Ras Mediates Effector Pathways Responsible for Pre-B Cell Survival, Which Is Essential for the Developmental Progression to the Late Pre-B Cell Stage

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Abstract

Ras is essential for the transition from early B cell precursors to the pro-B stage, and is considered to be involved in the signal cascade mediated by pre-B cell antigen receptors. To examine the role of p21ras in the late stage of B cell differentiation, we established transgenic mice (TG) expressing a dominant-inhibitory mutant of Ha-ras (Asn-17 Ha-ras) in B lineage cells at high levels after the early B cell precursor stage. Expression of p21Asn-17 Ha-ras was associated with a prominent reduction in the number of late pre-B cells, but had little effect on proliferation of early pre-B cells. Inhibition of p21ras activity markedly reduced the life span of pre-B cells, due, at least in part, to downregulation of the expression of an antiapoptotic protein, Bcl-xL. Thus, the apparent role for p21ras activity in pre-B cell survival may explain the decreased numbers of late pre-B cells in Asn-17 Ha-ras TG. Consistent with this possibility, overexpression of Bcl-2 in Asn-17 Ha-ras TG reversed the reduction in the number of late pre-B cells undergoing immunoglobulin light chain gene (IgL) rearrangement and progressing to immature B cells. These results suggest that p21ras mediates effector pathways responsible for pre-B cell survival, which is essential for progression to the late pre-B and immature B stages.

Key words: B cell development • life span • immunoglobulin gene rearrangement • Bcl-xL • Bcl-2

Introduction

Committed B cell precursors undergo differentiation through several critical check points into surface immunoglobulin (sIg) positive immature B cells (for a review, see reference 1). The earliest recognizable B cell precursors were identified as developing through two distinct developmental pathways, the pre-B pathway and the pre-B cell antigen receptor (pre-BCR) pathway. The pre-B pathway is characterized by the expression of sIgM but the absence of sIgD, while the pre-BCR pathway is characterized by the expression of sIgD but the absence of sIgM. The pre-B cell antigen receptor (pre-BCR) is a cell surface complex composed of the surrogate light chain (SL) and the pre-BCR-associated protein (BLNK), which is essential for pre-B cell development. The pre-B cell antigen receptor (pre-BCR) is responsible for signal transduction and is activated by binding of BLNK to the pre-BCR. This signal transduction induces the expression of Bcl-xL, an antiapoptotic protein, which is essential for pre-B cell survival. Thus, the pre-B cell antigen receptor (pre-BCR) pathway is essential for the survival of pre-B cells. The pre-B cell antigen receptor (pre-BCR) pathway is essential for the survival of pre-B cells.
fied in bone marrow (BM) cells expressing CD43 and a low level of B220 (2, 3), and these cells progress to the pro-B stage. Successful IgH gene rearrangement and transient activation of surrogate light chain (SL) genes at the pro-B cell stage lead to expression of the pre-B cell antigen receptor (pre-BCR), which plays a pivotal role in allelic exclusion and in the pre-B transition (4–8). After expansion at the pre-B transition, pre-B cells increase the expression of the large isoform of Bcl-x (Bcl-xL; see references 9–11) and the novel finding that p21(Asn-17) activity could be essential for pre-B cell survival, but not for proliferation of cells during the transition from the pro-B to early pre-B stages.

Materials and Methods

Establishment of Asn-17 Ha-ras Tg. The plasmid containing Asn-17 Ha-ras was a gift of Dr. G. M. Cooper (Dana-Farber Cancer Institute, Boston, MA). The coding region of Asn-17 Ha-ras was amplified by PCR, and the 0.6-kb DNA fragment was subcloned in the expression vector containing the promoter of the V(α)186.2 gene (30), E(+) (Ec0r1-X bal 0.8-kb fragments), 3′ Eκ (X bal–Sacl 0.8-kb fragments), SV40 intron (small t antigen), and polya signals. The final construct was microinjected into pronuclei of C57BL/6 fertilized eggs. We bred transgene-positive founder mice with C57BL/6 mice, and established three independent transgenic lines, designated N-17-52, N-17-75, and N-17-95. These lines were maintained by breeding of heterozygous Tg with nontransgenic littermates in sister–brother mating. To establish double Tg, we crossed Tg from either N-17-52 or N-17-75 to those from either N-17-52 or N-17-75. To determine the genotypes of offspring of littermates, we purified tail DNA and provided for Southern blot analysis by using HindIII as restriction enzyme and a 1.5-kb DNA fragment of SV40 polya DNA as a probe. In addition, the bd-2 transgene was introduced into the background of Asn-17 Ha-ras by crossing N-17-95 Tg with Bcl-2 Tg, which were purchased from The Jackson Laboratory.

FACS® Analysis. To analyze the early B cell precursors, BM cells were incubated with biotinylated anti-μ, anti-δ, anti-CD23 (BD PharMingen), anti-NK1.1 (BD PharMingen), antilymphoblasts (TER119), and anti-Gr-1 (BD PharMingen) mAbs. After washing, the cells were incubated with biotinylated anti-NK1.1, anti–Gr-1, anti–CD43, anti–CD23, anti–Gr-1, and anti–Gr-1 mAbs, followed by staining with biotinylated anti-μ, anti-δ, anti-CD23, anti–Gr-1, and anti–Gr-1 mAbs, followed by staining with biotinylated anti-μ, anti-δ, anti-CD23, anti-NK1.1, antilymphoblasts, and anti–Gr-1, followed by staining with anti-B220PE, anti-CD43FITC, and anti–BP-1FITC mAbs. After washing, cells were incubated with Texas red–coupled UltraAvidin (avidinTEX; Leinco Technologies, Inc.). To analyze immature and circulating B cells in the BM, we stained cells with anti-B220PE, anti-HSAPE, biotinylated anti-CD23, FITC–coupled anti-μ (anti-μFITC), and anti–sFITC mAbs, followed by incubation with avidinTEX. Cells were resuspended in staining buffer containing propidiodide (5 μg/ml) and analyzed with a FACS Vantage™ (Becton Dickinson) equipped with appropriate filters for five-color analysis in a dual argon laser (488 nm)/dye laser (599 nm) system (30).

A pre-BCR expression. BM cells were stained with ethidium monoazide (EMA; Molecular Probes) and a mixture of anti-μFITC, anti-δFITC, anti-CD23FITC, anti-B220TEX, and anti–BP-1FITC mAbs. After washing, the cells were left for 20 min under UV light for irreversible photolytic coupling of EMA to the cellular DNA. Thereafter, the cells were washed with PBS and fixed with
and littermate controls (LM) from N-17-95 using a MACS column (Miltenyi Biotec). To purify pre-B cells and recirculating NK1.1, and anti-CD90). After washing, cells were incubated with 0.5% saponin, followed by incubation for 30 min with Hoechst 3342 (1 μg/ml; Sigma-Aldrich) at room temperature. DNA contents were analyzed with a FACS Vantage® equipped with the appropriate filters for four-color analysis in a dual argon laser (488 nm)/UV laser (351/360 nm) system. Excitation of the Hoechst 3342 was carried out by UV light with a maximum wavelength of 351/360 nm, and fluorescence of the Hoechst 3342 was achieved by UV transmission at 424 ± 22 nm.

Purification of BM B Cells. To enrich B cells, we incubated BM cells with biotinylated mAbs anti-Gr-1, anti-Mac-1, anti-NK1.1, and anti-CD90. After washing, cells were incubated with streptavidin-coated microbeads and separated on a MACS column (Miltenyi Biotec). To purify pre-B cells and recirculating B cells, we stained enriched B cells with anti-B220APC, anti-HSA PE mAbs. To purify early B cell precursors, we stained enriched B cells with anti-B220PE, anti-CD43FITC, anti-HSA PE mAbs. After washing, cells were incubated with avidin TEX. Viable cells were sorted under the forward and side scatter lymphocyte gate with 0.5% saponin, followed by incubation for 30 min with 5-bromo-2′-deoxyuridine (BrdU) (Sigma-Aldrich). After injection, pre-B cells were purified from individual animals by FACS® sorting and provided for staining with or without FITC-conjugated anti-BrdU mAb (Becton Dickinson), or in a buffer containing 1% NP-40, 137 mM NaCl, 1 mM MglCl, 0.1 mM CaCl2, 20 mM Tris-HCl (pH 9.0), 10% glycerol, 0.3 mg papain, 30 μg/ml pancreas extract, 2 μg/ml chymotrypsin, 0.5 μg/ml thermolysin, 20 μg/ml trypsin for analysis of Bcl-xL. The same amount of protein in each sample was separated by SDS-PAGE on a 12 or 15% gel and transferred to a polyvinylidene (PVDF) membrane (Millipore). Blots were incubated with rat anti-Ras mAb (Y13-259; a gift from Dr. Nakafuku, Nara Institute of Science and Technology, Ikoma, Japan), mouse anti-R as mAb (Ab-3; Oncogene Research Products), or mouse anti-Bcl-xL mAb (Transduction Laboratories). After washing, blots were incubated with anti-rat Ig or anti-mouse Ig antibody coupled with peroxidase (Amersham Pharmacia Biotech). After washing, p21V0 or Bcl-xL was visualized by the ECL system (Amersham Pharmacia Biotech) as described previously (32). In addition, the blot was probed with mouse anti-β-tubulin mAb (Sigma-Aldrich).

In vitro MAP kinase assay. Resting B cells were purified from pooled spleens of Asn-17 Ha-As T G and age-matched wild-type C57BL/6 mice and stimulated with Fab13 goat anti-mouse IgM polyclonal antibody (Jackson ImmunoResearch Laboratories). These cells were provided for an in vitro kinase assay for endogenous ERK activity, as described previously (32). Semiquantitation of mRNA by reverse transcription PCR. Messenger RNA of sorted cells was purified, and first-strand cDNA was synthesized. Aliquots of cDNA were serially diluted fivefold and provided for reverse transcription (RT)-PCR by using a Premix Taq (Takara Shuzo Co., Ltd.) or Super Taq Premix (Sawady Technology). As a control, β-actin cDNA was amplified by primers, CCAAGGCAACACGCAGAAAG and TCTCATGTTGCTAGGACCA. Ha-ras cDNA was amplified by a set of primers, TGACATCCAGCTGATCCAG and TGGCAGCTCATGACCC. In some experiments, additional amplification was performed by using nested primers TGCCATCAACAACCAAG and GACGCCAGTCACACTGGT. The reaction conditions used for each primer set were as follows: β-actin, 94°C for 1 min, 58°C for 1 min, 72°C for 1.5 min, 40 cycles; H-ras and Ha-ras nested, 94°C for 1 min, 60°C for 1 min, 72°C 1.5 min, 45 cycles and 25 cycles, respectively.

Semiquantitation of R effarored IgG and IgE. High molecular mass genomic DNA was extracted by the proteinase K method from purified pre-B cells. The PCR cycle was repeated 26–32 times. PCR reactions were performed to detect rearrangements of IgG as described previously (33). PCR products were detected by Southern blot hybridization to specific radiolabeled J probe (1.7-kb Sphl–Pstl fragment) or ethidium bromide staining. To detect bd-6, we used the following set of primers: mBCL-6EX5, 5′-GGCTGTTGAATCTGTGGTTGTC-3′, and mBCL-6EX6, 5′-AGTGATCGTCTCACTGCTGTT-3′.

**Results and Discussion**

Establishment of T G expressing A sn-17 Ha-ras T G transgene in B cells. We constructed the transgene by insertion of the dominant-inhibitory mutant of A sn-17 Ha-ras (25) in an expression cassette consisting of the Vα1 gene promoter (Vp), Eμ, and 3′ Eκ (Fig. 1A). The transgene was injected into fertilized eggs of C57BL/6 mouse origin, and three independent transgenic lines, designated N-17-52, N-17-75, and N-17-95, were analyzed. As shown in Fig. 1B, R T-PCR analysis showed the greater expression of ras transcripts in pre-B (top) and pro-B cells (middle) in T G from line N-17-95 than LM. In contrast, this transcript was undetectable in early B cell precursors (bottom, a) without additional amplification by nested PCR (bottom, c). These

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results suggested that the transgene could be fully activated in BM B cells after the early B cell precursor stage.

Immunoblot analysis showed a remarkable expression of p21<sup>ras</sup> in pro-B cells (B<sub>220</sub><sup>dim</sup>CD43<sup>dull</sup>CD<sup>-</sup>1<sup>+</sup>) and circulating B cells in the BM of N-17-95. Similar results were obtained by analysis of resting B cells of Tg N-17-52 and N-17-75 (data not shown), suggesting that expression of p21<sup>ras</sup> may significantly inhibit endogenous p21<sup>ras</sup> activity in B cells.

Fig. 1 E shows that ERK activation by IgM stimulation was reduced in resting B cells of TG from line N-17-95 by 30-50% of the wild-type C57BL/6 B cells. Similar results were obtained by analysis of resting B cells of Tg N-17-52 and N-17-75 (data not shown), suggesting that expression of p21<sup>ras</sup> may significantly inhibit endogenous p21<sup>ras</sup> activity in B cells.

Expression of Asn-17 H-ras Transgene Preferentially Reduced the Number of Pro-B Cells and Late Pre-B Cells in the BM. As shown in Fig. 2, we evaluated B cell development in the BM of Asn-17 H-ras Tg by five-color flow cytometry (30), according to the strategy established by Hardy and colleagues (2, 3). To analyze early B cell precursors (B<sub>220</sub><sup>dim</sup>CD43<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup>), we excluded non-B lineage cells, Ig<sup>-</sup> cells, and plasma cells in B<sub>220</sub><sup>dim</sup> cells (Fig. 2 a), and monitored CD43<sup>+</sup>CD11<sup>+</sup> cells (Fig. 2 b). To analyze pro-B cells (B<sub>220</sub><sup>dim</sup>CD43<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup>), we gated B<sub>220</sub><sup>dim</sup>CD43<sup>+</sup> cells in a B<sub>220</sub><sup>+</sup>CD<sub>11b</sub><sup>+</sup>CD<sub>11c</sub><sup>+</sup> population (Fig. 2 e), and BP-1<sup>+</sup> pre-B cells were analyzed (Fig. 2 f). To analyze pre-B cells (B<sub>220</sub><sup>dim</sup>CD43<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup>), we gated B<sub>220</sub><sup>+</sup>CD<sub>11b</sub><sup>+</sup>CD<sub>11c</sub><sup>+</sup> cells in a B<sub>220</sub><sup>+</sup>CD<sub>11b</sub><sup>+</sup>CD<sub>11c</sub><sup>+</sup> population (Fig. 2 e), and BP-1<sup>+</sup> pre-B cells were analyzed (Fig. 2 f). To analyze pre-B cells (B<sub>220</sub><sup>+</sup>CD43<sup>+</sup>).
HSA(high), we excluded immature and mature B cells and non-B lineage cells (Fig. 2 g) and monitored B220dullCD43dull− cells (Fig. 2 h), which can be divided into BP-1− and BP-1+ fractions (Fig. 2 i).

TG at ages between 10 and 15 wk from N-17-95 (Fig. 2 and Fig. 3 A), N-17-75 (Fig. 3 B), and N-17-52 (Fig. 3 C) displayed a reduction in the number of pro-B cells by 40-50% of LM. The number of early B cell precursors was comparable between TG and LM, consistent with the results illustrated in Fig. 1 B. Among pre-B cell subsets, TG from N-17-95 and N-17-75 reduced the number of BP-1− cells, four- to fivefold below the level of LM, more prominently than the reduction in the number of BP-1+ cells (Fig. 3 C). A similar phenotype was observed in N-17-52 TG, although the reduction in pro-B cells was less marked (Fig. 3 C). Preferential reduction in the number of pro-B and BP-1− pre-B cells was also observed in young N-17-95 TG (Fig. 3 D). RT-PCR analysis suggested that transgenic BP-1+ and BP-1− pre-B cells expressed Asn-17 Ha-ras transcripts at comparable levels (data not shown), excluding the possibility that preferential reduction in the number of BP-1− pre-B cells was due to high levels of transgene expression in this subset.

We crossed N-17-95 TG with either N-17-52 or N-17-75 and analyzed B cell development in the BM of the offspring of the littermates (Fig. 4). The reduction in the number of B cells was dose sensitive, except for the early B cell precursors (pro-pre-B) that almost sustained the number at comparable levels in single and double TG as well as LM. The dose effect was more pronounced in N-17-95/N-17-75 double TG (Fig. 4 B) than in N-17-95/N-17-52 double TG (Fig. 4 A). Although the number of BP-1− pre-B cells was reduced in N-17-95/N-17-75 double TG, by 3-fold below that of LM, the reduction in the number of pro-B and BP-1− pre-B cells was more prominent, 15- and 30-fold below the level in LM, respectively (Fig. 4 B). Thus, a preferential reduction in the number of BP-1− cells

Figure 3. Preferential reduction in the number of pro-B and BP-1− pre-B cells in the BM of Asn-17 Ha-ras TG. BM cells were prepared from individual TG and LM from N-17-95 (A), N-17-75 (B), or N-17-52 (C) of 10–15 wk of age and N-17-95 at 5–6 wk of age (D). The number of early B cell precursors (pre-pro-B), pro-B, and BP-1+ and BP-1− pre-B cells was estimated by FACS®, and the number per 10⁵ viable BM cells was recalculated (y-axis). The results show the average number of cells in TG (gray bars) and LM (white bars), summarized from five to eight independent experiments. Circles represent the results for individual animals.

Figure 4. Preferential reduction in the number of pro-B and late pre-B cells by expression of Asn-17 Ha-ras transgene at different gene dosages. A set of offspring with the genotype of N-17-52 (b), N-17-95 (c), N-17-52/N-17-95 (d), and nontransgenic control (a) in A, or those with the genotype of N-17-95 (b), N-17-95/N-17-95 (c), N-17-52/N-17-95 (d) and nontransgenic control (a) in B were obtained by crosses between N-17-95 and N-17-52 (A) or N-17-95 and N-17-75 (B), respectively. BM cells were prepared from individual animals at 12–13 wk of age and subjected to FACS® analysis. Bars represent the average number of early B cell precursors (pre-pro-B), pro-B cells (pro-B), and BP-1+ and BP-1− pre-B cells (pre-B), immature B cells (immature B), and recirculating B cells (mature B) in the BM of TG (white bars) and LM (gray bars). Filled circles represent the number of cells in individual animals. The results were summarized from three independent experiments using animals of the same age.
in pre-B cell subsets was a common feature in animals that expressed the Asn-17 H a-ras transgene at different doses.

BP-1 is expressed at the pro-B to pre-B transition, whereas the expression is downregulated at the pre-B to immature B transition (2, 34). We observed that 30–50% of BP-1+ pre-B cells were labeled with BrdU by a single injection of the reagent, three- to fourfold above the level of BP-1+ pre-B cells (data not shown). Assuming that BP-1+ and BP-1− cells could be enriched by early and late pre-B cells, respectively, we investigated whether pre-B cells were more susceptible at the late stage than at the early stage to any effect caused by inhibition of p21ras activity. As shown in Fig. 5 A, we analyzed the expression of pre-BCR in Ig−CD23−B220full B cells of N-17-95 TG, N-17-95/N-17-52 double TG, and nontransgenic LM by using mAb SL156 (31), which recognizes a conformational epitope on the μ and SL chain complexes. Ig−CD23−B220full B cells contained pre-B cells and small numbers of pro-B and the earliest B cell precursor cells (data not shown).

As shown in Fig. 5 B, pre-BCR+ cells were detected in the BP-1+ and BP-1− subsets of the B220full Ig−CD23−B cells. Because the pre-BCR is transiently expressed at the late pro-B to early pre-B transition (35), we speculated that pre-BCR+ BP-1− cells would be at the late pro-B stage, in the process of maturation to BP-1+ pre-B cells. The number of pre-BCR− BP-1− cells was prominently reduced by the expression of Asn-17 H a-ras transgene in a dose-dependent manner, whereas a reduction in the number of pre-BCR+ and pre-BCR− BP-1− cells was less marked. Because pre-BCR+ pre-B cells enter the S phase of cell cycle at a high frequency (35), these results support the notion that inhibition of p21ras activity does not have much effect on proliferating cells at the late pro-B to the early pre-B transition.

The BP-1+ cells consisted of a minor population in immature B cells at comparable levels between LM and TG from N-17-95 or N-17-95/N-17-52 (data not shown), excluding the possibility that inhibition of p21ras caused an abnormality in the expression of BP-1. In addition, we did not detect any B cells with an aberrant phenotype in either the BM or spleen of the TG within the limitations of the FACS® analysis.

Inhibition of p21ras activity caused a preferential reduction in the number of pre-BCR+ BP-1− pre-B cells. BM cells were prepared from a set of offspring with the genotype of N-17-95 (#95), N-17-95/N-17-52 (#95 × #52), and nontransgenic controls (LM). These cells were stained with EMA and with anti-B220 

anti-Ig 

anti-CD23 mAbs. After washing, the cells were fixed with formaldehyde, permeabilized with saponin, and stained with biotinylated mAb SL156, followed by streptavidin at the second step. The cells were then analyzed by flow cytometry using FITC- or PE-conjugated mAbs. As shown in Fig. 5 C, 13–30% of B220full Ig−CD23−B220full cells in BM of N-17-95 TG, N-17-95/N-17-52 double TG, and LM using flow cytometry (Fig. 6, A and B). As summarized in Fig. 6 C, 13–30% of B220full HSA+ pre-B cells entered the S phase of cell cycle in the BM of LM, at a comparable level to that of pre-B cells (35). Although B220full HSA+ pre-B cells in TG and LM were composed of pre-B cells, immature B cells, and pro-B cells (83–86, 12–15, and 1.3–3%, respectively), proliferating B220full HSA+ pre-B cells could comprise largely pre-B cells, because immature B cells are mostly at a resting state in TG and LM (36; data not shown). As shown in Fig. 6 C, the number of proliferating cells was reduced in N-17-95 TG, by 60% of LM, whereas the reduction in the number of resting B220full HSA+ pre-B cells was more significant, threefold below the level found in LM. As shown in Fig. 6, D and E, the number of resting B220full HSA+ pre-B cells was remarkably reduced in N-17-95/N-17-52 double TG, seven- to eightfold below the level in LM. In contrast, the number of cycling B220full HSA+ pre-B cells was almost comparable between N-17-95 TG and N-17-95/N-17-52 double TG. These results raise the possibility that the late pre-B transition, but not the early pre-B transition, could be highly susceptible to an effect caused by the inhibition of p21ras activity.

As shown in Fig. 6 F, to investigate further the role of p21ras in the proliferation and accumulation of pre-B cells, we injected a single dose of BrdU into TG and LM from N-17-95. Pre-B cells were purified from the BM of individual animals at 2, 40, and 62 h after injection and stained with anti-BrdU mAb, followed by examination under confocal microscopy (30). In agreement with the results depicted in Fig. 6, B and D, the number of pre-B cells incorporating BrdU 2 h after injection was comparable between

Figure 5. Inhibition of p21ras activity caused a preferential reduction in the number of pre-BCR+ BP-1− pre-B cells. BM cells were prepared from a set of offspring with the genotype of N-17-95 (#95), N-17-95/N-17-52 (#95 × #52), and nontransgenic controls (LM). These cells were stained with EMA and with anti-B220, anti-Ig, anti-CD23 mAbs. After washing, the cells were fixed with formaldehyde, permeabilized with saponin, and stained with biotinylated mAb SL156, followed by staining with APC-coupled streptavidin at the second step. The cells were then analyzed by a lymphocyte gate on forward by side light scatter (A, b) after exclusion of EMA− cells from analysis by gating (A, a). B220low Ig−CD23− cells were gated out (A, c) and B220full cells stained with or without BP-1 were analyzed for the expression of SL156 (A, d). In B, the number of pre-BCR− cells in BP-1− or BP-1+ pre-B cells in the mice

with the genotype of #95 × #52 (striped bars), #95 (gray bars), and LM (white bars), summarized from two independent experiments. Circles represent the data from individual animals.
TG and LM, whereas in TG the number of nonlabeled pre-B cells was reduced approximately fourfold below that of LM (Fig. 6 F, a). BrdU-labeled pre-B cells increased in number in the BM of LM 40 h after injection (Fig. 6 F, b), indicating accumulation of pre-B cells after several successive proliferations at the transition from late pro-B to early pre-B cells (35, 36). The number of labeled cells in TG was half that in LM 40 h after injection (Fig. 6 F, b), followed by a remarkable reduction in number 62 h after injection, 10-fold below the level of LM (Fig. 6 F, c), suggesting that inhibition of p21ras activity may affect the life span of pre-B cells. The number of labeled cells in the immature B cells was significantly low in TG relative to LM 40 and 62 h after injection (data not shown), excluding the possibility that transgenic pre-B cells progress to the stage of immature B cells within periods shorter than required for the cells in the LM.

Expression of Bcl-xL Was Downregulated in Transgenic Pre-B Cells. Bcl-xL plays a critical role in regulating pre-B cell survival (9–11). To investigate whether the short life span of transgenic pre-B cells might reflect the low levels of Bcl-xL expression, we analyzed the expression by Western blotting analysis in pre-B cells that were purified from the pooled BM of TG and LM of N-17-95. Fig. 7 A shows that pre-B cells expressed Bcl-xL, as previously reported (11), and the level of expression was reduced in transgenic pre-B cells, two- to threefold below the level in LM. RT-PCR analysis showed that BP-1 and BP-1' pre-B cells expressed bcl-xL transcripts at comparable levels in both N-17-95 TG and LM, but the level of expression was significantly low in TG relative to LM (data not shown). These results support the notion that p21ras activity is associated with pre-B cell survival, at least in part through regulation of Bcl-xL expression.

Bcl-xL expression reaches its maximum at the small pre-B stage (10). Although the mechanism responsible for upregulation of Bcl-xL at the pre-B stage remains unknown, pre-BCR-mediated signaling would be one of the candidate elements. Considering that the pre-BCR and BCR may activate several common signaling molecules (37), including Ras (27, 28), we set out to determine to what extent the expression of p21Asn-17 ras has an inhibitory effect in the upregulation of Bcl-xL in transgenic B cells via BCR stimulation. As reported previously (11), the expression of Bcl-xL proteins was upregulated in B cells upon IgM stimulation (Fig. 7 B); however, the level was reduced in transgenic B cells to approximately two- to threefold below that of LM. In addition, the expression of Bcl-xL mediated by
of BP-1
in independent experiments. (C) Coexpression of Bcl-2 restored the number of cultured B cells, respectively. Shown is a representative result from three independent experiments. (B) Expression of Bcl-xL protein in splenic B cells measured by dot blot analysis for detection of Bcl-xL (arrowhead). H and L represent the IgH and IgL chains of cells, which might occur at the pre-B stage.

Bcl-xL expression in B cells mediated by extracellular stimulation with the phenotype HSA<sup>+</sup>B220<sup>dull</sup>CD43<sup>dull</sup>BP-1<sup>+</sup> (see Fig. 2 B). Although the nature of IgH<sup>+</sup>SA<sup>+</sup>B220<sup>dull</sup>CD43<sup>dull</sup> cells remains unknown at present, these cells were barely detectable in the BM of N-17-95 TG and LM (data not shown).

We observed that the number of early B cell precursors was indistinguishable between offspring of crosses between N-17-95 and Bcl-2 TG (data not shown). We could draw no definite conclusion on the effect of Bcl-2 in the number of pro-B cells in Asn-17 Ha-ras TG. Bcl-2 TG and Bcl-2/N-17-95 TG prominently expanded Ig<sup>+</sup> B cells with the phenotype HSA<sup>+</sup>B220<sup>dull</sup>CD43<sup>dull</sup>BP-1<sup>+</sup> cells (see Fig. 2 B). Although the nature of Ig<sup>+</sup>SA<sup>+</sup>B220<sup>dull</sup>CD43<sup>dull</sup>BP-1<sup>+</sup> cells remains unknown at present, these cells were barely detectable in the BM of N-17-95 TG and LM (data not shown).

As depicted in Fig. 7 C and summarized in Fig. 7 D, the number of BP-1<sup>+</sup> pre-B and immature B cells was comparable between Bcl-2 TG (Fig. 7, C, d and h, and D, d) and LM (Fig. 7, C, a and e, and D, a), as reported previously (39). The number of BP-1<sup>+</sup> pre-B cells was significantly reduced in N-17-95 TG (Fig. 7, C and D, b), whereas coexpression of Bcl-2 restored the number from 13 to 55% of that in LM, on average (Fig. 7, C and D, c). The number of pro-BCR<sup>+</sup> cells in BP-1<sup>+</sup> and BP-1<sup>+</sup> pre-B cells was almost comparable between Bcl-2/N-17-95 double TG and N-17-95 TG (data not shown), supporting the notion that coexpression of Bcl-2 did not affect the accumulation of

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pre-B cells at the early stage. The number of immature B cells in Bcl-2/N-17-95 double TG (Fig. 7, C, g, and D, c) was approximately twofold above the level in N-17-95 TG (Fig. 7, C, f, and D, b); this effect was statistically significant (P < 0.05). Taken together, these results suggest the possibility that overexpression of Bcl-2 in N-17-95 TG may alleviate the reduction in the number of pre-B cells capable of progression to immature B cells.

Maximal induction of Igk rearrangement occurs at the late pre-B cell stage (14). The Igk rearrangement is preceded by an activation of the chromatin structure in the Igk locus sufficient for substrate accessibility, which is probably mediated by signaling through the pre-BCR (12–14). To delineate the maturation stage of pre-B cells in Bcl-2/N-17-95 double TG, we analyzed genomic DNA samples from purified pre-B cells by a semiquantitative PCR to measure the relative level of Igk rearranged to the normal level. Considering that cell survival is regulated by the Bcl-2 member of proteins that consists of anti- and proapoptotic members (for reviews, see references 38 and 41), the results might imply that p21ras provides multiple effector pathways for pre-B cell survival, in addition to regulation of antiapoptotic activity. In this context, it has previously been suggested that p21ras activates Akt/protein kinase B (PKB) kinase via PI-3K and ERK via Raf-1, which control the activity of proapoptotic effects in different types of cells in vitro (for a review, see reference 42). Antiapoptotic molecules may function by inhibiting the process of caspase activation, whereas proapoptotic proteins may independently regulate a common apoptotic pathway or function by heterodimerization with antiapoptotic proteins (38, 41). Therefore, it is conceivable that exogenous Bcl-2 activity in Asn-17 ras TG would be overridden by proapoptotic activity, which could be downregulated by p21ras in pre-B cells in a normal state. Whether p21ras regulates pre-B cell survival through multiple effector pathways remains to be elucidated.

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