The Transcription Factor Early Growth Response 1 (Egr-1) Advances Differentiation of Pre-B and Immature B Cells

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Summary

In mature B lymphocytes, the zinc finger transcription factor early growth response 1 (Egr-1) is one of the many immediate-early genes induced upon B cell antigen receptor engagement. However, its role during earlier stages of lymphopoiesis has remained unclear. By examining bone marrow B cell subsets, we found Egr-1 transcripts in pro/pre-B and immature B lymphocytes, and Egr-1 protein in pro/pre-B–I cells cultured on stroma cells in the presence of interleukin (IL)-7. In recombinase-activating gene (RAG)-2–deficient mice overexpressing an Egr-1 transgene in the B lymphocyte lineage, pro/pre-B–I cells could differentiate past a developmental block at the B220low BP-12 stage to the stage of B220low BP-11 pre-B–I cells, but not further to the B220low BP-11 IL-2R1 pre-B–II cells. Therefore, during early B lymphopoiesis progression from the B220low BP-11 IL-2R1 pre-B–I stage to the B220low BP-11 IL-2R1 pre-B–II stage seems to occur in at least two distinct steps, and the first step to the stage of B220low BP-11 pre-B–I cells can be promoted by the overexpression of Egr-1 alone. Wild-type mice expressing an Egr-1 transgene had increased proportions of mature immunoglobulin (IgM)1 B220high and decreased proportions of immature IgM1 B220low bone marrow B cells. Since transgenic and control precursor B cells show comparable proliferation patterns, overexpression of Egr-1 seems also to promote entry into the mature B cell stage. Analysis of changes in the expression pattern of potential Egr-1 target genes revealed that Egr-1 enhances the expression of the aminopeptidase BP-1/6C3 in pre-B and immature B cells and upregulates expression of the orphan nuclear receptor nur77 in IgM1 B cells.

Key words: Egr-1 • transcription factor • B cell development • BP-1 • nur77

Antigen binding to surface (s)IgMs in B cells initiates a signal cascade which in the context of secondary signals leads to proliferation and differentiation of mature resting B lymphocytes into plasma or memory cells. Changes in the activity and expression of transcription factors translate activating signals into the modulated expression pattern of downstream genes. One of these transcription factors is called early growth response 1 (Egr-1;1 also known as Krox-24, NGFl-A, Tis-8, zif268, pAT225, or Z-225 [1–3]). Egr-1 is induced very rapidly in many different cell types and tissues, including fibroblasts (1), monocytes (4), lymphocytes (5, 6), kidney (7), neurons (3), and brain (8), in response to a wide range of signals (1–3, 5, 9). In mature B lymphocytes, transient Egr-1 expression is rapidly induced upon stimulation by B cell antigen receptor (BCR) cross-linking (5, 10), whereas signals resulting from Fc receptor cross-linking inhibit induction (11, 12). Thus, the broad spectrum of Egr-1 expression and the diverse modes of Egr-1 induction suggest that Egr-1 functions as a transcriptional regulator that links common biochemical signaling pathways to the rapid modulation of downstream gene expression.

Mature peripheral B lymphocytes originate from bone marrow precursor cells that are ordered according to their phenotype, gene expression, Ig gene rearrangement, and
proliferative and developmental potential into the pro-B, pre-B, and immature B lymphocyte subsets (13–17). Transcriptional regulation plays a critical role during B cell development (for a review, see reference 18) as shown by gene targeting of multiple transcription factors. Mutations in these factors that obliterate their activity were shown to arrest B lymphopoiesis at defined stages of maturation (19–29).

Little is known about the expression and function of Egr-1 during early steps of B cell differentiation. Here we report that Egr-1 expression can be detected already in pre-B cells isolated from bone marrow and in fetal liver-derived pre-B cell cultures. These results suggested that Egr-1 might also have a regulatory function in early stages of B lymphopoiesis. However, mice deficient for Egr-1 fail to show defects in lymphocyte or monocyte maturation, most probably because the missing Egr-1 activity is masked by other members of the Egr transcription factor family (30, 31). To bypass the complementing activity of Egr-2, Egr-3, or Egr-4, we studied B lymphocyte differentiation in transgenic mice overexpressing Egr-1 in B cells in normal and recombinase-activating gene (RAG)-2–deficient mice. Since the RAG-2 mutation prevents rearrangement of Ig genes (32), precursor B cells are developmentally arrested in the stage of B220BP-1–pro/pre-B–1 cells (33, 34). Analysing Egr-1 transgenic RAG-2–deficient mice, we found that pro/pre-B–1 cells overcame the RAG-2–/- induced differentiation block at the stage of B220BP-1–pro/pre-B–1 cells and differentiated into B220BP-1–pre-B–1 cells. Comparing B lymphocyte maturation in the bone marrow of normal transgenic and control animals, we found that Egr-1 transgenic mice had increased their fraction of mature cells. Because Egr-1–enhanced progression of developing thymocytes was also found in transgenic mice overexpressing Egr-1 in T cells (35), we propose that Egr-1 activity promotes maturation of B and T lymphocytes.

Materials and Methods

Pre-B Cell Cultures. Fetal liver cells of day 15–18 embryos were removed and plated onto irradiated ST-2 feeder cells in Iscove’s medium containing IL-7 and 10% FCS. Cells were cultured as described previously (36). Cells from transgenic lines were identified by PCR. For further analyses, nonadherent cells were collected and washed twice in ice-cold PBS. Samples from wells containing only ST-2 feeder cells were treated in parallel and served as controls.

Mice. The detailed description of the generation of Egr-1 transgenic mice using the BALB/c embryonic stem cell line BALB/c-1 will be described elsewhere. Egr-1 transgenic mice of the IA7 line were transferred to a special pathogen–free unit by backcrossing female Egr-1 transgenic IA7 mice twice with C57BL/6. Egr-1 transgenic DNA, 5′-CTTCCGTTTGGGGGCTGGGACA-3′ and 5′-CGCTGCTGGTGCCTGGC-3′, as transgene-specific primer pair. The RAG-2–/- phenotype was verified by FACS$^\alpha$ analysis of peripheral blood cells.

RNA Isolation, Northern Blot, and PCR Analysis. RNA was extracted using the guanidinium thiocyanate method as described (37). For Northern blotting, 10 µg of total RNA was separated in a 1% agarose gel containing 7% formaldehyde, transferred onto nylon filters, and fixed by UV cross-linking. Filters were prehybridized (50% deionized formamide, 5 × SSC, 5 × Denhardt’s solution, 50 mM Na$_2$PO$_4$·$\alpha$H, 7.10 mM Na$_2$PO$_4$·$\alpha$H, 0.1% SDS, 0.1 mg/ml denatured salmon sperm DNA) for 2 h at 42°C. For detection of Egr-1–specific transcripts, [α-32P]dATP–labeled probes were prepared from a 1.6-kb EcoRI-HindIII fragment from plasmid pMS33 (a gift from V. Sukhatme) containing the Egr-1 cDNA by the oligonucleotide priming method (38). The probe was added to the prehybridization and filters were incubated overnight, washed with 0.2 × SSC, 0.1% SDS at 42°C, and exposed to X-ray films. Egr-1 expression was analyzed by PCR using cDNA reverse transcribed from total RNA with Superscript II (GIBCO BRL, Eggenstein, Germany) and the Egr-1–specific primers 5′-GCAGATCTCTGACCCGTTG3′ and 5′-CCGAGCTTTGCCTGGGATA-3′ as described by T. Miyazaki (35). PCR was performed using Taq polymerase (MBI Fermentas, Inc., Amherst, NY) using 1/25 of the cDNA reaction as template at an annealing temperature of 54°C.

Immunoblot Analysis. Bone marrow cells from six femurs were isolated and resuspended in FACS buffer (0.1% sodium azide, 3% FCS in PBS). B220–specific biotin–labeled antibody R A3.3A1 (39) was added and incubated for 30 min on ice. Cells were washed, magnetic streptavidin–labeled beads (Dynal, Oslo, Norway) were added, and B cells were isolated. Quality of the sorting process was verified by flow cytometric analysis. The B cells were resuspended in 30 µl lysing buffer (1% NP-40, 150 mM NaCl, 10 mM Tris·HCl, pH 7.0, 0.1 mM PMF·SC) and incubated on ice for 10 min. Cell debris was removed by centrifugation (10 min, 4°C, 22,000 × g), and the extract was separated by SDS-PAGE (8%) and transferred onto nitrocellulose membrane (Hybond C extra, Amersham Pharmacia Biotech, Uppsala, Sweden). Egr-1 was detected using the antisera C19 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 25 ng/ml followed by peroxidase–conjugated goat anti-rabbit IgG (F(ab’)$_2$) (Dianova GmbH, Hamburg, Germany) at 200 ng/ml. Expression of nur77 was analyzed using a mouse IgG anti-nur77 mAb (a gift of B. Osborne, University of Massachusetts, Amherst, MA) followed by peroxidase–conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Inc., Birmingham, AL). IgM was detected by a goat anti–mouse IgM peroxidase–labeled serum (Southern Biotechnology Associates, Inc.). Signals were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech).

Flow Cytometry. Flow cytometry was carried out as described previously (40) using the following antibodies: R S3.1–biotin for murine IgM$^\alpha$ (41), 6C3–biotin for BP-1, 7D4–biotin for IL-2R$\alpha$ chain, 2B8–biotin for c-kit, S7–biotin for leukosialin, AM S9.1–biotin for IgD$, R A3.682–PE for B220, IM 7–biotin for Pgp-1, 3E2–PE for intercellular adhesion molecule 1 (ICAM-1) (all from Pharmingen Europe, Hamburg, Germany), and biotinylated PB493 (42) to stain immature B lymphocytes. Cells were counterstained using PE- or APC–conjugated streptavidin (PharMingen Europe). Unspecific binding to Fc receptors was blocked by adding unlabeled mouse FcγR–specific mAb 2G4.2. Dead cells were excluded by staining with propidium iodide. Using a FACS$\alpha$ alibur$^\alpha$ and CellQuest$^\alpha$ software (Becton Dickinson, San Jose, CA), 3–5 × 10$^4$ cells were acquired according to their forward/side scatter pattern and analyzed. To analyze Egr-1 expres-
tion in bone marrow B cell subsets, $3.4 \times 10^5$ pre-B and $7 \times 10^4$ immature B cells were isolated from both femurs of a 5-wk-old BALB/c mouse by cell sorting at $4^\circ C$ according to their IgM$^+$/PB493 staining pattern using a FACStar$^+$ cell sorter and LYSIS II$^+$ software (Becton Dickinson).

Bromodeoxyuridine Treatment and Staining. Bone marrow cells were labeled with bromodeoxyuridine (BrdU; Sigma, Deisenhofen, Germany) starting with a single injection of 1 mg/ml i.p. BrdU and feeding mice continuously with drinking water containing 1 mg/ml BrdU for 48 h as described (43). During the labeling period, the drinking water was protected from light. Simultaneous detection of surface staining and BrdU labeling was done as described (44). After surface marker staining, cells were resuspended in 500 $\mu$M NaCl, 1.2 ml ice-cold 95% ethanol was added, and the cells were incubated 30 min on ice. Cells were washed and resuspended in 1 ml fixation buffer (1% paraformaldehyde and 0.01% Tween in PBS). After incubation for 30 min at room temperature, the cells were incubated for 30 min in DNase I solution (50 KU DNase I in 4.2 mM MgCl$_2$ 0.15 M NaCl, pH 5). Cells were washed, 10 $\mu$l anti-BrdU antibody (Becton Dickinson) was added, and the cells were then incubated for 30 min and washed.

Electrophoretic Mobility Shift Assays. Gel shift was carried out using recombinant Egr-1 as described (45) with double-stranded radiolabeled oligonucleotides from the nur77 and BP-1 promoter regions carrying putative Egr-1 binding sites (bold): 5'-TTC-CAAGTTCTCCCTCAGCCCTCCTC-3' for BP-1 (position -753 to -729), 5'-GTCAGGGCCGGCCGGCGCCCTCC-3' for nur77 (position -66 to -50), and 5'-GGATC-CAGCGGCGCGAGCGCGGCGGCG-3' for Egr-1.

Results

Egr-1 Expression in Pre-B and Immature B Cell Precursors. BCR cross-linking has previously been reported to induce Egr-1 expression in mature B cells, but not in immature B lymphocytes or in immature B cell lines (5, 46, 47). We addressed the question of whether unstimulated pre-B and immature B cells express Egr-1 by analyzing sorted B cell subsets from murine bone marrow. Transcription of the Egr-1 gene was found by PCR in both IgM$^{-}$ (pre-B) and IgM$^{+}$ PB493$^{+}$ immature B cells (Fig. 1 A). Likewise, Egr-1 protein was detected by immunoblotting in IgM$^{-}$ pre-B cells isolated from fetal liver and expanded in culture on ST-2 stroma cells in the presence of IL-7 (Fig. 1 B). Both results show transcription of the Egr-1 gene and translation of Egr-1 mRNA into detectable amounts of protein as early as the pre-B cell stage before BCR surface expression.

Egr-1 Expression in Transgenic and Normal Mice. These results suggested that Egr-1 function might also be important during early stages of B lymphopoiesis. To test this hypothesis, we generated transgenic mice expressing Egr-1 specifically in B lymphocytes using an Ig heavy chain promoter/enhancer construct. Four different founder mice showing Egr-1 germline transmission were obtained. By breeding to BALB/c mice, we established the Egr-1 transgenic lines IA7, IB10, IC4, and ID4 (to be published elsewhere). At first, we compared Egr-1 expression between transgenic and BALB/c control mice by Northern and immunoblotting (Fig. 2). Spleen cells of the line IA7 expressed 10-fold more Egr-1 mRNA than the control littermates, whereas the other lines showed Egr-1 expression levels of about two- to threefold above unstimulated spleen cells (Fig. 2 A). Since transgenic IC4 mice expressed only low levels of Egr-1 they were abandoned. Carrying out most of the experiments with mice from lines IA7, IB10, and ID4, we found only small variations between these transgenic lines. Testing Egr-1 protein expression, we found high levels in purified B220$^{+}$ bone marrow B cells as well as in cultivated pre-B cells isolated from fetal liver (Fig. 2, B and C, respectively). These results show that transgenic Egr-1 is expressed during similar stages of B cell maturation, but at far higher levels than endogenous Egr-1.

Egr-1 Expression Promotes At Least Two Different Stages of B Cell Development. To examine whether enhanced Egr-1 expression has an effect on early stages of B cell development, we backcrossed the IA7 transgenic mice to a RAG-2-deficient background. The RAG-2 mutation prevents rearrangement of the Ig genes (32) and therefore blocks B cell maturation at the pro/pre-B→I stage (16, 34). These cells carry the surface markers c-kit and CD43; $<$5-15% express BP-1 and $<$1% express the IL-2R$\alpha$ chain (data not shown). Phenotypically, these pro/pre-B→I cells correspond to fraction B as classified by Hardy et al. (13). FACScan analysis of control and Egr-1 transgenic RAG-2-deficient mice revealed an unchanged expression pattern for c-kit and CD43, but a three- to fourfold increase in the fraction of BP-1$^{+}$ cells, compared with control littermates (Fig. 3). Phenotypically, these BP-1$^{+}$ c-kit$^{+}$ CD43$^{+}$ pre-B lymphocytes are defined as fraction C cells (13), and progression into this stage normally requires RAG-2 expression and Ig heavy chain gene rearrangement (16, 34), suggesting that Egr-1 might support the maturation of fraction B pre-B cells even in the absence of RAG-2 activity. The reduced cell size of transgenic BP-1$^{+}$ B220$^{+}$ lymphocytes as reflected by changes in forward/side scatter (Fig. 3 A, C,

![Figure 1](image-url)

**Figure 1.** Expression of Egr-1 during different stages of B cell maturation. (A) Egr-1 mRNA expression. Bone marrow cells of a 5-wk-old BALB/c mouse were stained for B220, PB493, and IgM$^+$ expression. Pro/pre-B cells (B220$^{+}$, PB493$^{+}$, and IgM$^+$) and immature B cells (B220$^{+}$, PB493$^{+}$, and IgM$^-$) were sorted and RNA was extracted. Analysis of Egr-1 transcripts by reverse transcription PCR was performed as described by Miyazaki (35). Lane 1 shows expression of Egr-1 in pro/pre-B cells and lane 2 in immature B cells. Anti-IgM-stimulated splenocytes (lane 3) serve as a positive control. In lane 4, cDNA was omitted from the PCR. (B) Expression of Egr-1 protein. BALB/c fetal liver B cells (day 16) were cultivated in the presence of IL-7 on ST-2 stroma cells. Cellular lysates of 10$^6$ cells were examined for Egr-1 expression by immunoblotting using the Egr-1-specific antibody C19 and developed with horseradish peroxidase–coupled goat anti–rabbit IgG. In cultivated pre-B cells, Egr-1 protein expression is easily detected (lane 2). As a negative control an equal amount of ST-2 feeder cells was loaded in lane 1.
mice. In parallel, samples were stained with a horseradish peroxidase-conjugated goat anti-mouse IgM antibody to standardize for the different amounts of protein loaded per lane (b). (C) Increased expression of Egr-1 protein in transgenic pre-B cells. Pre-B cell cultures and immunoblots were performed as described for Fig. 1B. Transgenic pre-B cells (IA7, lane 1) express higher levels of Egr-1 than an equal amount of wild-type BALB/c pre-B cells (lane 2). Egr-1 protein is undetectable in whole cell lysates of corresponding numbers of ST-2 feeder cells (lane 3).

Figure 2. Expression of transgenic Egr-1. (A) Comparison of Egr-1 mRNA levels in BALB/c and Egr-1 transgenic spleen cells. RNA was extracted from splenocytes, and Northern blot analysis was performed using a probe specific for endogenous and recombinant Egr-1 mRNA. Because the endogenous and transgenic Egr-1 mRNA species migrate with different electrophoretic mobilities, they are easily identified on Northern blots (data not shown). To standardize for the amounts of mRNA, filters were rehybridized with a GAPDH-specific probe. The relative intensity of the Egr-1 expression in BALB/c and in four transgenic lines (IA7, IB10, IC4, and ID4) was determined using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). (B) Expression of Egr-1 protein in B cells of the bone marrow. B220⁺ B cells were purified from the bone marrow of three BALB/c and three ID4 transgenic mice using streptavidin-loaded magnetic beads and the B220-specific, biotinylated antibody R A3.3A1. Whole cell lysates with equal amounts of protein were analyzed for Egr-1 expression by immunoblotting using the Egr-1-specific antibody C-19 (a). Lane 1 shows Egr-1 expression in BALB/c bone marrow cells, lane 2 in ID4 mice, lane 3 shows an equal amount of wild-type BALB/c pre-B cells.

Figure 3. Egr-1 induces development of BP-1⁺ B220⁻ bone marrow cells. Bone marrow cells of IA7 transgenic mice with a RAG-2 background and control littermates were stained with BP-1- and B220-specific antibodies and analyzed by flow cytometry. Dead cells were excluded by gating for propidium iodide-negative cells, and 5 × 10⁴ cells were acquired according to their forward/side scatter (FSC, SSC) profile. As shown for one individual example in A, IA7 mice (b) have about three times more BP-1⁺ cells than control littermates (a). Changes in the forward/side scatter pattern document that most of the B220⁺ BP-1⁺ cells (74% small cells, d) are smaller than the majority of BP-1⁻ cells (37% small and 54% large cells, c). B compiles the BP-1 staining pattern for six RAG-2-deficient IA7 mice and six control littermates.

Figure 4. Normal proliferation of B cells in Egr-1 transgenic mice. The enlarged population of BP-1⁺ pre-B cells in transgenic RAG-2-deficient mice and the higher percentage of mature cells in transgenic RAG-2⁺ animals could result from an increased proliferative potential of B cell precursors. Because dividing B lymphocytes can be traced by incorporation of the nucleotide analogue BrdU (49), we compared the proportion of BrdU⁺ cells in the pre-B, immature B, mature B cell subsets between control littermates and transgenic mice after 2 d of in vivo labeling. The similar percentages of BrdU⁺ cells in all B cell subsets of both groups of mice suggest that the expression of transgenic Egr-1 does not change the proliferative potential of B cells (Fig. 5).

Regulation of downstream genes. The increased maturation of transgenic B cells would be expected to correlate with changes in the expression of genes controlled directly or indirectly by Egr-1. Using a panel of antibodies, we screened transgenic bone marrow B cells for alterations in the expression pattern of B cell–specific surface markers by flow cytometry. Changes were not observed except for a slight increase in the percentage of BP-1⁺ cells and in BP-1 expression levels in all transgenic lines (Fig. 6A). As discussed above, the proportion of BP-1⁺ cells might relate to the Egr-1–enhanced transition of fraction B pre-B cells into the fraction C stage, but it would not explain higher B-P-1 surface levels. Therefore, we speculated that Egr-1 might regulate the transcription of the BP-1 gene and screened the BP-1 promoter for potential Egr-1 binding.
sites. Finding a (5′-GAGGGGGGAA) sequence ~1.6 kb upstream of the mRNA start (50) resembling an Egr-1 binding site (5′-GCGGGGGGCG), we analyzed by an electrophoretic mobility shift assay (EMSA) if recombinant Egr-1 binds to an oligonucleotide containing the putative Egr-1 recognition site from the BP-1 promoter. As shown in Fig. 6 C, labeled oligonucleotides containing a cognate Egr-1 binding site (lane 1) or the binding site from the BP-1 promoter (lane 15) produced a shifted DNA–protein complex with identical electrophoretic mobility. Their intensities were reduced only by adding an excess of unlabeled oligonucleotides with an Egr-1 binding site but not by competing with an Sp-1 binding site (lanes 2–5 and 16–19, respectively). Likewise, only the addition of Egr-1– but not of Sp-1–specific antibodies retarded the migration of the complex (lanes 6 and 7, 20 and 21). Therefore, the forced expression of Egr-1 in transgenic B cells may not only help pre-B cells to proceed from fraction B into fraction C but may also enhance the expression of BP-1, which is normally upregulated during this transition.

The nur77 gene (also called NGFI-B [3] or N10 [51]) encoding an orphan nuclear receptor represents one of the transcription factors found to be induced by Egr-1 (52). Analyzing nur77 expression in purified B220+ bone marrow cells of transgenic (ID4) and control littermates by Western blotting, we found nur77 to be expressed only by transgenic but not by control B cells (Fig. 6 B). Since upregulated nur77 expression was not found in cultivated transgenic spl pre-B cells (data not shown), the induction of the nur77 gene seems to be confined to pre-B-II or IgM+ bone marrow cells. To analyze if Egr-1 could directly induce nur77 transcription, we tested by EMSA the binding of recombinant Egr-1 to an oligonucleotide of the nur77 promoter (position −74 to −50) containing a cognate Egr-1 binding site. As shown in Fig. 6 C, binding of recombinant Egr-1 produced a DNA–protein complex (lane 8). The specificity of Egr-1 binding was demonstrated by a competition assay using an excess of an oligonucleotide carrying an Egr-1 binding site (lanes 11 and 12) and by the decreased mobility of the complex upon the addition of an Egr-1–specific antibody (lane 13).

Since it was shown recently that nur77 activity is involved in the induction of apoptosis during negative selection of thymocytes (53–56) and in the upregulation of CD95L expression (57), we looked for enhanced CD95L expression in Egr-1 transgenic mice compared with control littermates. In contrast to the results reported for nur77-expressing T cells, we did not find increased CD95L expression in Egr-1 transgenic B cells (data not shown).

In response to BCR-derived signals, Egr-1 is thought to modulate the expression pattern of downstream genes that promote further activation and differentiation of B lymphocytes. Using variants of the B cell line WEHI-231, it was shown that Egr-1 induces the expression of CD44 and of intracellular adhesion molecule 1 (ICAM-1 [58, 59]). Therefore, we tested the expression pattern of both surface markers in our transgenic mice, but did not detect differences when compared with BALB/c controls (data not shown).

### Discussion

Egr-1 A accelerates B cell Maturation. Mature B cells respond to signals resulting from antigen receptor engagement by immediately inducing Egr-1 transcription (5), but the role of Egr-1 in earlier stages of B cell development has
not been defined. The different stages and the order of B cell development are well characterized, allowing the precise typing of bone marrow B cells according to the expression of characteristic cell surface markers, the rearrangement of Ig genes, and the proliferative and differentiation potential of B cell precursors (13, 14, 16, 60, 61). By analyzing Egr-1 expression in bone marrow–derived B lymphocyte subsets and by testing Egr-1 expression in cultured, fetal liver–derived pre-B cells, we have shown that Egr-1 is also expressed in pre-B cells lacking sIgM as well as in immature sIgMa B cells in the absence of sIgM-induced signals. These observations suggest that Egr-1 might also have a regulatory function in pre-B cell development. By studying transgenic mice overexpressing Egr-1 from the pre-B stage on, we have found higher proportions of mature B cells and fewer immature B cells in transgenic animals than in control littermates. To identify if early stages of B lymphopoiesis are sensitive to Egr-1 activity, we arrested B cell development at the stage of pro/pre-B–I cells by backcrossing the Egr-1 transgenic line IA7 to mice deficient in RAG–2. Since the null mutation in the RAG–2 gene prevents rearrangement of Ig genes (32), B cell precursors do not receive stimulating signals required for developmental progression beyond the stage of B220low CD43– BP-1– pro/pre-B cells (16, 62), also defined as fraction B (13). Comparing the phenotype of bone marrow pro/pre-B cells from transgenic and control mice, we found a three- to fourfold increased population of BP-1+ pre-B cells in Egr-1 transgenic mice. Since the transcription activation function of Egr-1 seems to enhance BP-1 expression in more mature B cell subsets, the increase in BP-1+ pre-B cells could also reflect the induction of BP-1 expression only and not Egr-1–induced differentiation. However, this seems to be less likely because transgenic BP-1+ cells were found to be smaller than BP-1+ cells, consistent with further maturation. Therefore, these results suggest that forced expression of Egr-1 in BP-1– pro/pre-B cells induces progression into the stage of BP-1+ pre-B cells (fraction C). Since these cells failed to upregulate the IL-2Rα chain and heat-stable antigen, two markers characteristic for pre-B–II cells (fraction C' and D (13, 16)), overexpression of Egr-1 in pro/pre-B–I cells seems to be sufficient to induce differentiation to fraction C, but not to more mature stages of B lymphopoiesis.

Progression of pro/pre-B cells developmentally arrested by a mutation in the RAG–2 gene into more mature pre-B cell stages is also induced by in vivo cross-linking of the Igα/β heterodimer using Ig-α–specific mAbs (63). Under those conditions, anti-Ig-α–treated pro/pre-B–I cells become smaller in size and acquire IL-2Rα expression in addition to BP-1. Since they also downregulate c-kit (CD117) and CD43, they are considered as small pre-B–II cells. In the same report, it was shown that Ig-β cross-linking stimulates tyrosine phosphorylation of several substrate...
proteins, including Ig-α, Syk, and Vav, and the activation of mitogen-activated protein kinase extracellular signal-regulated kinase (ERK). Based on these results, Nagata et al. (63) proposed that the signal cascade initiated by Ig-β activation evokes differentiation signals similar to those delivered by the pre-BCR in normal B cell development. For mature B cells it is known that BCR engagement upregulates Egr-1 transcription through a signal cascade including p21/ras and mitogen-activated protein kinase (ERK [10, 64]), and for other cell types it has been shown that ERK activation induces Egr-1 transcription (65). Since RAG-2-deficient pro/pre-B-1 cells overexpressing Egr-1 do not reach the same developmental stage as anti-Ig-β-stimulated cells, it seems likely that Egr-1 activity substitutes only part of the differentiation signal originating from the pre-BCR.

Analyzing later stages of B cell development in RAG-2+/+ Egr-1 transgenic mice, we observed lower proportions of immature and increased proportions of mature bone marrow B cells compared with their wild-type littermates, whereas there was no increased proliferation of transgenic pre-B or immature B cells detectable. These findings are consistent with the current model of the development from immature to mature B cells (66, 67). Immature B cells leave the bone marrow and enter the spleen where about half of them reach the mature stage (42). Mature bone marrow B cells are thought to be part of the recirculating pool. This would suggest that Egr-1 influences this migration at one or several steps.

Egr-1 expression was also found in CD4+CD8− double negative thymocytes by Miyazaki (35). Overexpression of transgenic Egr-1 in a RAG-2−/− background allowed thymocytes to bypass the RAG-2−/− dependent block at the IL-2R−/− Pgp-1− double negative stage and develop into immature CD8 single-positive cells, but not further to the CD4+CD8− double-negative cell stage. In cortical CD4+CD8+ thymocytes, Egr-1 expression was reported by Shao et al. (68) to be dependent on TCR engagement, suggesting that high level expression of Egr-1 in the thymus might be a consequence of thymocyte selection. The high coincidence of Egr-1 expression in analogous B and T cell precursor subsets and the increased differentiation of pro/pre-B-1 cells and thymocytes in Egr-1 transgenic mice suggest that Egr-1 activity regulates similar functions in both types of lymphocytes.

Downstream Target Genes. Searching for potential downstream target genes responding to Egr-1, we found increased expression of the nuclear orphan receptor nur77 in bone marrow B cells from transgenic mice but not in cultivated transgenic pro/pre-B-1 cells. Since we also could demonstrate binding of recombinant Egr-1 to a cognate Egr-1 binding site present in the nur77 promoter, it seems likely that Egr-1 directly induces nur77 expression in B lymphocytes before the mature B cell stage. It was reported that nur77 activity is involved in the regulation of thymocyte apoptosis (53, 54) by inducing CD95L expression (57). However, in the Egr-1 transgenic mice, we could detect neither upregulation of CD95L expression by bone marrow B cells nor an increased frequency of apoptotic cells (Warnatz, K., unpublished results). On the other hand, cellular responses other than apoptosis may be linked to nur77 function, since it is also induced upon antigen receptor ligation in B and T cells during proliferative responses (52, 69). Besides nur77 promoter, we also found enhanced expression of BP-1 in pre-B-II and in IgM+ bone marrow cells. Similar to the nur77, we could also demonstrate binding of recombinant Egr-1 to a sequence from the BP-1 promoter resembling an Egr-1 binding site. Therefore, it seems that Egr-1 activity promotes not only development to the stage of BP-1+ cells (fraction C), but also an increased surface expression of BP-1 on BP-1+ B cells. Although it is known that BP-1 acts as an aminopeptidase catalyzing the hydrolysis of acidic amino acid residues from the NH2 termini of proteins (70), its role in B lymphopoiesis remains to be clarified (71, 72).

Conclusions. Here we provide evidence that Egr-1 supports at least two distinct steps of B cell maturation, the progression into the pre-B and into the mature B cell stage. Since Egr-1 activity is also sufficient to promote the development of double negative thymocytes into immature single-positive CD8low cells (35), as well as macrophage in vitro differentiation (73, 74), this transcription factor seems to play an important role in the differentiation of three major hematopoietic cell types.

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