Figure 2). There is a dose-dependent reduction of B cell progenitors at all three stages of development in *lckEμH-rasN17* transgenic mice. Fractions A–F denote specific stages in B cell development as characterized by Hardy *et al.* (1991). (B) A bar histogram depicting the total number of pro- and pre-B cells ( $B220^{hi}IgM^{-}$ ), immature B cells ( $B220^{lo}IgM^{+}$ ) and mature recirculating B cells ( $B220^{hi}IgM^{+}$ ) in *lckEμH-rasN17* mice. Shown are the means  $\pm$  SD of seven pairs of 8- to 12-week-old *lckEμH-rasN17* transgenic and littermate control mice from the A9227.2 line. (C) Total bone marrow cells from 12-week-old *lckEμH-rasN17* transgenic and littermate control mice from the A9227.2 line were stained with PE-conjugated anti-CD43, FITC conjugated anti-B220 and biotinylated anti-heat stable antigen (HSA). The biotinylated antibody was detected using streptavidin-tricolor. A representative single-parameter histogram describing HSA expression versus cell number is shown for cells that fall within the pre-determined  $B220^{+}CD43^{+}$  gate. Only fraction A cells remain in high-expressing *lckEμH-rasN17* mice.

### *p21<sup>H-rasN17</sup>* perturbs B cell development prior to formation of the pre-B cell receptor

To define the specific stages at which B cell development was affected by expression of the *lckEμH-rasN17* transgene, total bone marrow cells were stained with antibodies to B220 and surface IgM. As shown in Figure 3A and B, these analyses documented an impressive, transgene dose-dependent reduction in the representation of  $B220^{+}IgM^{-}$  cells (pro- and pre-B cells),  $B220^{lo}IgM^{+}$  cells (immature B cells) and  $B220^{hi}IgM^{+}$  (long-lived recirculating B cells) in the bone marrow. We observed a similar phenotype in mice examined as early as 2 weeks of age, at which time B lineage cells are largely of fetal origin. This phenotype cannot result from the known ability of *p21<sup>H-rasN17</sup>* to impair T cell function, since T cells are not required for these early steps in B cell maturation (Mombaerts, 1995). Moreover, B cell development is not perturbed in mice expressing *p21<sup>H-rasN17</sup>* under the control of the *lck* proximal promoter alone (data not shown).

To pinpoint precisely the earliest stage at which B cell development is perturbed by expression of *p21<sup>H-rasN17</sup>*, we utilized a scheme outlined by Hardy *et al.* (1991), in which seven discrete developmental stages in B lymphopoiesis were defined by flow cytometry (see Figure 8). In normal mice the pro- and pre-B cell compartments, which consist of  $B220^{+}IgM^{-}$  cells, can be divided into two populations: large, cycling cells that express the CD43 molecule (representing Hardy fractions A–C') and small, resting pre-B cells which lack CD43 (representing Hardy fraction D). The  $B220^{+}CD43^{+}$  compartment can be further divided into fractions A, B, C and C' by expression of BP-1 and heat-stable antigen (HSA). As shown in Figure 3C, transgenic mice from a line (9227.2) expressing high levels of *p21<sup>H-rasN17</sup>* produce  $B220^{+}CD43^{+}$  cells which do not express HSA (corresponding to fraction A), but not cells which do express HSA (corresponding to fractions B and C). In addition, the transgenic B cell progenitors do not acquire BP-1 or CD19 (data not shown). Hence B

cell progenitors cannot transit from fraction A to fractions B and C in the presence of significant levels of dominant-negative Ras. While the representation of transgenic B cell progenitors in fraction A increases relative to that in fraction B, the total number of cells in fraction A actually decreases relative to that observed in control mice. This may reflect inhibition of an earlier developmental transition, from a still incompletely defined progenitor, which permits maturation of fraction A cells (Cumano and Paige, 1992; Li *et al.*, 1996). While it is possible that blockade of Ras signaling triggers a death pathway in fraction A cells, B220<sup>+</sup>IgM<sup>-</sup> B cell progenitors in *lckEμH-rasN17* transgenic mice do not show increased apoptosis, as judged using flow cytometric measurement of 7-aminocytomycin D (7AAD) incorporation, relative to controls (data not shown). We note, however, that apoptotic cells may escape detection as a result of rapid phagocytosis (Osmond *et al.*, 1994). Hence we cannot ascertain the mechanism underlying this decline in the numbers of fraction A cells in *lckEμH-rasN17* animals.

Mice which cannot form a pre-B cell receptor, e.g. due to disruption of the membrane exon of the  $\mu$  heavy chain (Kitamura *et al.*, 1991), disruption of the RAG-1 or RAG-2 genes required for antibody gene assembly (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992), or disruption of the Ig $\beta$  chain (Gong and Nussenzweig, 1996), have normal or increased numbers and percentages of cells in fractions A–C', and the proportions of each population are normal relative to control mice. Because there is a decrease in the representation of cells in fractions A–C' in *lckEμH-rasN17* transgenic mice, disruption of normal p21<sup>ras</sup> function must act prior to the assembly of the pre-B cell receptor. Indeed, cells in Fraction A have not yet completed the process of heavy chain gene rearrangement (Li *et al.*, 1993). Hence p21<sup>ras</sup> ordinarily sustains an early transition in B lineage development.

#### **p21<sup>H-rasN17</sup> expression leads to a partial depletion of peripheral B lymphocytes**

Despite the dramatic attenuation of B lineage maturation in the bone marrow cells of *lckEμH-rasN17* mice, some mature B lymphocytes nevertheless emerge to populate the periphery. Figure 4 documents the transgene dose-dependent reduction in the percentage (Figure 4A) and total number (Figure 4B) of B lymphocytes in the spleens of these mice. This represents a 5-fold average decrease in splenic B cell numbers in 6- to 12-week-old mice from the 9227.2 line. There is also a higher representation of immature IgM<sup>hi</sup>IgD<sup>lo</sup> cells within the spleen of *lckEμH-rasN17* mice relative to littermate control mice (Figure 4A). As mice age, the number of B cells in transgenic mice approaches that of littermate control mice, perhaps reflecting peripheral expansion of transgenic B cells which escape the early developmental block. Importantly, these cells still express comparable levels of p21<sup>H-rasN17</sup>.

Ligation of the B cell antigen receptor on mature B cells is known to lead to activation of p21<sup>ras</sup> within 1–2 min, as indicated by its accumulation in the activated GTP-bound state (Harwood and Cambier, 1993; Lazarus *et al.*, 1993), and its co-capping with membrane-bound immunoglobulin (Graziadei *et al.*, 1990). In T cells, expression of p21<sup>H-rasN17</sup> substantially disrupts signaling from the TCR (Rayter *et al.*, 1992; Baldari *et al.*, 1993;

Woodrow *et al.*, 1993; Swan *et al.*, 1995). Given the structural similarities between the B and T cell antigen receptors (reviewed in Weiss and Littman, 1994), it seemed likely that p21<sup>ras</sup> might play an important role in delivering proliferative signals from the B cell antigen receptor as well, though to our knowledge no previous studies have specifically addressed this question. As shown in Figure 4C and D, splenocytes from *lckEμH-rasN17* mice proliferated about half as well as did normal cells in response to treatment with anti-IgM or LPS, both potent B cell mitogens. While it is possible that the impaired mitogenesis of *lckEμH-rasN17* splenic B cells in response to B cell mitogens may reflect the higher representation of immature B cells in these mice, we favor the view that expression of dominant-negative p21<sup>H-rasN17</sup> in B cells, as has been shown in T cells, inhibits mitogenesis triggered by ligation of the antigen receptor.

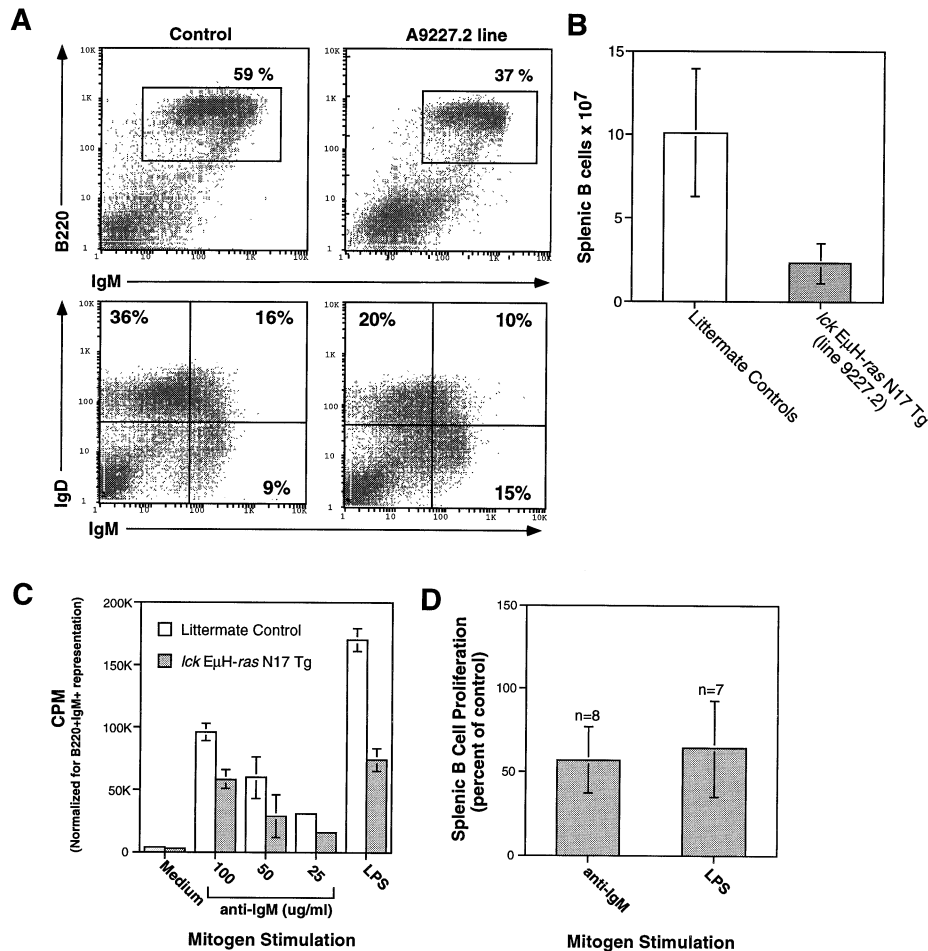
#### **Impaired development of B1 lymphocytes in transgenic mice expressing p21<sup>H-rasN17</sup>**

The peritoneum of mice is populated in part by a distinct subset of B lymphocytes (B1a cells) which differ from conventional B lymphocytes (B2 cells) in that they express higher levels of IgM, lower levels of IgD and the CD5 surface antigen. Unlike conventional bone marrow-derived B2 lymphocytes, B1a lymphocytes arise from precursors found in the fetal liver and omentum (for review see Kantor and Herzenberg, 1993; Hardy *et al.*, 1994). To determine whether the development of B1a B cells is also perturbed by the expression of p21<sup>H-rasN17</sup>, total peritoneal cells were stained for surface expression of CD5 and IgM. As shown in Figure 5A and B, transgenic animals have a lower representation of peritoneal CD5<sup>+</sup>IgM<sup>+</sup> B1a cells relative to CD5<sup>+</sup>IgM<sup>+</sup> B2 B cells as compared with control animals, and lower numbers of peritoneal B cells overall (data not shown). This decrease in the ratio of peritoneal B1a to conventional B2 cells imposed by expression of the *lckEμH-rasN17* transgene indicates that B1a cell development also proceeds via a Ras-regulated mechanism. Moreover these results, in conjunction with the observed reduction of B cell progenitors in 2-week-old mice, suggest that both fetal and adult B lymphopoiesis require Ras.

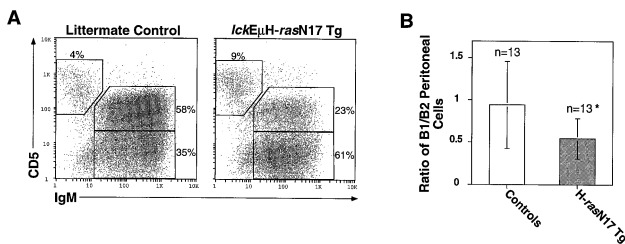
#### **An activated Raf transgene drives Pro-B cell development and rescues maturation in lckEμH-rasN17 transgenic mice**

We have shown that expression of p21<sup>H-rasN17</sup> arrests most B cell development at the transition from pre-pro-B cells (Hardy fraction A) to pro-B cells (Hardy fractions B and C). Studies of Ras family signal transduction demonstrate that p21<sup>ras</sup> can activate several downstream effector molecules including the serine/threonine kinase Raf-1, PI-3 kinase, other low molecular weight GTPases and perhaps Ras-GAP. Any of these molecules might serve to communicate Ras-derived signals to regulatory mechanisms controlling B cell maturation. However, activation of the Raf/MAPKK/MAPK serine/threonine kinase cascade clearly conveys Ras-derived signals in T lymphocytes (reviewed in Pastor *et al.*, 1995), which suggested that B lineage maturation, though arrested at a different stage by H-rasN17, would also require these kinases.

To test this conjecture, we generated a construct encod-

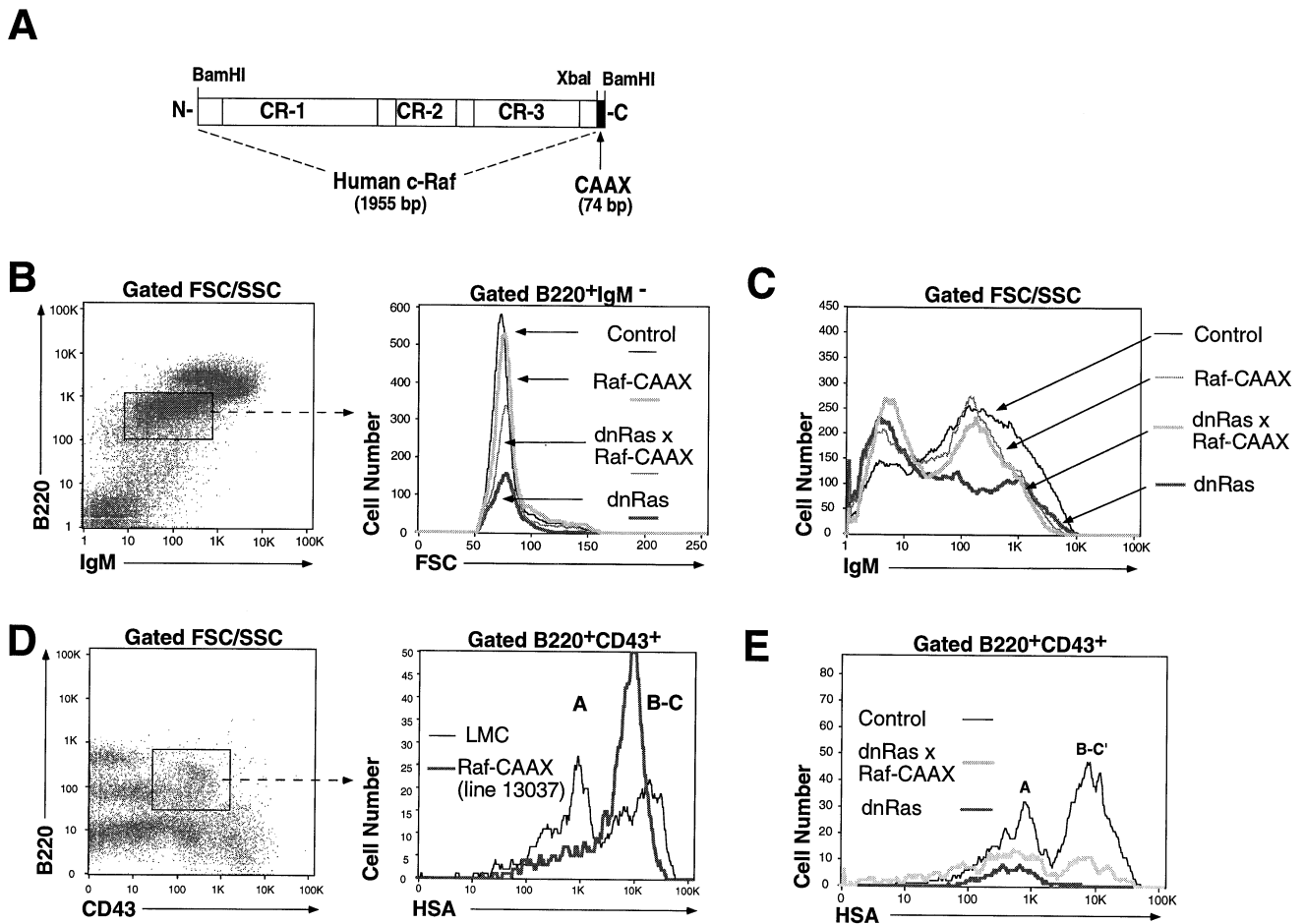


**Fig. 4.** Splenic B cells are reduced and mitogenic responses impaired in *lckEμH-rasN17* transgenic mice. (A) Total splenocytes were stained with PE-conjugated anti-B220 plus FITC-conjugated anti-IgM or PE-conjugated anti-IgM plus FITC-conjugated anti-IgD. Shown is a representative two-dimensional histogram for lymphocyte-sized cells from 8-week-old *lckEμH-rasN17* transgenic and control spleens from the A9227.2 line. (B) The total number of B220<sup>+</sup>IgM<sup>+</sup> splenic B cells from seven 6- to 12-week-old *lckEμH-rasN17* transgenic and littermate control mice from the A9227.2 line is displayed. (C) Total splenocytes or purified splenic B cells were stimulated with either 50, 25, or 12.5 μg/ml of anti-IgM (Fab')<sub>2</sub>, or LPS at 25 μg/ml, or media only for 48 h. Shown is a representative bar histogram depicting the average incorporation of [<sup>3</sup>H]thymidine by *lckEμH-rasN17* transgenic (A9227.1 line) versus littermate control B cells. (D) Splenic B cell proliferation in response to 50 μg/ml anti-IgM (eight pairs of mice) or LPS (seven pairs of mice) is shown as percent of the response of control mice analyzed simultaneously, over five separate experiments. Each bar represents the means ± SD. Mice analyzed ranged from 14–32 weeks of age, and were from the 9227.2 line.



**Fig. 5.** Representation of peritoneal B1a B Cells in Eμ/*lck-H-rasN17* Transgenic Mice. Total cells isolated from the peritoneal cavities of *lckEμH-rasN17* transgenic (9227.2 line) and littermate control mice were stained with PE-conjugated anti-CD5, and FITC-conjugated anti-IgM. (A) The percentage of cells in each region is shown for representative *lckEμH-rasN17* transgenic and littermate control mice. 20 000 events from a pre-defined forward- and side-scatter lymphocyte gate are shown. (B) The relative ratio of B1a (CD5<sup>+</sup>, IgM<sup>+</sup>) to B2 (CD5<sup>-</sup>, IgM<sup>+</sup>) cells isolated from the peritoneal cavities of 13 pairs of 8- to 13-week-old *lckEμH-rasN17* transgenic and littermate control mice is shown. The open bar (control mice) or filled bar (transgenic mice) represents the mean ± SD. The representation of B1a peritoneal cells in *lckEμH-rasN17* mice is half that observed in normal mice (\**P* = 0.02 by student's two tailed *t*-test).

ing an activated version of the serine/threonine kinase c-Raf-1, containing a farnesylation signal (CAAX) at its carboxyl-terminus (Figure 6A). This sequence directs Raf to membranes, mimicking in part the Ras-mediated event that ordinarily initiates Raf activation (Leever *et al.*, 1994; Stokoe *et al.*, 1994). The Raf-CAAX construct was cloned into the p1026X vector and a *NotI* fragment was injected into (C57BL/6/J X DBA/2J) F2 mouse zygote pronuclei as previously described. Three transgenic founders were generated, two of which were backcrossed to C57BL/6 breeders to generate lines (designated A12122<sup>lo</sup> and A13037<sup>hi</sup>). While transgene-derived Raf-CAAX protein is difficult to detect by immunoblotting, both lines express transgene-derived mRNA (data not shown). Several animals from the higher expressing Raf-CAAX transgenic line A13037 developed thymic tumors by 20 weeks of age, a result consistent with what has been observed when Raf-CAAX is expressed under control of the thymocyte-specific p1017 vector (J.Alberola-Illa, personal communication). Neoplastic transformation does not occur in cells from the lower-expressing *lckEμRaf*



**Fig. 6.** An *lckEμ*Raf-CAAX transgene partially rescues development of bone marrow B lymphocyte progenitors in *lckEμ*H-*ras*N17 transgenic mice. (A) The Raf-CAAX transgene consists of human c-Raf-1 cDNA with a farnesylation signal connected to its C-terminus. This sequence targets Raf to the membrane, mimicking the Ras-mediated event that normally initiates Raf activation (Leevers *et al.*, 1994). The Raf-CAAX cDNA was cloned into the BamHI site of the p1026X vector and transgenic mice were generated as previously described (Garvin *et al.*, 1990). (B) *lckEμ*Raf-CAAX line A12122 was bred to *lckEμ*H-*ras*N17 line A9227.2. Total bone marrow cells from 8- to 14-week-old single- or double-transgenic mice from the resulting cross were stained with PE-conjugated anti-B220 and FITC-conjugated anti-IgM. 20 000 cells from a pre-defined forward- and side-scatter lymphocyte gate were analyzed. Gated B220<sup>+</sup>IgM<sup>-</sup> cells were analyzed for forward-scatter (FSC), which is an indication of cell size. Shown is a representative single parameter histogram comparing forward-scatter and cell number. The smaller population consists largely of small pre-B cells while the larger population consists of pro-B and large pre-B cells. (C) Shown is a single-parameter histogram comparing IgM expression and cell number for total bone marrow cells isolated from offspring of the above cross. (D and E) Total bone marrow cells were stained with FITC-conjugated anti-B220, PE-conjugated anti-CD43 and biotinylated anti-HSA. The biotinylated antibody was visualized by addition of streptavidin-tricolor. The single parameter histogram of HSA expression was determined from a pre-defined B220<sup>+</sup>CD43<sup>+</sup> gate. All of the above histograms are representative of five experiments performed using five different litters.

CAAX transgenic line, A12122, and hence this line was used for most subsequent experiments.

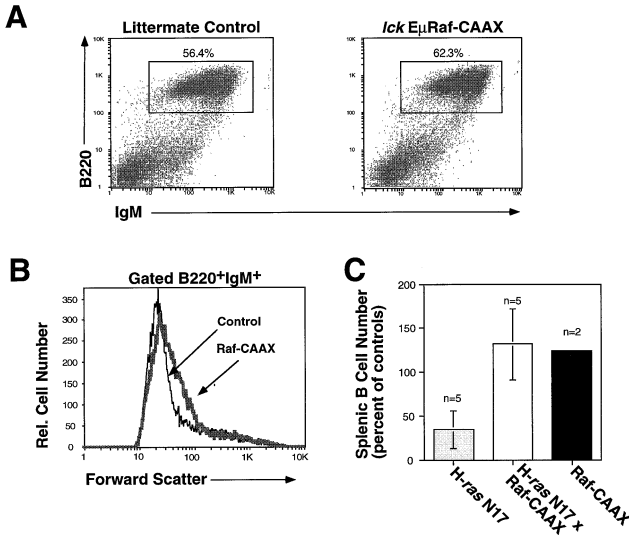
To determine whether expression of Raf-CAAX in B cell progenitors could rescue development in animals which co-express p21<sup>H-*ras*N17</sup>, we bred the non-tumorigenic *lckEμ*Raf-CAAX line A12122 to the *lckEμ*H-*ras*N17 mice (line 9227.2) and analyzed offspring from five different litters. As shown in Figure 6B, a partial rescue of B cell maturation was observed in doubly-transgenic *lckEμ*Raf-CAAX/*lckEμ*H-*ras*N17 mice, as judged by an increase in B220<sup>+</sup>IgM<sup>-</sup> large pro- and pre-B cells, and small pre-B cells when compared with singly transgenic *lckEμ*H-*ras*N17 mice. B220<sup>+</sup>IgM<sup>+</sup> immature B cells also emerged in proportion to the rescue of pro- and pre-B cells (Figure 6C).

Three-color analysis of B220, CD43 and HSA expression demonstrated that the *lckEμ*Raf-CAAX transgene powerfully directs maturation of fraction A cells to the

fraction B-C compartment, as determined by a relative increase in HSA<sup>+</sup> cells and a decrease in HSA<sup>-</sup> cells. This effect is most apparent in the high-expressing Raf-CAAX line (Figure 6D). In doubly transgenic mice, partial resolution of the H-*ras*N17-induced B cell maturational arrest is observed: both the number of fraction A cells, and the proportion of fraction B-C cells, increase substantially in the presence of the *lckEμ*Raf-CAAX transgene. That this rescue of the H-*ras*N17-mediated developmental blockade is only partial appears to reflect a quantitative effect of Raf-CAAX expression; doubly transgenic mice generated using the higher-expressing A13037 Raf-CAAX line show complete rescue of fraction B-C representation in the bone marrow (data not shown).

Expression of the Raf-CAAX transgene in mature splenic B cells does not significantly perturb the normal representation of splenic B cells (Figure 7A), though a modest increase in B cell size, as determined by forward

light-scatter characteristics, was noted (Figure 7B). Levels of surface IgM and IgD were also not affected by expression of Raf-CAAX (data not shown). When crossed with *lckEμH-rasN17* transgenic mice, complete rescue of splenic B220<sup>+</sup>IgM<sup>+</sup> B lymphocyte number to the level of non-transgenic littermate control mice, was observed (Figure 7C). This suggests that the deficiency in B cell number in the periphery of *lckEμH-rasN17* transgenic mice results primarily from an inability to stimulate Raf signaling, which, we infer, directs further development of immature B cell progenitors.



**Fig. 7.** Peripheral B cell number is rescued in *lckEμH-rasN17* transgenic mice by expression of Raf-CAAX transgene. (A) Total splenocytes from *lckEμRaf-CAAX* transgenic mice (12122 line) were stained with PE-conjugated anti-B220 and FITC-conjugated anti-IgM. Shown is a representative two-dimensional histogram from an *lckEμRaf-CAAX* transgenic and a littermate control mouse. (B) Relative cell size of splenic B cells from *lckEμH-rasN17* transgenic and littermate control mice was determined by measuring forward scatter of cells that fall within a predetermined B220<sup>+</sup>IgM<sup>+</sup> and forward/side-scatter lymphocyte gate. (C) Shown are the means ± SD of the number of splenic B cells (B220<sup>+</sup>IgM<sup>+</sup> cells) of singly and doubly transgenic mice as a percentage of control animals. The bar histogram compiles the results of five experiments. The above experiments were performed using 8- to 14-week-old *lckEμRaf-CAAX* (line 12122) and *lckEμH-rasN17* (line 9227.2) mice.

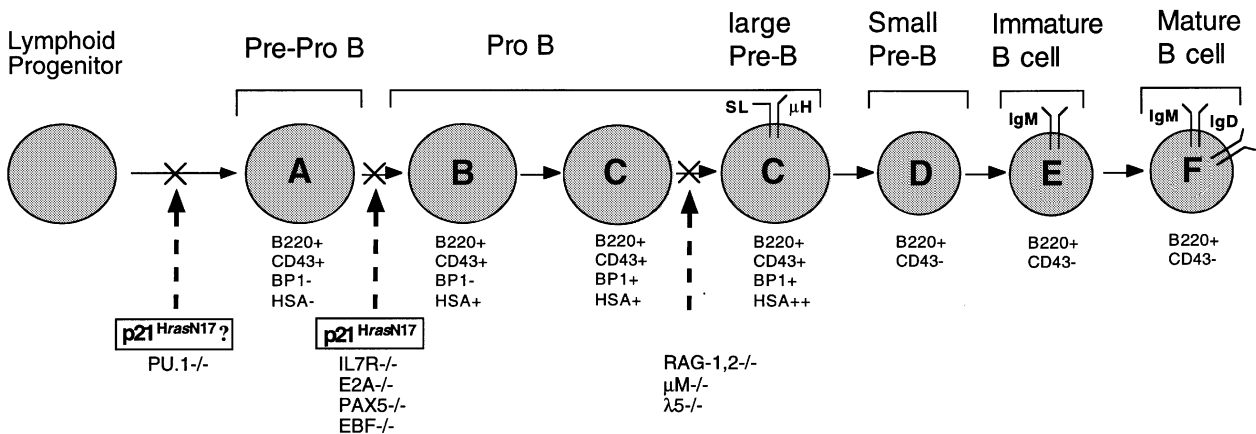
**Discussion**

***p21<sup>ras</sup> regulates a critical transition during B lymphocyte development***

B lymphocyte development proceeds via a series of highly ordered maturational steps that initiate with the rearrangement of V, D and J gene segments of the immunoglobulin heavy chain locus. Thereafter, assembly of a pre-B cell receptor containing the μ heavy chain, a surrogate light chain complex, and the Igα and Igβ signaling proteins, stimulates rearrangement and expression of light chain genes, leading to the appearance of surface IgM (Karasuyama *et al.*, 1996). Our studies demonstrate that *p21<sup>ras</sup>* regulates B lymphopoiesis at a pivotal stage in this process—the maturation of the earliest recognizably committed B cell progenitor, the pre-pro-B cell. Indeed, progression to pro- and pre-B cell stages can be almost completely inhibited using dominant-negative Ras. Moreover, since the Eμ enhancer first becomes active in pre-pro-B cells, and since the number of such (fraction A) cells is also reduced in *lckEμH-rasN17* mice, the generation of all B220<sup>+</sup> progenitors can be said to rely, at least in part, on signals transduced via the Ras GTPase.

***Raf-1 delivers Ras-mediated signals during early B cell development***

Biochemical and genetic studies have together defined the Raf/MAPKK/MAP kinase cascade as a major conduit whereby Ras-derived signals are conveyed to the nucleus. Our studies provide genetic evidence for the coherence of this pathway in B lymphocytes by showing that expression of an activated form of Raf rescued B cell development in transgenic mice which co-expressed a dominant-negative form of Ras. Thus, the developmental transition from pre-pro-B cells to pro-B cells depends, in significant part, on the ability of Ras to activate Raf-1. Since the MAP kinase kinases (Mek-1,2) are the only known targets of Raf, we conclude that the MAP kinase cascade delivers Ras-derived signals during this maturational process. In preliminary studies, we have observed that expression of a kinase-inactive form of Mek-1 using the p1026x vector leads to a perturbation in B cell development at the same stage as does expression of *p21<sup>H-rasN17</sup>* (data not shown).



**Fig. 8.** A model of a role for *p21<sup>ras</sup>* in regulating B lymphocyte development. Shown are steps in the B cell maturation sequence defined using monoclonal antibodies. The points at which the *lckEμH-rasN17* transgene exerts its effects are noted, as are defined defects observed in mice bearing the indicated gene disruptions. Adapted from Hardy *et al.* (1991).



Hence Ras acts with Raf and Mek to regulate B lymphopoiesis at this critical transition point.

### **Ras activation during B cell development**

Ras interacts with, and delivers signals from, a variety of transmembrane receptors, including those which act via non-receptor protein tyrosine kinases (e.g. the B cell antigen receptor or the cytokine receptors), receptors which interact with heterotrimeric G proteins (Van Corven *et al.*, 1993), and transmembrane protein tyrosine kinases (for review see Pronk and Bos, 1994). Of the receptors which interact with tyrosine kinases, only the IL-7 receptor is known to be essential for early B cell development *in vivo*. Mice lacking the high affinity IL-7 receptor  $\alpha$  chain (Peschon *et al.*, 1994), the  $\gamma_c$  chain shared amongst IL-2, IL-4, IL-7, IL-9 and IL-15 receptors (Cao *et al.*, 1995), or JAK3 (Nosaka *et al.*, 1995; Park *et al.*, 1995; Thomis *et al.*, 1995) a Janus family kinase known to deliver signals from the shared  $\gamma_c$  chain (Russell *et al.*, 1994), manifest a block in B cell development much like that which we report here. Similarly, injection of anti-IL7 (Grabstein *et al.*, 1993) or anti-IL-7 receptor antibodies (Sudo *et al.*, 1993) results in a complete abrogation of B cell development at the pre-pro-B cell stage. Hence disruption of either IL-7 or Ras signaling blocks B cell development at a similar point. However, to our knowledge no studies directly demonstrate Ras activation by the IL-7 receptor. In this context, recent evidence indicates that maximal STAT activation, as ordinarily occurs when JAK kinases phosphorylate these transcription factors, also requires MAP kinase-mediated serine phosphorylation (David *et al.*, 1995; Wen *et al.*, 1995; Zhang *et al.*, 1995). Hence we propose either that IL-7 itself activates Ras in B cell progenitors, or that activation of Ras by some other factor permits definitive IL-7 receptor-induced STAT signaling in these cells. Interestingly, the receptor tyrosine kinases c-Kit and flk2/flt3 (Hunte *et al.*, 1995; Brasel *et al.*, 1996; Veiby *et al.*, 1996) are known to act synergistically with IL-7 as pro-B cell growth factors, and c-Kit ligand stimulates the accumulation of Ras-GTP in bone marrow cells (Duronio *et al.*, 1992; Baker *et al.*, 1994). While mice deficient in either c-Kit-ligand or c-Kit itself manifest only mild defects in early B lymphopoiesis (Takeda *et al.*, 1997), 6-day-old mice deficient in both c-kit and  $\gamma_c$  chain have a severe reduction in splenic B lymphocyte number (~700-fold) relative to wild-type mice (Rodewald *et al.*, 1997). In contrast, mice deficient in the  $\gamma_c$ , or c-kit alone, manifest a much more modest decrease in B cell number (28-fold and 7-fold respectively). Thus, c-kit and IL-7 act synergistically *in vivo* to drive B cell development. Mice expressing p21<sup>H-rasN17</sup> have a more severe reduction in pro-B cells than is evident in mice deficient in the either IL-7 receptor  $\alpha$ -chain, or c-kit alone (Figure 3 and data not shown). These results strongly suggest that Ras conveys signals distal to both IL-7 and c-kit in B lymphocyte progenitors. Alternatively, Ras may regulate the ability of B cell progenitors to respond to growth factor signals.

Finally, chemokine receptors may also contribute to a pre-regulated signaling pathway in B cells. One member of the C-X-C family of chemokines, pre-B cell-growth-stimulating-factor/stromal cell-derived-factor-1 (PBSF/SDF-1), stimulates proliferation of B cell progenitors

*in vitro* (Nagasawa *et al.*, 1994), and is essential for generation of pro- and pre-B cells *in vivo* (Nagasawa *et al.*, 1996). Since Ras can assist in delivering signals from G protein coupled receptors (Van Corven *et al.*, 1993), defective PBSF/SDF-1 signaling may contribute to the early arrest in B cell maturation in *lckH-rasN17* transgenic mice.

### **Mechanisms whereby Ras might control B cell maturation**

Progression through the B cell maturation sequence requires the activation of numerous genes, any or all of which might receive input from p21<sup>ras</sup>. Of the transcription factors known to be activated through p21<sup>ras</sup>, the best characterized are members of the Ets (Wasylyk *et al.*, 1993; Cahill *et al.*, 1996) and AP-1 families, which respond to Ras-induced stimulation of the Raf/MAPKK/MAPK cascade. For example, two members of the Ets family, *pointed* and *yan*, play pivotal roles in determining the fate of *Drosophila* R7 photoreceptor cells, which develop via a Ras-regulated process (Dickson, 1995). Furthermore, dominant-negative forms of Ets family members, including PU-1, c-Ets-1 and c-Ets-2, inhibit Ras-induced activation of transcription and revert Ras-mediated transformation in NIH 3T3 fibroblasts (Wasylyk *et al.*, 1994). Numerous developmentally regulated B lymphocyte-specific transcriptional elements, e.g. the E $\mu$ ,  $\kappa 3'$  and  $\lambda$  enhancers and the J-chain, TdT,  $\lambda 5$  and mb-1 promoters, contain recognition sites for Ets family proteins (for review see Hagman and Grosschedl, 1994). Hence p21<sup>ras</sup> could regulate pro-B cell development by controlling Ets-factor activation. Consistent with this idea, mice deficient in the Ets family member PU-1 exhibit a profound block in B cell development similar to that imposed by expression of dominant-negative p21<sup>ras</sup> (McKercher *et al.*, 1996).

Other transcription factors may also serve as downstream targets of Ras-derived signals during B lymphopoiesis. For example, mice deficient in the E2A-encoded basic helix-loop-helix (bHLH) proteins E12 and E47 (Bain *et al.*, 1994; Zhuang *et al.*, 1994, 1996), or expressing high levels of the Id protein, an inhibitor of bHLH proteins (Sun, 1994), manifest a block in B cell development similar to what we observe in *lckE $\mu$ H-rasN17* transgenic mice. Remarkably, mice deficient in Pax-5 (Urbanek *et al.*, 1994) and Early B cell Factor (EBF) expression (Lin and Grosschedl, 1995) also exhibit a similar phenotype.

Together these studies suggest that numerous regulatory inputs converge to direct a single, key event in B lymphopoiesis, the maturation of pro-B cells. It is at this point that many of the genes that encode proteins that typify B cells first become expressed. By analogy to well-characterized invertebrate systems, we suspect that Ras activation, acting through an already partially defined set of transcription factors, regulates the differentiation of all B lineage cells by controlling progression through this developmental checkpoint. While we cannot rigorously exclude the possibility that p21<sup>H-rasN17</sup> simply inhibits expansion, rather than differentiation, of early committed B cell progenitors, this seems less likely. For example, the representation of cycling pro- and pre-B cells from *lckH-rasN17* transgenic animals (based on incorporation of Hoechst dye or BrdU *in vivo*), is equivalent to that of littermate control mice (data not shown). Moreover, pre-

B cells which express p21<sup>H-rasN17</sup> undergo relatively normal expansion when compared with littermate controls (data not shown). These data complement results obtained in studying T cell maturation where CD4<sup>-</sup>8<sup>-</sup> (double-negative) pre-T cells which express significant levels of p21<sup>H-rasN17</sup> also undergo normal expansion following a pre-T cell receptor signal (Swan *et al.*, 1995; Figure 1 and data not shown). These results suggest that in both B and T lymphocytes, Ras selectively regulates certain critical transition points, while leaving others intact. Finally, the highly selective ability of Raf to rescue the transition of fraction A cells to fractions B and C while leaving other developmental steps largely unperturbed strongly argues against a non-specific role of Ras and Raf in proliferative expansion of B lineage cells.

### Differential regulation of T and B cell development

In thymocytes, inhibition of the Ras/Raf/MEK/MAP kinase cascade imposes a profound block on positive selection, a process that occurs after assembly of the definitive T cell receptor. By comparison, we observe only a slight decrease in the representation of mature B cells in the spleen of *lckEμH-rasN17* mice. We suspect that this difference between signaling pathways in T and B cell development conveys broader lessons regarding the normal physiology of lymphopoiesis. T cell production declines dramatically in early adult life, and the T cell receptor repertoire, once elaborated, is largely immutable (Zheng *et al.*, 1994). Hence regulatory mechanisms must act to ensure efficient emergence of cells with ideal functional properties. In contrast, B cell production continues throughout adulthood, and the B cell repertoire sustains late modifications, the result of somatic hypermutation and receptor editing (Nemazee, 1996; Scharff *et al.*, 1997), through mechanisms unrelated to the generative process itself. In this context, control of the number of cells entering the maturation pathway, becomes especially important.

Our results, coupled with evidence from other studies (reviewed above) indicate that the maturation of pre-pro-B cells constitutes a pivotal control point in B lymphopoiesis. These studies permit elaboration of a coherent model for early B cell development, wherein cytokine- and chemokine-derived signals act via the Ras/Raf/MEK/MAP kinase cascade to control the emergence of pro-B cells in the bone marrow. Manipulation of this process could, in principle, be used to enhance B cell recovery, or engraftment, following ablative chemotherapy.

## Materials and methods

### Expression constructs and transgenic mouse generation

The p1026x vector was created by ligating a 0.92 bp fragment of the immunoglobulin Eμ heavy chain enhancer into the *Xba*I site within the *lck* proximal promoter portion of the p1017 vector (Chaffin *et al.*, 1990). Surprisingly, transgenic mice that were initially created using this construct produced both functional human growth hormone as well as products encoded by the H-*ras*N17 cDNA (data not shown), possibly due to re-initiation of translation. Therefore, the *Bgl*III site in the fourth exon of hGH was cleaved, filled in and religated, resulting in the generation of an in-frame stop codon. This treatment, yielding the mutant hGx cassette, has been shown to prevent expression of functional growth hormone protein in similar transgenic constructs (R.Palmiter, personal communication). The H-*ras*N17 transgene was produced by ligating the 0.72 kb H-*ras*N17 cDNA (containing 27 bp of 5' untranslated region,

the entire coding region and 170 bp of 3' untranslated region sequence) into the *Bam*HI cloning site of the p1026x vector (Swan *et al.*, 1995). The Raf-CAAX transgene was generated by digesting human c-Raf-1 cDNA (pKS, Stratagene, La Jolla, CA) with *Tth*III and *Xba*I. A synthetic double-stranded oligonucleotide (5'-ACGTCCCGAGGCTCGTCTTCT-3' + 5'-CTAGAGAAGACAGGCAGCGTCGGGGAC-3') was then ligated to this site, thereby adding an *Xba*I site in-frame to the 3' end of the Raf-1 cDNA. The resulting construct was then digested with *Xba*I and *Not*I and a synthetic oligonucleotide (5'-CTAGAAAAATGTCG-AAGGACGGAAAGAAAAAGAAGAAGTCCAAAACCAAGTGCATCATGTAGGATCCGC3' plus 5'-GGCCGCGGATCCTACATG-ATGACGCACATTGGTTTGGACRTTCTTCTTTTCTTTTCCGT-CCTTCGACATTTTT) added which encodes the CAAX sequence [described by Stokoe *et al.* (1994)], and a stop codon. The entire Raf-CAAX cDNA was then excised from the Bluescript pKS vector by *Bam*HI digestion and subcloned into the *Bam*HI site of the p1026x vector. For transgenic mouse generation, purified *Not*I fragments containing the *lckEμH-rasN17* transgene or the *lckEμRaf-CAAX* transgene were injected at 5 ng/μl into (C57BL/6×DBA2J) F2 mouse zygote pronuclei as previously described (Garvin *et al.*, 1990). Transgenic founders were detected by dot-blot hybridization of genomic tail DNA with an hGH probe (Chaffin *et al.*, 1990). Founders were then bred to C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) to generate stable lines of transgenic mice. Mice were housed under SPF conditions and were analyzed between 2 and 16 weeks of age.

### Cell preparation

Single cell suspensions of bone marrow cells were prepared by flushing two femurs per mouse with 5 ml Hanks' Balanced Salt Solution (HBSS) without phenol-red (Gibco BRL, Germantown, MD) supplemented with 3% fetal calf serum (HyClone Laboratories, Logan, UT). Splenocytes were prepared by crushing spleens between the frosted ends of glass microscope slides (Fisher Scientific, Pittsburgh, PA) into the above media. Peritoneal cells were collected by injecting and withdrawing 5 ml HBSS plus 3% FCS from the peritoneal cavities of mice, immediately following euthanasia. Erythrocytes were depleted by ammonium chloride lysis (Mishell and Shiigi, 1980). Cells were collected by centrifugation for 10 min at 3000 r.p.m. and resuspended in HBSS plus 3% FCS for further analysis.

### Flow cytometric analysis

Prior to staining with primary and secondary reagents, bone marrow cells and splenocytes were incubated in 5% normal mouse serum (Jackson Laboratories, Bar Harbor, ME) for 20–30 min, followed by two washes with staining media (HBSS supplemented with 5% bovine serum albumin and 0.1% azide). Two- and three-color flow cytometric analyses were performed by staining 1×10<sup>6</sup> total bone marrow cells, splenocytes or peritoneal cells with various combinations of the following monoclonal antibodies specific to murine antigens: phycoerythrin (PE)-conjugated anti-CD45R/B220, fluorescein isothiocyanate (FITC)-conjugated anti-B220 (CalTag Laboratories, San Francisco, CA); PE-conjugated anti-CD43, biotin-conjugated anti-heat stable antigen, PE-conjugated anti-GR-1, FITC-conjugated anti-MAC1, PE-conjugated anti-CD61, Biotin-conjugated anti-Forsman antigen and PE-conjugated anti-Ly5 (Pharmingen, San Diego, CA); FITC-conjugated anti-IgM, biotin-conjugated anti-IgD (Southern Biotechnology Associates, Birmingham, AL). Biotinylated reagents were detected by further staining with streptavidin-tricolor (CalTag). Cells were stained as previously described (Gross *et al.*, 1995) and 20 000 events within a pre-defined lymphocyte forward- and side-scatter lymphocyte gate were collected and stored in list mode files using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) with Lysis II software, and analyzed using Reproman Software version 2.07 (Truefacts Software, Seattle, WA). Four-color analysis was performed by staining bone marrow cells with tricolor-conjugated anti-CD45R/B220 (CalTag), FITC-conjugated anti-BP-1 (Pharmingen), PE-conjugated anti-CD43 and biotin-conjugated anti-heat stable antigen, followed by incubation with streptavidin-AMCA (Molecular Probes Inc., Eugene, OR). For four-color analysis, 500 000 events within a lymphocyte size gate were collected and stored in list mode using a FACStar flow cytometer (Becton Dickinson) equipped with a 424 filter, and analyzed as described above.

### Proliferation assays

For mitogen stimulations, splenic B cells were partially purified by incubating erythrocyte-depleted splenocytes in anti-Thy1.2 (Sigma, St Louis, MO) at 10 μg/ml per 1×10<sup>8</sup> cells for 40 min on ice, followed by the addition of guinea pig complement (Gibco BRL) at 1:15 dilution

for 1 h at 37°C. Live cells were enriched by separation over 100% fetal calf serum. Splenic B cells were typically enriched to 80–95% using this method, as judged by subsequent staining for B220 expression. Purified splenic B cells were stimulated as previously described (Appleby *et al.*, 1995).

#### RNA blot analysis

Tissues were collected from transgenic mice and immediately frozen on dry ice. Total RNA was then isolated from each tissue using RNazol B (Tel-Test 'B' Inc, Friendswood, TX). 15 µg of total RNA (in 5 µl of DEPC water +0.5% SDS) from each tissue was heated at 65°C in 50% formamide, 10% formalin, 10% MOPS buffer for 10 min and cooled on ice. RNA was subsequently fractionated by electrophoresis and blotted onto a nylon membrane (Hybond N, Amersham, Arlington Heights, IL) using standard techniques. Subsequent hybridization with a either a heat-denatured <sup>32</sup>P-labelled H-rasN17 cDNA probe, or a mouse efl $\alpha$  cDNA probe (Allen *et al.*, 1992), was performed at 42°C for 12 h. The membrane was then washed for 15–45 min twice in 0.1% SSC (0.015 M NaCl, 0.0015 M Na<sup>+</sup> citrate) plus 0.1% SDS buffer at 60°C and exposed on X-ray film (Eastman Kodak, Rochester, NY) for 1 to 3 days at –70°C.

#### Immunoblot analysis

Bone marrow and splenocyte lysates were collected as previously described (Cooke *et al.*, 1991). Total protein was quantitated using a protein assay (Bio-Rad Laboratories, Hercules, CA) and 20 µg/sample separated by 12% SDS–PAGE followed by electrophoretic transfer onto a Hybond C-Extra membrane (Amersham). The membranes were probed with a pan-Ras-specific antibody (ras 10, Ab-3; Oncogene Sciences, Cambridge, MA) and visualized using the enhanced chemiluminescence system (Amersham) as previously described (Swan *et al.*, 1995). Levels of protein were quantitated using a densitometer (Bio-Rad, Model 65670 Imaging Densitometer) with background levels determined from selected areas of each lane on the autoradiograph.

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