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# The Role of MAPKs in B Cell Receptor-induced Down-regulation of Egr-1 in Immature B Lymphoma Cells*

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**Cross-linking of the B cell receptor (BCR) on the immature B lymphoma cell line BKS-2 induces growth inhibition and apoptosis accompanied by rapid down-regulation of the immediate-early gene *egr-1*. In these lymphoma cells, *egr-1* is expressed constitutively and has a prosurvival role, as Egr-1-specific antisense oligonucleotides or expression of a dominant-negative inhibitor of Egr-1 also prevented the growth of BKS-2 cells. Moreover, enhancement of Egr-1 protein with phorbol 12-myristate 13-acetate or an *egr-1* expression vector rescued BKS-2 cells from BCR signal-induced growth inhibition. Nuclear run-on and mRNA stability assays indicated that BCR-derived signals act at the transcriptional level to reduce *egr-1* expression. Inhibitors of ERK and JNK (but not of p38 MAPK) reduced *egr-1* expression at the protein level. Transcriptional regulation appears to have a role because *egr-1* promoter-driven luciferase expression was reduced by ERK and JNK inhibitors. Promoter truncation experiments suggested that several serum response elements are required for MAPK-mediated *egr-1* expression. Our study suggests that BCR signals reduce *egr-1* expression by inhibiting activation of ERK and JNK. Unlike ERK and JNK, p38 MAPK reduces constitutive expression of *egr-1*. Unlike the immature B lymphoma cells, normal immature B cells did not exhibit constitutive MAPK activation. BCR-induced MAPK activation was modest and transient with a small increase in *egr-1* expression in normal immature B cells consistent with their inability to proliferate in response to BCR cross-linking.**

The immediate-early gene *egr-1* (also known as *NGFI-A*, *krox24*, *zif268*, and *tis8*) encodes a transcription factor containing three tandem zinc finger motifs that bind to GC-rich DNA elements in the promoters of a range of target genes to activate their transcription (1). *egr-1* expression is elicited in response to a diverse variety of signals, including growth factors, cytokines, lipopolysaccharide, serum, irradiation, stress, hypoxia, and urea, in many cell types, including neuronal cells, epithelial cells, fibroblasts, myeloid cells, and T and B lymphocytes (2–9). In many studies, Egr-1 was found to be associated with cell proliferation, differentiation, and transformation (3, 10–14). Egr-1 was also shown to induce apoptosis in certain cell types in response to irradiation by activating p53 (15) or PTEN expression (16). Yu *et al.* (17) reported that differential post-translational modification of Egr-1 (acetylation *versus* phosphorylation) is likely responsible for its different roles in promoting growth *versus* apoptosis in response to serum and UV irradiation. Moreover, Egr-1 is shown to be important for production of pro-inflammatory cytokines such as interleukin-1$\beta$, interleukin-13, and tumor necrosis factor as well as chemokines (18–20).

In normal mature B lymphocytes, signaling through the B cell receptor (BCR)[2] induces rapid and transient expression of *egr-1* (6), but its importance for subsequent B cell activation and proliferation is unknown. In contrast, BCR engagement in immature B lymphoma cells fails to induce *egr-1* expression, and the lymphoma cells undergo growth inhibition and apoptosis (21). Thus, BCR-induced positive *versus* negative signals are reflected in the differential expression of *egr-1*. Interestingly, an immature B lymphoma cell line (BKS-2) constitutively expresses high levels of Egr-1, and antisense oligodeoxynucleotides (ODNs) to Egr-1 inhibit BKS-2 cell growth (14). BCR signaling, which causes growth arrest and apoptosis of BKS-2 cells, also down-regulates *egr-1* (14). The down-regulation of Egr-1 and growth inhibition caused by anti-IgM antibody are reversed by CpG ODN (22). These data suggest a positive correlation between the levels of Egr-1 and growth of B lymphoma cells. An examination of the microarray data published by Alizadeh *et al.* (23) revealed that *egr-1* expression is elevated in ~35% of human diffuse large B lymphoma cells, supporting the notion that Egr-1 is important for B lymphoma cell growth.

Several studies in different cell lines have shown that the rapid induction of Egr-1 with various stimuli is mediated

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[2] The abbreviations used are: BCR, B cell receptor; ODNs, oligodeoxynucleotides; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; EGFP, enhanced green fluorescent protein; ICAM-1, intercellular adhesion molecule 1; FCS, fetal calf serum; FACS, fluorescence-activated cell sorter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PMA, phorbol 12-myristate 13-acetate; SREs, serum response elements.

through ERK1/2. In cardiomyocytes, estrogen induces the rapid induction of Egr-1 mRNA through activation of ERK1/2 (24). In human monocytes, the rapid induction of Egr-1 by lipopolysaccharide is dependent on the Ras/Raf-1/MEK/ERK pathway (19). In erythroleukemia cells, the erythropoietin-induced expression of *egr-1* is mediated through the ERK1/2 pathway (25). In T cell hybridoma, ERK activation is required for induction of *egr-1* promoter activity by T cell receptor stimulation (26). In splenic B cells, the induction of Egr-1 in response to BCR signaling is also mediated through the Ras/Raf-1/MAPK pathway (27). However, factors that govern the constitutive expression of *egr-1* in certain lymphoma cells or mechanisms that result in BCR-mediated down-regulation of Egr-1 are not known.

Here, we found that constitutive expression of *egr-1* in B lymphoma cells is dependent on constitutively active ERK and JNK pathways. The growth of B lymphoma cells is inhibited by blocking the Egr-1 upstream pathways (ERK and JNK) or inhibiting Egr-1 directly by retrovirus-mediated expression of the dominant-negative construct WT1-EGR1 (28). Furthermore, we found that Egr-1 expression is regulated by MAPK at the level of transcription and that the down-regulation of Egr-1 by BCR is through down-regulation of ERK and JNK activities.

## EXPERIMENTAL PROCEDURES

*Reagents*—The MEK1/2 inhibitors PD98059 and U0126 and the p38 MAPK inhibitor SB203580 were purchased from Calbiochem. SP600125, an anthrapyrazolone inhibitor of JNK, was obtained as a gift from Dr. B. Bennett (Celgene, San Diego, CA). All three inhibitors were dissolved in $Me_2SO$ to make a 20 mM stock solution and diluted in culture medium before use. Phospho-specific antibodies against JNKs ($Thr^{183}/Tyr^{185}$), ERKs ($Thr^{202}/Tyr^{204}$), and p38 ($Thr^{180}/Tyr^{182}$) were obtained from Cell Signaling Technology, Inc. (Beverly, MA). The antibody to total p38 was also obtained from Cell Signaling Technology, Inc. Antibodies to total JNK1, ERK, Egr-1 (clone C-19), and the N-terminal domain of the Wilms tumor protein WT1 (clone F-6) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-β-actin monoclonal antibody was obtained from Sigma. The effectiveness and specificity of PD98059 for MEK1/2 and SP600125 for JNK have been demonstrated by previous studies (29–32).

*DNA Plasmids and Antisense ODNs*—The *egr-1* expression plasmid pBX-Egr1 containing the full-length Egr-1 cDNA driven by the SV40 promoter has been described (33). The dominant-negative Egr-1 construct WT1-EGR1 was cloned into the retroviral vector LZRSpBMN-linker-internal ribosomal entry site-enhanced green fluorescent protein (EGFP) encompassing a ribosomal entry site allowing for cap-independent translation of EGFP (40). LucA–D constructs containing different *egr-1* promoter regions linked to a firefly luciferase gene have been described previously (5). To create the p903luc construct, the *egr-1* promoter sequence from −903 to +65 was excised from the pBL903 plasmid described by McMahon and Monroe (34) by XbaI and ligated into the NheI-treated promoterless firefly luciferase vector pGL3b (pluc) from Promega (Madison, WI). To create the p395luc construct, the *egr-1* promoter sequence from −395 to +65 was excised from the pBL395 plasmid described by McMahon and Monroe (34) by XbaI and SalI double digestions and ligated into the NheI- and XhoI-treated plasmid pGL3b. To create the p242luc construct, the *egr-1* promoter sequence from −242 to +65 was PCR-amplified from the pBL395 vector, and the PCR fragment was treated with XhoI and SalI and ligated into the XhoI-treated pGL3b vector. To create the actin promoter-driven *Renilla* luciferase construct (pActinRluc), the *Renilla* luciferase gene was excised from the pRL-SV40 vector (Promega) by HindIII and BamHI enzyme digestions and ligated into the pHβ-Apr1 plasmid (a gift from Dr. Daret St. Clair, University of Kentucky) that had been treated with the same two enzymes. All DNA clones were confirmed by DNA sequencing. In experiments aimed at blocking *egr-1* expression, a phosphorothioate-capped antisense Egr-1 ODN (AS2AS295, 5′-CTTGGCCGCTGCCAT-3′), a control phosphorothioate-capped nonsense Egr-1 ODN (NS281, 5′-GAGCGACCAGGCCCTACCGT-3′), and a phosphorothioate-capped antisense ODN against ICAM-1 (ISIS3082, 5′-TGCATCCCCCAGGCCACCAT-3′) were used. The antisense ODNs for Egr-1 and ICAM-1 have been described previously (35, 36).

*Mice, Cell Lines, and Cell Preparation*—Neonatal (9–11 days old) and young adult (2–3 months old) BALB/c mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Female CBA/N (Xid) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific pathogen-free conditions in micro-isolator cages. B cells were purified by two methods. For MAPK experiments, splenic cells were pooled from at least one litter of neonatal mice and purified by negative selection using magnetic cell sorter (MACS®) B cell enrichment MicroBeads (Miltenyi Biotec, Bergisch Gladbachor, Germany). As previously found in our laboratory (37), the splenic B cells from neonatal mice were predominantly immature B cells. The purified B cells were ~90% B220 and AA4.1 double positive as determined by flow cytometry. The purified B cells were rested in IF-12 medium (1:1 mixture of Iscove's modified Dulbecco's medium and Ham's F-12 medium) in a 37 °C incubator at 5% $CO_2$ for 1 h and then treated with or without 20 μg/ml anti-IgM antibody for different time periods. Cells were harvested and lysed for Western blot analysis as described below. For Egr-1 RNA analysis, immature and mature B cells from neonatal and young adult mice, respectively, were enriched by panning with goat anti-IgM antibody immobilized on tissue culture-treated dishes as described previously (37). Purified B cells were treated with anti-IgM antibody for different time periods and harvested for RNA extraction as described below.

The isolation and characterization of the immature B lymphoma cell line BKS-2 has been described (38). Briefly, BKS-2 cells were grown in female CBA/N (Xid) mice as splenic tumors by intravenous injection. These cells attained maximal growth ($2–6 \times 10^8$ cells/mouse spleen) in ~7–10 days and were collected for experimental use at this stage. Depletion of host residual T cells was performed using a mixture of anti-Thy1.2, anti-CD4 (L3T4), and anti-CD8 (Lyt2) antibodies, followed by rabbit complement treatment as described previously (39). BKS-2 cells were cultured in IF-12 medium and 10% fetal calf serum (FCS; Atlanta Biologicals, Norcross, GA). CH12.Lx lym-

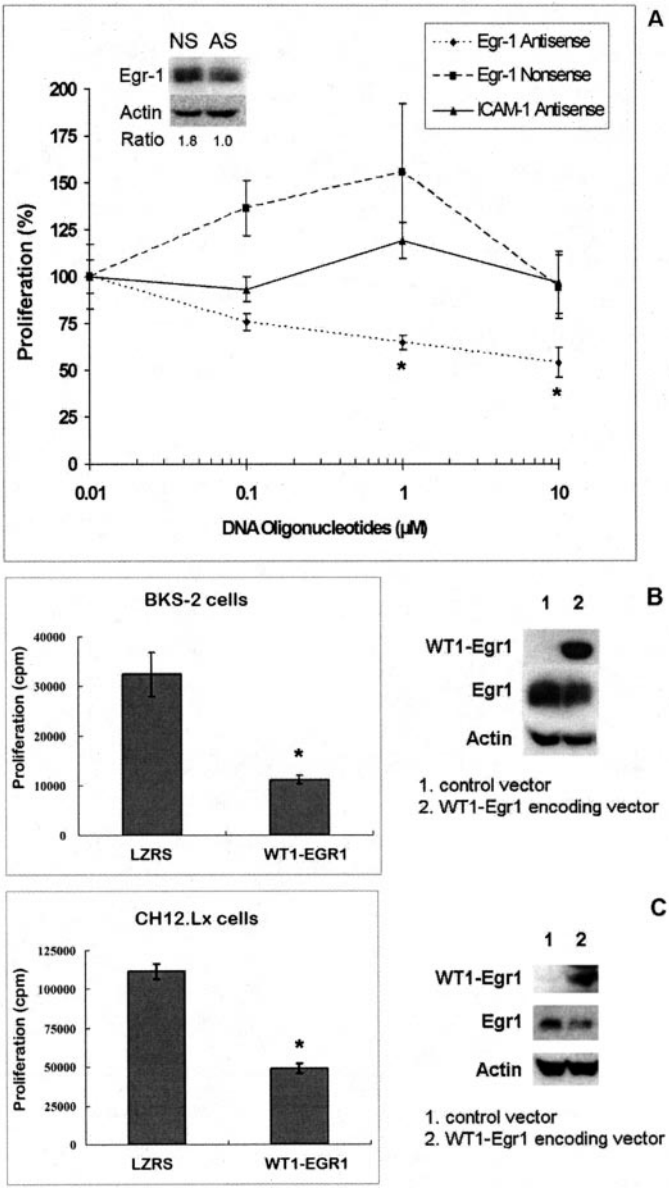


FIGURE 1. **Inhibition of Egr-1 protein or function causes growth inhibition of BKS-2 cells.** *A*, an antisense Egr-1 oligomer without the tetra-G motif inhibits the growth of BKS-2 cells. BKS-2 cells (2–3 × $10^4$/well) were cultured with the indicated concentrations of nonsense (■) or antisense (◆) Egr-1 oligomer or an antisense oligomer to ICAM-1 (▲) for 48 h. Cells were pulsed with [$^3$H]thymidine during the last 4 h of culture. Data points indicate the mean ± S.E. of the percent [$^3$H] incorporation compared with the medium group of triplicate cultures from a representative experiment. The actual counts for the medium group were 66,962 ± 11,612 cpm. The first point at 0.01 μM is really with no oligomer, but it was given this value because the log scale does not allow a zero value. This experiment was repeated two times with similar results. *, $p < 0.02$ for antisense Egr-1 *versus* either nonsense Egr-1 or antisense ICAM-1 data points. *Inset*, Western blot analysis of Egr-1 expression after BKS-2 cells were treated with 2 μM nonsense (*NS*) or antisense (*AS*) Egr-1 oligomer for 24 h. The same blot was stripped and reprobed for β-actin as a loading control. The ratio of the densitometry of the Egr-1 band to the actin band is shown below the Western blot. *B* and *C*, retrovirus-mediated expression of WT1-EGR1, a dominant-negative inhibitor of EGR1, inhibits the growth of BKS-2 and CH12.Lx lymphoma cells, respectively. *Left panels*, BKS-2 (*B*) or CH12.Lx (*C*) lymphoma cells were transfected with either the empty retroviral vector LZRS expressing only EGFP or the vector expressing both EGFP and WT1-EGR1. The GFP$^+$ cells were sorted 72 h post-transfection and cultured for 48 h. Cells were pulsed with [$^3$H]thymidine during the last 4 h of culture. Data points indicate the mean counts ± S.E. of triplicate cultures from a representative experiment. This experiment was repeated three times with similar results. *, $p < 0.005$ *versus* the LZRS control vector. *Right panels*, Western blot analysis examining the expression of WT1-EGR1 and endogenous Egr-1 pro-teins for both cell lines. Cells (5 × $10^6$) were harvested 48 h post-transfection and used for Western analysis. An antibody against the N-terminal domain of the Wilms tumor protein WT1 (clone F-6) was used to detect WT1-EGR1 protein.

phoma cells were obtained from Dr. Gail Bishop (University of Iowa) and cultured in RPMI 1640 medium supplemented with 10% FCS.

*Proliferation Assay*—BKS-2 cells (3 × $10^4$/well) were cultured in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) in 200 μl of IF-12 medium and 10% FCS. Cultures were treated with varying doses of SP600125, PD68059, or SB203580 or an equivalent concentration of $Me_2SO$; incubated at 37 °C and 5% $CO_2$ for a total of 48 h; and pulsed with 1 μCi of [$^3$H]thymidine (PerkinElmer Life Sciences) during the last 4 h of the culture period. The cells were harvested onto filter mats using a cell harvester (Packard Instrument Co., Meriden, CT). The levels of radionucleotide incorporation were measured with a Matrix 96 β-radiation counter (Hewlett-Packard, Downers Grove, IL). Results are presented as the means ± S.E. of triplicate cultures. The percent control response is defined as (cpm in the treated group/cpm in the untreated group) × 100.

*Retroviral Transfection of B Lymphoma Cells and Fluorescence-activated Cell Sorter (FACS) Analysis*—Retroviral vectors were transiently transfected into Phoenix-E packaging cells using the Lipofectamine Plus transfection system (Invitrogen) according to the manufacturer's protocol. Virus-containing supernatants were harvested from transfected Phoenix-E cells for transfection of B lymphoma cells. B lymphoma cells were washed and resuspended in serum-free Opti-MEM I (low serum medium; Invitrogen), and single cell suspensions at a concentration of 1 × $10^6$ cells/ml were spin-infected for 2 h at 30 °C in 2 ml of Polybrene (Sigma)-treated virus-containing supernatant (40). At the end of the 2-h infection period, the virus-containing supernatant was discarded, and fresh medium was added to the B lymphoma cells. For Western analysis, 5 × $10^6$ cells were harvested 48 h post-transfection, and cell pellets were lysed in 100 μl of cell lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, l mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM $Na_3VO_4$, 1 μg/ml leupeptin, and 1 mM PMSF). For proliferation assay, GFP$^+$ cells were sorted by a FACS MoFlo flow cytometer (DakoCytomation, Fort Collins, CO) 72 h post-transfection and used as described above.

*Nuclear Run-on Assay*—Nuclei were isolated from BKS-2 cells treated with or without anti-IgM antibody for 1 h, and nuclear run-on assay was performed as described previously (41). The newly synthesized mRNA molecules were labeled by incubating nuclei with [$^{32}$P]UTP. Labeled RNA was hybridized to filters that had been cross-linked with Egr-1, c-Myc, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA or the BlueScript empty vector. The amounts of nascent Egr-1, c-Myc, and GAPDH mRNAs present in cells upon different treatments were analyzed by dot blot analysis. The amounts of Egr-1 and c-Myc mRNAs were normalized to that of GAPDH mRNA.

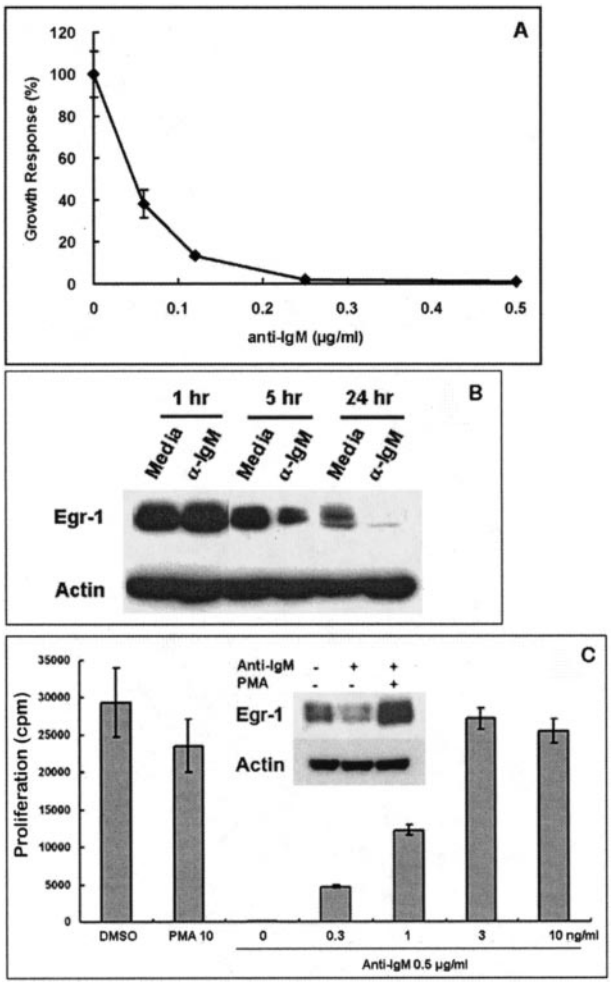


FIGURE 2. **PMA overcomes anti-IgM antibody-mediated growth inhibition by up-regulating Egr-1.** *A*, anti-IgM antibody inhibits the growth of BKS-2 cells. BKS-2 cells ($3 \times 10^4$/well) were cultured with the indicated concentrations of anti-IgM antibody for 48 h. Cells were pulsed with [$^3$H]thymidine during the last 4 h of culture. Data points indicate the mean counts ± S.E. of triplicate cultures from a representative experiment of three independent experiments. *B*, time course of Egr-1 protein expression following anti-IgM antibody cross-linking. BKS-2 cells ($4 \times 10^6$) were cultured in the absence or presence of 5 μg/ml anti-IgM antibody for 1, 5, and 24 h. Cells were harvested and lysed. Protein lysates were analyzed by Western blotting using anti-Egr-1 antibody. The same blot was stripped and reprobed for β-actin as a loading control. *C*, PMA reverses the BCR-mediated growth inhibition of BKS-2 cells and up-regulates Egr-1 protein. BKS-2 cells ($3 \times 10^4$/well) were cultured in the absence or presence of the indicated concentrations of PMA and 0.5 μg/ml anti-IgM antibody for 48 h. Cells were pulsed with [$^3$H]thymidine during the last 4 h of culture. Data points indicate the mean counts ± S.E. of triplicate cultures from a representative experiment of three independent experiments. *Inset*, Western blot of protein lysates from BKS-2 cells treated with or without 0.5 μg/ml anti-IgM antibody in the absence or presence of 3 ng/ml PMA for 5 h. *DMSO*, dimethyl sulfoxide.

*Western Blot Analysis*—After different treatments, BKS-2 cells ($4 \times 10^6$) were cultured at $1 \times 10^6$/ml in 6-well plates (Costar). Cell pellets were lysed in 90 μl of cell lysis buffer on ice for 30 min, and Western blotting was performed as described (14, 31). The blots were developed with Pico chemiluminescence substrate (Pierce) and exposed to Kodak X-Omat films, which were scanned with a Hewlett-Packard ScanJet 6300C flat-bed scanner. Alternatively, the blots were scanned by a Eastman Kodak Image Station 2000RT. For reprobing, membranes were stripped using a solution containing 62.5 mM Tris-HCl, 2% SDS, and 100 mM β-mercaptoethanol at 62 °C for 10 min. The relative integrated absorbance of the protein bands was estimated using Scion Image software. Band intensities were normalized by dividing the intensity of phosphorylated protein by that of total protein or by dividing the intensity of the protein of interest by that of β-actin.

*Quantitative RT-PCR*—After various treatments, BKS-2 cells ($5 \times 10^6$) were used for RNA extraction with the QIAamp RNA blood mini kit (Qiagen Inc.). 2 μg of total RNA was used to make cDNA with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Real-time PCR was performed on an ABI Prism 7000 system using TaqMan-based *egr-1*-specific primers and probe (Applied Biosystems, Foster City, CA). The GAPDH- or β-actin-specific primers and probe were used to control for loading (Applied Biosystems).

*Transfection of Lymphoma Cells and Luciferase Assay*—The *egr-1*-encoding plasmid pBX-Egr1 or various *egr-1* promoter-driven firefly luciferase constructs (p903luc, p395luc, p242luc, and LucA–D) were introduced into BKS-2 cells by electroporation. For electroporation, BKS-2 cells were washed and resuspended in cold Opti-MEM I. The cells were then mixed with the indicated amount of DNA and electroporated at 250 mV, 960 microfarads, and 200 ohms with a Gene Pulser electroporator (Bio-Rad). For ectopic *Egr-1* expression experiments, 1 day post-electroporation, BKS-2 cells transfected with pBX-Egr1 or a control vector were counted, and an equal number of cells with the indicated treatment were used to set up the proliferation assay as described. For luciferase assay, BKS-2 cells were washed once and rested at 37 °C in IF-12 medium containing 10% FCS for 1 h post-electroporation. After that, BKS-2 cells were plated at ~$1 \times 10^5$/well into 96-well flat-bottom microtiter plates in 200 μl of IF-12 medium containing 10% FCS with various treatments added and incubated at 37 °C for 6 h. Cells were spun down and lysed in the plate with 20 μl of cell lysis buffer at room temperature for 5 min. Firefly and *Renilla* luciferase activities were measured on an LMax luminometer (Molecular Devices Corp., Sunnyvale, CA) using a firefly and *Renilla* luciferase assay kit (Biotium Inc., Hayward, CA).

## RESULTS

*Inhibition of Egr-1 by Antisense ODNs or Retrovirus-mediated Expression of WT1-EGR1 Inhibits B Lymphoma Cell Growth*—A previous study showed that *egr-1* is constitutively expressed in BKS-2 lymphoma cells and that inhibition of *egr-1* by specific antisense ODNs causes growth inhibition and apoptosis of BKS-2 (14). Because the antisense ODNs used in this study contained tetra-G motifs (GGGG) that could form triple helical structures leading to sequence-independent inhibition (42, 43), we used two additional approaches to study the importance of Egr-1 for lymphoma cell growth. First, an *egr-1*-specific antisense ODN without the tetra-G motif was used. Western blot analysis showed that the Egr-1 antisense ODN caused a reduction in endogenous Egr-1 protein (Fig. 1*A*, *inset*). Cell proliferation measured by [$^3$H]thymidine incorporation showed that this Egr-1 antisense ODN inhibited the growth of BKS-2 cells (Fig. 1*A*). Used as controls, both an Egr-1 nonsense ODN and an antisense ODN against ICAM-1 did not cause growth inhibition of BKS-2 cells. Second, retrovirus-mediated expression of WT1-EGR1, a domi-

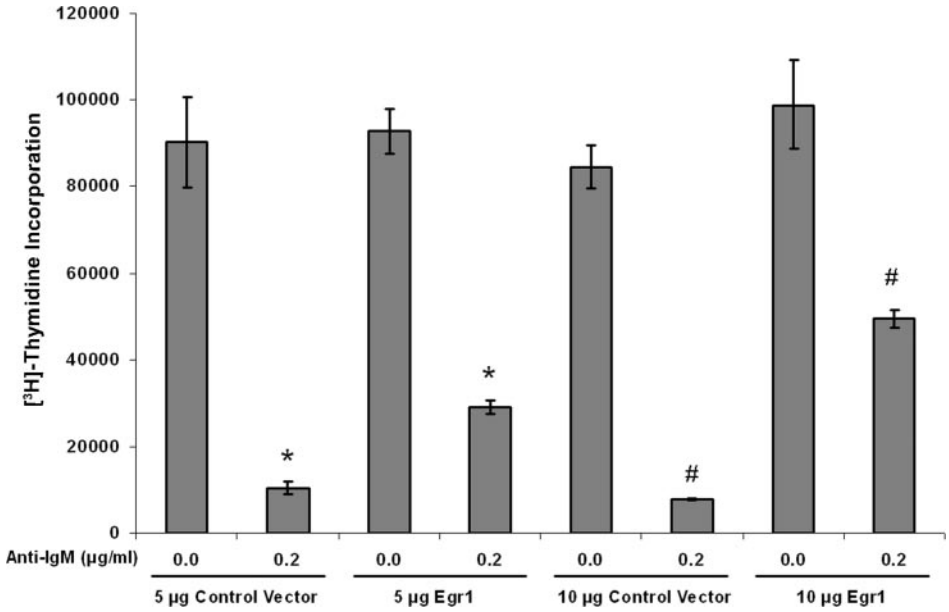


FIGURE 3. **Ectopic expression of Egr-1 partially overcomes anti-IgM antibody-induced growth inhibition.** BKS-2 cells were transfected with either a control vector plasmid (pEGFP-N1) or an *egr-1*-encoding plasmid (pBX-Egr1) by electroporation. 1 day after electroporation, cells were counted, and an equal number of cells ($2 \times 10^4$/well) were plated in 200 $\mu$l of medium supplemented with 10% FCS in a 96-well plate with or without the indicated anti-IgM antibody treatment. Cell proliferation was determined as described in the legend to Fig. 2. Data points indicate the mean counts ± S.E. of triplicate cultures. * and #, $p < 0.001$ for BKS-2 cells transfected with pBX-Egr1 compared with BKS-2 cells transfected with the control vector. This experiment was repeated once with a similar outcome.

nant-negative inhibitor of Egr-1 containing the repressor domain from the Wilms tumor gene and the three zinc finger DNA-binding motifs from Egr-1 (28), inhibited the basal proliferation of both BKS-2 (Fig. 1*B*) and CH12.Lx (Fig. 1*C*) lymphoma cells. Western blot analyses using an antibody against the N-terminal domain of WT1 protein detected the expression of a ~45-kDa protein, corresponding to the molecular mass of WT1-EGR1, in both BKS-2 and CH12.Lx cell lines transfected with the WT1-EGR1-encoding retrovirus, but not with the GFP-only control vector (Fig. 1, *B* and *C*). Interestingly, irrespective of the WT1-EGR1 protein expression status, Egr-1 protein was expressed in both BKS-2 and CH12.Lx cells (Fig. 1, *B* and *C*). The level of Egr-1 was slightly reduced upon WT-EGR1 expression. Because WT1-EGR1 is a functional inhibitor of Egr-1 (inhibits the downstream targets of Egr-1), it may indirectly inhibit *egr-1* expression. These two experiments firmly establish the importance of Egr-1 for B lymphoma cell growth.

*Up-regulation of Egr-1 by Phorbol 12-Myristate 13-Acetate (PMA) Rescues BCR-mediated Growth Inhibition of BKS-2 Cells*—Anti-IgM antibody-mediated cross-linking of BCR induces growth arrest and apoptosis of several immature B lymphoma cells, including BKS-2 (38, 44). In agreement with the previous results, rat anti-mouse IgM monoclonal antibody (AK11) inhibited BKS-2 proliferation in a dose-dependent manner (Fig. 2*A*). Western blot analysis showed that, at early time point (1 h), BCR cross-linking did not appreciably affect Egr-1 protein expression compared with the untreated group (Fig. 2*B*). At later time points (5 and 24 h), BCR cross-linking significantly down-regulated Egr-1 protein compared with the untreated group (Fig. 2*B*). Consistent with its nature as an immediate-early gene, Egr-1 protein in the untreated group underwent down-regulation with time. This suggests that, being an immediate-early gene, *egr-1* expression is tightly regulated in lymphoma cells. We postulate that the expression of *egr-1* may be cell cycle-dependent and required only during early phases of the cell cycle. Once it exerts its function, Egr-1 is down-regulated to allow cells to progress into other phases of the cell cycle.

Direct inhibition of Egr-1 reduced the growth of BKS-2 cells, whereas anti-IgM antibody-mediated growth inhibition of BKS-2 cells was accompanied by down-regulation of Egr-1. In this study, we found that PMA, a potent protein kinase C activator, was able to rescue the anti-IgM antibody-induced growth inhibition of BKS-2 cells in a dose-dependent manner (Fig. 2*C*). Although the ability of PMA to rescue B lymphoma from BCR-induced growth inhibition has been reported before in WEHI-231 cells (45), its effect on *egr-1* expression in B lymphoma cells has not been reported. We examined whether PMA can overcome anti-IgM antibody-induced down-regulation of Egr-1 protein in BKS-2 cells. Indeed, we found that the anti-IgM antibody-induced down-regulation of *egr-1* expression was reversed by PMA treatment, which was accompanied by robust proliferation of BKS-2 cells even in the presence of anti-IgM antibody (Fig. 2*C*). These data suggested that protein kinase C is upstream of Egr-1 and support that Egr-1 has an important role in promoting the growth and proliferation of B lymphoma cells.

*Ectopic Expression of Egr-1 Partially Rescues Anti-IgM Antibody-induced Growth Inhibition*—To further establish the importance of Egr-1 for B cell proliferation, a plasmid encoding the full-length Egr-1 cDNA driven by the SV40 promoter was transiently transfected into BKS-2 cells by electroporation. After 1 day, cells were washed and treated with or without anti-IgM antibody for 48 h, and proliferation was measured. As shown in Fig. 3, 0.2 $\mu$g/ml anti-IgM antibody strongly inhibited the growth of BKS-2 cells, and BKS-2 cells transfected with the Egr-1 plasmid exhibited partial restoration of proliferation in comparison with BKS-2 cells transfected with the control vector. This increase in proliferation is statistically significant ($p < 0.001$) and was dose-dependent on the Egr-1 plasmid. We also performed similar experiments using WEHI-231 cells and demonstrated that ectopic expression of Egr-1 in WEHI-231 cells also partially reversed anti-IgM antibody-mediated growth inhibition (data not shown). These experiments collectively suggest that Egr-1 has a pro-growth role in B lymphoma cells.

*Egr-1 mRNA Stability Is Not Affected by BCR Cross-linking*—Because Egr-1 plays an important role in B lymphoma cell growth, we next wanted to understand how Egr-1 mRNA is

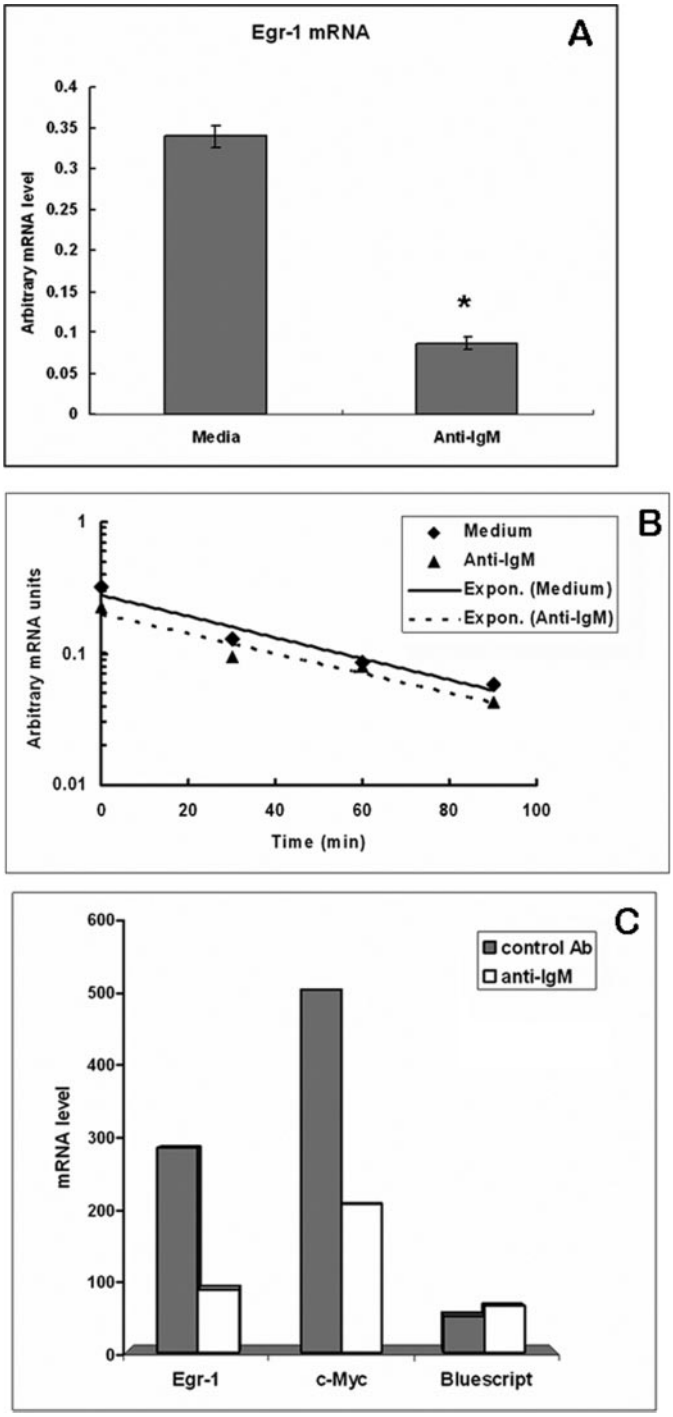


FIGURE 4. **Regulation of *egr-1* by BCR cross-linking is at the transcriptional level.** *A*, Egr-1 mRNA is down-regulated by anti-IgM antibody. BKS-2 cells were cultured with or without anti-IgM antibody for 30 min. Total RNA was isolated, and Egr-1 and GAPDH mRNAs were quantified by real-time PCR as described under "Experimental Procedures." The results are representative of three independent experiments. *, $p < 0.01$ *versus* the medium group. *B*, Egr-1 mRNA stability is not affected by BCR cross-linking. BKS-2 cells were cultured with or without anti-IgM antibody for 1 h and then treated with 5 $\mu$g/ml actinomycin D for 30, 60, and 90 min. Total RNA was isolated, and Egr-1 and GAPDH mRNAs were quantified by real-time PCR as described under "Experimental Procedures." The Egr-1 level was normalized to the GAPDH level and is plotted *versus* time. Two exponential (*Expon.*) curves were fitted to the data points. The slopes of the two lines are 0.018 and 0.017, and $R^2$ is $>0.94$ for both lines. A representative of two independent experiments is shown. *C*, transcription of *egr-1* is reduced by anti-IgM antibody (*Ab*). Nuclei were isolated from BKS-2 cells treated with or without anti-IgM antibody for 1 h. Nuclei were incubated with [$^{32}$P]UTP to label the mRNA being newly synthesized. Labeled RNA was hybridized to filters containing Egr-1 and c-Myc cDNAs or empty vector. The amount of mRNA hybridizing to each target was analyzed by dot blot analysis.

regulated in BKS-2 cells by BCR cross-linking. Consistent with previous Northern results (14), real-time PCR analysis showed that anti-IgM antibody treatment of BKS-2 cells induced down-regulation of Egr-1 mRNA compared with the medium group (Fig. 4*A*). To determine whether BCR-mediated down-regulation of Egr-1 mRNA is due to its effect on Egr-1 mRNA stability, Egr-1 mRNA levels were measured after blocking the transcription. BKS-2 cells were treated with or without anti-IgM antibody for 1 h, and then 5 $\mu$g/ml actinomycin D was added to block the transcription. Total RNA was extracted from BKS-2 cells collected at different time points following actinomycin D treatment. Egr-1 mRNA in each sample was quantified by real-time PCR and normalized to GAPDH mRNA. Least-square analysis was used to fit a straight line to the data points. The two lines appear parallel (Fig. 4*B*). The rate of Egr-1 mRNA decay was very similar with or without anti-IgM antibody treatment, suggesting that Egr-1 mRNA stability is not appreciably affected by BCR cross-linking.

*egr-1 Transcription Is Down-regulated by BCR Cross-linking*—To determine whether BCR-mediated down-regulation of Egr-1 mRNA and expression is regulated at the level of transcription, we used a nuclear run-on assay to measure the transcription rate of *egr-1* in the presence and absence of anti-IgM antibody cross-linking. Nuclei were isolated from BKS-2 cells treated with or without anti-IgM antibody for 1 h and used for *in vitro* transcription. As shown in Fig. 4*C*, the transcription of *egr-1* and c-*myc* was down-regulated by ~70% and ~60%, respectively, by anti-IgM antibody compared with the control antibody-treated group, suggesting that *egr-1* transcription is affected by BCR cross-linking.

*High Egr-1 Protein Expression Levels in BKS-2 Lymphoma Cells Are Dependent on JNK and ERK Activities*—The p21$^{ras}$/Raf-1/MAPK pathway has been implicated in the induction of *egr-1* by BCR signaling in normal splenic B lymphocytes (27). It is not clear whether MAPK activation has a role in the constitutive expression of *egr-1* in B lymphoma cells. We used specific pharmacological inhibitors to block each MAPK activity to determine its role in constitutive *egr-1* expression in transformed B cells. By Western blot analysis, we found that inhibition of JNK activity by SP600125 partially reduced Egr-1 protein (~40% reduction) (Fig. 5). Treatment of BKS-2 with PD98059, an inhibitor of MEK1/2 upstream of ERK, strongly inhibited Egr-1 protein (~80% reduction) (Fig. 5). Interestingly, treatment of BKS-2 with SB203580, a p38 MAPK inhibitor, resulted in up-regulation of Egr-1 protein (~40% increase). These data suggest that the three MAPKs differentially regulate Egr-1 protein levels. JNK and ERK are positive regulators of Egr-1, whereas p38 MAPK is a negative regulator.

*Blocking ERK Activity Inhibits the Growth of BKS-2 Cells*—The role of JNK in the growth and survival of B lymphoma cells has been studied before (31). It was also shown that PD98059 does not appreciably inhibit the growth of BKS-2 cells (31). Because ERK is a major regulator of Egr-1 expression, we examined the effect of ERK on BKS-2 cell growth more carefully. In

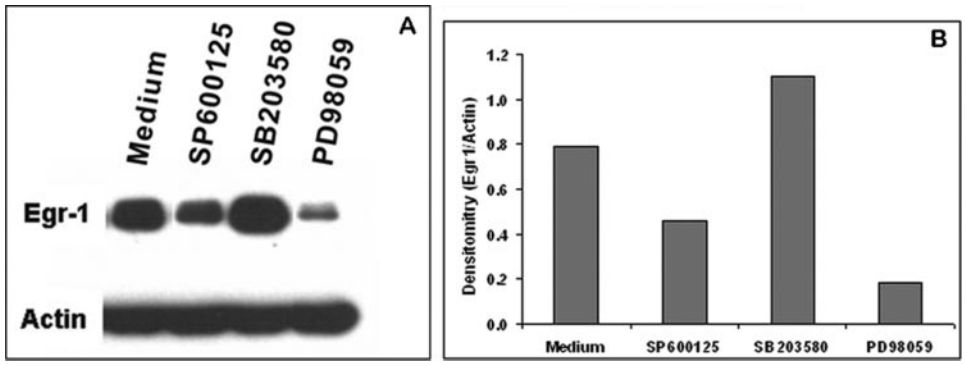


FIGURE 5. *A*, constitutive Egr-1 protein expression is regulated by MAPK in BKS-2 cells. BKS-2 cells ($4 \times 10^6$) were treated with the JNK inhibitor SP600125, the p38 MAPK inhibitor SB203580, or the MEK1/2 inhibitor PD98059 at 20 $\mu$M for 5 h at 37 °C. Cells were harvested and lysed. Protein lysates were analyzed by Western blotting using anti-Egr-1 antibody. The same blot was stripped and reprobed for $\beta$-actin as a loading control. *B*, densitometry of the Egr-1 band *versus* the actin band for the actual Western blot. The results are representative of three experiments.

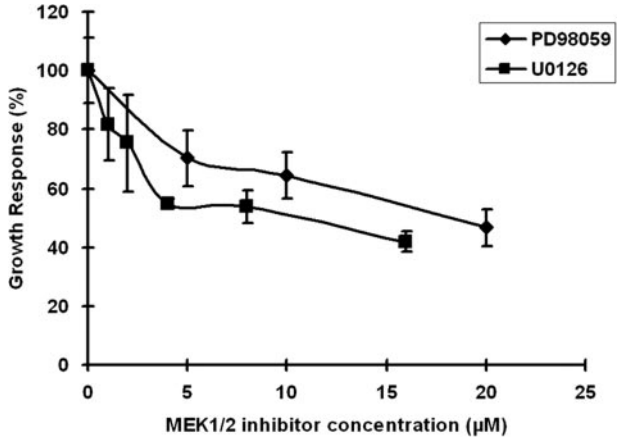


FIGURE 6. **Blocking ERK activity inhibits the growth of BKS-2 cells.** BKS-2 cells ($3 \times 10^4$/well) were cultured with the indicated concentrations of the MEK1/2 inhibitor PD98059 or U0126 for 48 h. Because of the instability of the inhibitors in culture, the MEK1/2 inhibitor was added at half of the indicated concentration at 0 and 24 h. Cells were pulsed with [$^3$H]thymidine during the last 4 h of culture. Data points indicate the mean ± S.E. of the percent [$^3$H] incorporation compared with the medium group of triplicate cultures from a representative experiment. This experiment was repeated three times with similar results. The actual counts for the medium group were 73,559 ± 8092 cpm.

the previous study, a single treatment of PD98059 was used. Gauld *et al.* (29) reported that neither PD98059 nor U0126 was stable in 2–3 day cultures of WEHI-231 cells. To inhibit MEK1/2 over a long period of time (48 h), in this study, we subjected the cells to multiple treatments with these reagents. As shown in Fig. 6, the growth of BKS-2 cells was inhibited by multiple treatments with two different pharmacological inhibitors of MEK1/2 (PD98059 and U0126) in a dose-dependent manner. U0126 was more effective than PD98059 as shown by the lower dose of U0126 required to reach the same extent of inhibition as PD98059. This suggests that sustained ERK activity is required for BKS-2 cell growth, presumably by activating downstream targets like Egr-1.

*Role of Five Serum Response Elements (SREs) in Constitutive egr-1 Transcription—egr-1* is constitutively expressed in BKS-2 cells presumably through constitutively active transcription. Analysis of the 903-bp *egr-1* promoter region by the computer program TESS (transcription element search system) (46) revealed multiple potential regulatory DNA elements (Fig. 7*A*), most notably the five SREs along with adjacent Ets motifs that have been demonstrated experimentally to play an important role in up-regulation of *egr-1* transcription in normal B cells by BCR cross-linking (34). To understand the contributions of the *cis*-DNA elements in the *egr-1* promoter region to constitutive *egr-1* transcription, DNA constructs containing different truncations of the *egr-1* promoter linked to a firefly luciferase gene (p903luc, p395luc, and p242luc) were used (Fig. 7*B*). BKS-2 cells were transiently transfected with the p903luc, p395luc, and p242luc DNA constructs, containing five, four, and two SREs, respectively, along with the pActinRluc construct, and the firefly and *Renilla* luciferase activities were measured at 6 h. To ensure equal transfection efficiency between groups, the *egr-1* promoter-driven firefly luciferase activity was normalized to the actin promoter-driven *Renilla* luciferase activity. Consistent with constitutive *egr-1* expression in BKS-2 cells, the *egr-1* promoter-driven luciferase gene was abundantly expressed in BKS-2 lymphoma cells (Fig. 7*B*). Compared with p903luc, there was an ~50–60% reduction in the normalized *egr-1* promoter activity for p395luc and an ~80% reduction in the normalized *egr-1* promoter activity for p242luc. Using luciferase expression as a measure of contribution to transcription by each SRE, SRE5 and the upstream region appear to contribute ~50–60% to constitutive Egr-1 transcription. SRE3/4 and SRE1/2 each contribute ~20% to constitutive Egr-1 transcription. To further investigate whether SRE5 and its adjacent Ets motif or the region farther upstream plays a more important role, BKS-2 cells were transiently transfected with another set of *egr-1* promoter-luciferase constructs (LucA–D) (Fig. 7*C*), and the firefly and *Renilla* luciferase activities were measured. LucA contains the 1.2-kb *egr-1* promoter region; LucB contains the *egr-1* promoter upstream region without any SREs; LucC contains five SREs; and LucD contains only the three distal SREs (Fig. 7*C*). Upon comparison of the *egr-1* promoter activities of LucB and LucC with that of LucA, it is evident that the majority of *egr-1* promoter activity is contributed by the five SREs and adjacent Ets motifs (~80%). This rules out a significant role played by the farther upstream region of the *egr-1* promoter (beyond 425 bp) in *egr-1* transcription (10~20%). Comparison of the promoter activities of LucC and LucD suggests that the removal of SRE1 and SRE2 caused a further ~20–30% reduction in *egr-1* transcription. These data collectively suggest that five SREs differentially contribute to *egr-1* transcription with the order of SRE5 > SRE1 + 2 ≅ SRE3 + 4.

*egr-1 Promoter Activity Is Dependent on JNK and ERK Activities*—To understand how ERK and JNK activate Egr-1 protein expression, we investigated whether transcriptional activation of *egr-1* is dependent on ERK and JNK activities. BKS-2 cells were transiently transfected with the p903luc DNA

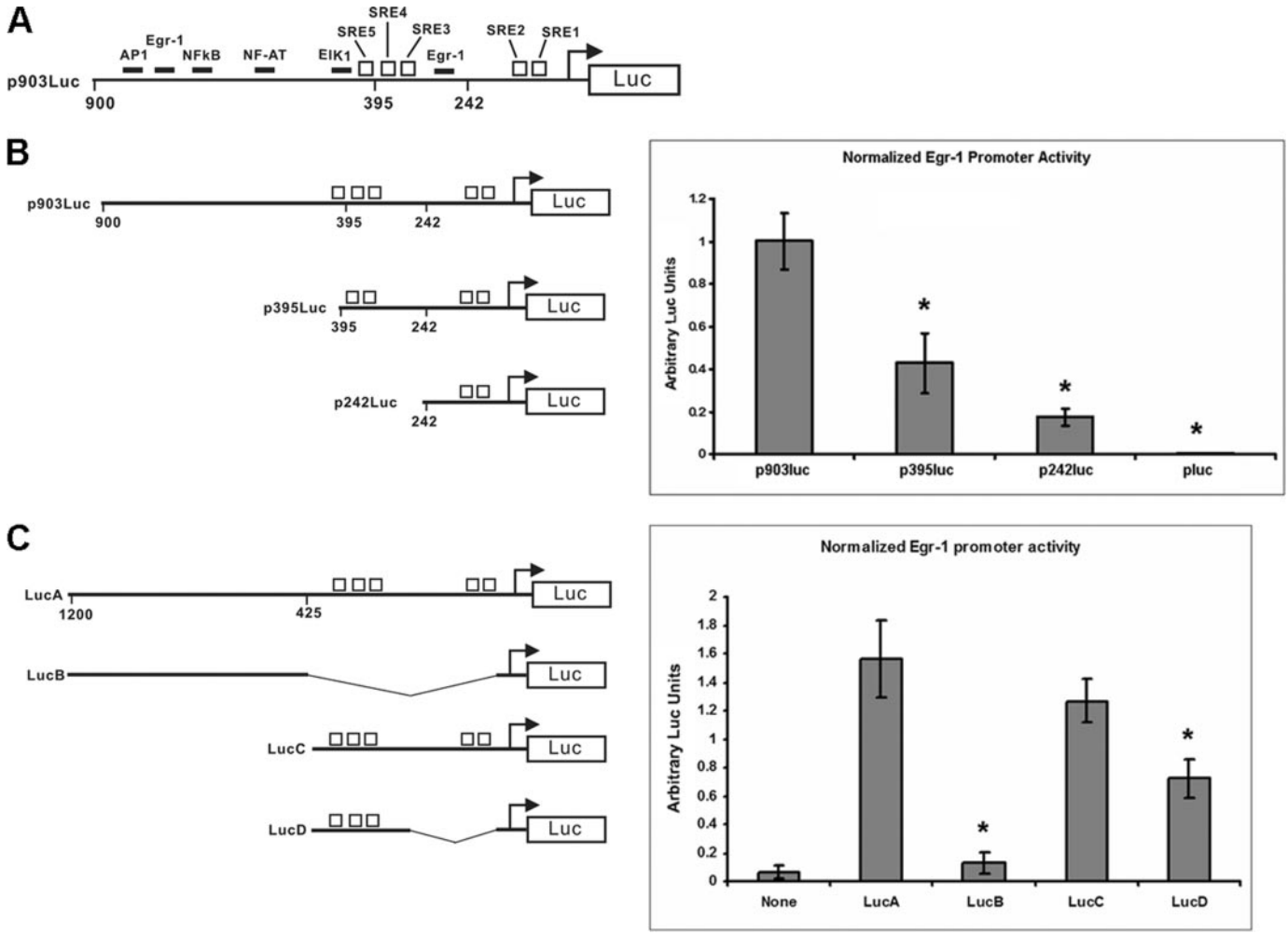


FIGURE 7. ***egr-1* transcription is dependent on the five SREs in the *egr-1* promoter region.** *A*, diagram of p903luc depicting some putative DNA elements in the 903-bp *egr-1* promoter region. *B*, diagram of the different truncations of *egr-1* promoter-luciferase (*Luc*) constructs with each of the five SREs shown as a *square* (*left panel*) and measured *egr-1* promoter activity (right panel). BKS-2 cells were transiently transfected with p903luc, p395luc, p242luc, or the promoterless firefly luciferase construct pluc along with pActinRluc by electroporation. The cells were rested for 1 h and incubated with IF-12 medium containing 5% FCS for 6 h. The cells were harvested and lysed. The cell lysates were assayed for luciferase activity as described under "Experimental Procedures." Data are representative of two independent experiments. *, $p < 0.01$ *versus* the p903luc group. *C*, diagram of the different truncations of *egr-1* promoter-luciferase constructs (LucA–D) (*left panel*) and measured *egr-1* promoter activity (*right panel*). The experiment details are similar to those described for *B*. *, $p < 0.01$ *versus* the LucA group.

construct and then treated with three MAPK inhibitors for 6 h, and luciferase activity was measured afterward. The *egr-1* promoter-driven reporter gene activity was reduced by ~50% upon PD98059 treatment and by ~20% upon SP600125 treatment, whereas SB203580 treatment slightly increased the reporter gene activity, which is statistically significant (Fig. 8*A*). Inhibition of both ERK and JNK activities had an additive effect on inhibition of *egr-1* transcription (~75% reduction) (Fig. 8*A*). This suggests that ERK and JNK activate *egr-1* transcription to allow constitutive expression of Egr-1 protein, whereas p38 MAPK slightly inhibits *egr-1* transcription.

*ERK Activates egr-1 Transcription through the Five SREs*—Because ERK has a major role in *egr-1* transcription, we further investigated what regions in the *egr-1* promoter are important in ERK-mediated transcriptional activation of *egr-1*. BKS-2 cells were transiently transfected with LucA–D along with pActinRluc and then treated with 25 $\mu$M PD98059 for 6 h. The promoter activities of LucA, LucC, and LucD were inhibited by PD98059 treatment, whereas that of LucB was not affected (Fig. 8*B*). LucA and LucC contain five SREs, and LucD contains three SREs, whereas LucB does not contain any SREs. This suggests that ERK activates *egr-1* transcription through the five SREs in the *egr-1* promoter region.

*BCR Cross-linking Down-regulates Three MAPK Activities in Immature B Lymphoma Cells but Up-regulates ERK Activity in Splenic Immature B Cells*—Because constitutive *egr-1* transcription and protein levels are dependent on ERK and JNK activities, we investigated whether BCR-induced Egr-1 down-regulation could be due to its effect on MAPK activation. The three MAPK activities in response to BCR cross-linking were measured by Western blotting. At 1 h after BCR cross-linking, there was a significant reduction in JNK and p38 activities (defined as the ratio of phospho-MAPK to total MAPK) and a small reduction in ERK activity (Fig. 9). The Egr-1 protein level was not appreciably down-regulated at 1 h. This could be due to a delay of the effect of BCR signaling on Egr-1 protein levels. Also because the p38 MAPK inhibitor enhanced Egr-1 expression (Fig. 5), this early reduction in p38 MAPK activity may have a role in BCR-induced stabilization of Egr-1 protein levels.

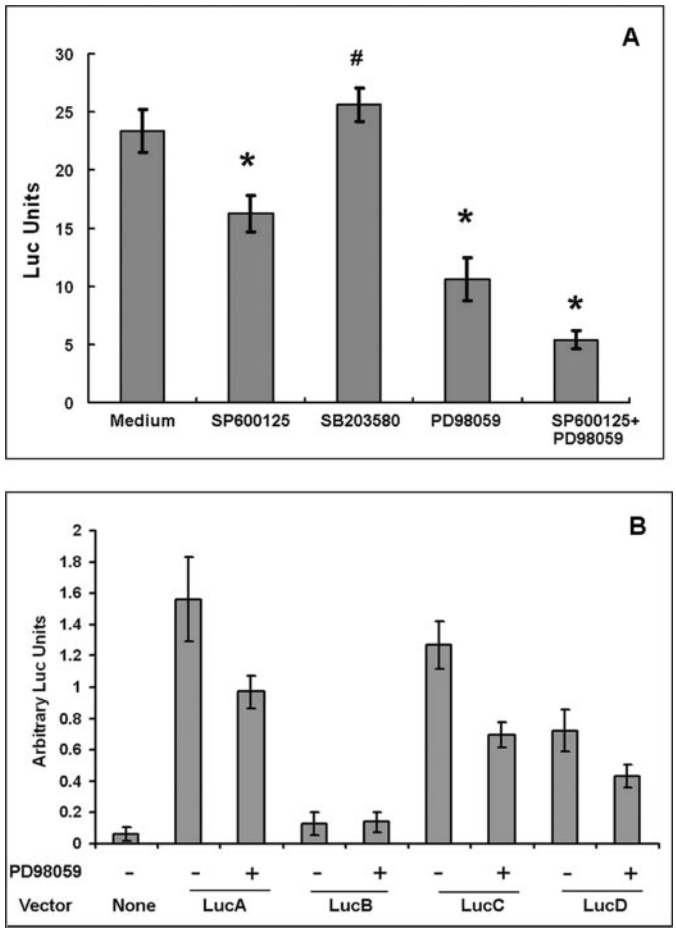


FIGURE 8. *A*, *egr-1* promoter activity is dependent on ERK and JNK activities. BKS-2 cells were transiently transfected with p903luc by electroporation. The cells were then rested for 1 h and treated with the JNK inhibitor SP600125, the p38 MAPK inhibitor SB203580, the MEK1/2 inhibitor PD98059, or the JNK and MEK1/2 inhibitors at 20 $\mu$M each for 6 h. The cells were harvested and lysed. The cell lysates were assayed for luciferase (*Luc*) activity as described under "Experimental Procedures." A representative of three independent experiments is shown. In these experiments, the same population of electroporated cells was used for different treatments. *, $p < 0.001$ *versus* the medium group; #, $p < 0.05$ *versus* the medium group. *B*, ERK activates *egr-1* transcription through the five SREs. BKS-2 cells were transiently transfected with LucA–D as indicated along with pActinRluc by electroporation. The cells were then rested for 1 h and treated with or without 20 $\mu$M PD98059 for 6 h. The cells were harvested and lysed. The cell lysates were assayed for luciferase activity as described under "Experimental Procedures." A representative of two independent experiments is shown.

At 5 h after BCR cross-linking, despite a decrease in p38 MAPK activity, ERK was further down-regulated, which, together with the reduced JNK activity, may account for the drop in Egr-1 protein upon BCR cross-linking (Fig. 2*B* and 9). At 24 h, JNK activity was almost undetectable, although some residual ERK activity could be detected. At this time point, p38 MAPK activity was similar in the anti-IgM antibody-treated and untreated groups because of down-regulation of p38 activity in the untreated group. The down-regulation of ERK and JNK activities at 24 h caused a significant reduction in Egr-1 protein (Fig. 2*B* and 9). These data suggest that BCR-mediated signaling down-regulates all three MAPK activities and that the down-regulation of ERK and JNK activities causes the down-regulation of Egr-1 protein.

The above data show that MAPKs are constitutively active in immature B lymphoma cells and that BCR cross-linking causes down-regulation of MAPK activities. To investigate whether the three MAPKs are also constitutively active in normal immature B cells and how MAPK activities are regulated by BCR cross-linking, we measured the levels of phospho-MAPK in normal immature B cells stimulated by BCR cross-linking for different time periods. Immature B cells were isolated from neonatal mice because, compared with young adult mice, splenic B cells from neonatal mice (9–12 days old) were predominantly immature as judged by B220, AA4.1, CD43, and IgM FACS staining (data not shown). Thus, immature B cells were purified from one litter of neonatal mice as described under "Experimental Procedures." As shown in Fig. 10, the levels of phosphorylated ERK, JNK, and p38 MAPK were low or undetectable without stimulation (0-h time point), in contrast to the constitutive activation of MAPK in immature B lymphoma cells such as BKS-2. Anti-IgM antibody-induced a transient increase in ERK activity at an early time point (25 min), but this increased activity of ERK was not sustained and was much reduced at 1–5 h, returning to the basal level at 24 h. There was a small induction of ERK activity at 5 h for the medium group, which was also not sustained at later time points. BCR cross-linking had only minor effects on the levels of phospho-JNK and phospho-p38 in immature B cells at all time points tested. A slight elevation of JNK and p38 activities was observed, which was not sustained. The data suggest that, in immature B cells, BCR cross-linking causes rapid but transient up-regulation of ERK with minimal effects on JNK and p38. More interestingly, there is a strong correlation between the induction of ERK activity and the induction of Egr-1 protein just as we observed for the lymphoma cell lines. ERK activity was maximum at 25 min, whereas Egr-1 reached its maximum at ~1 h (Fig. 10*B*). The reduction in ERK activity at later time points was followed by reduced levels of Egr-1 at 5 h and undetectable levels later on. Despite this Egr-1 expression, immature B cells are known to be deficient in proliferation in response to BCR cross-linking. So we wondered if this Egr-1 level is not optimum for proliferation. Immature B cells had one-half to one-third the amount of Egr-1 protein in BKS-2 cells (data not shown). For comparison with mature B cells, we performed real-time PCR analysis of Egr-1 mRNA in mature B cells from adults and immature B cells from neonates stimulated by BCR cross-linking. As shown in Fig. 10*C*, the Egr-1 message level in immature B cells was half of that in mature B cells between 30 min and 3 h.

## DISCUSSION

Previous studies in our laboratory demonstrated that the murine B lymphoma cell line BKS-2 constitutively expresses *egr-1* and that BCR cross-linking leads to strong growth inhibition accompanied by a reduction in Egr-1 mRNA (14, 22). This pro-growth-inducing property of Egr-1 in B lymphoma cells is in contrast to its role in suppressing tumor transformation in several human tumor cell lines, including fibrosarcoma, breast carcinoma, and glioblastoma cells, and its role in ionizing radiation-induced growth inhibition in human melanoma cells (15, 47, 48). Hence, we further investigated the prosurvival role of Egr-1 in B lymphoma cells. In this study, we have demonstrated

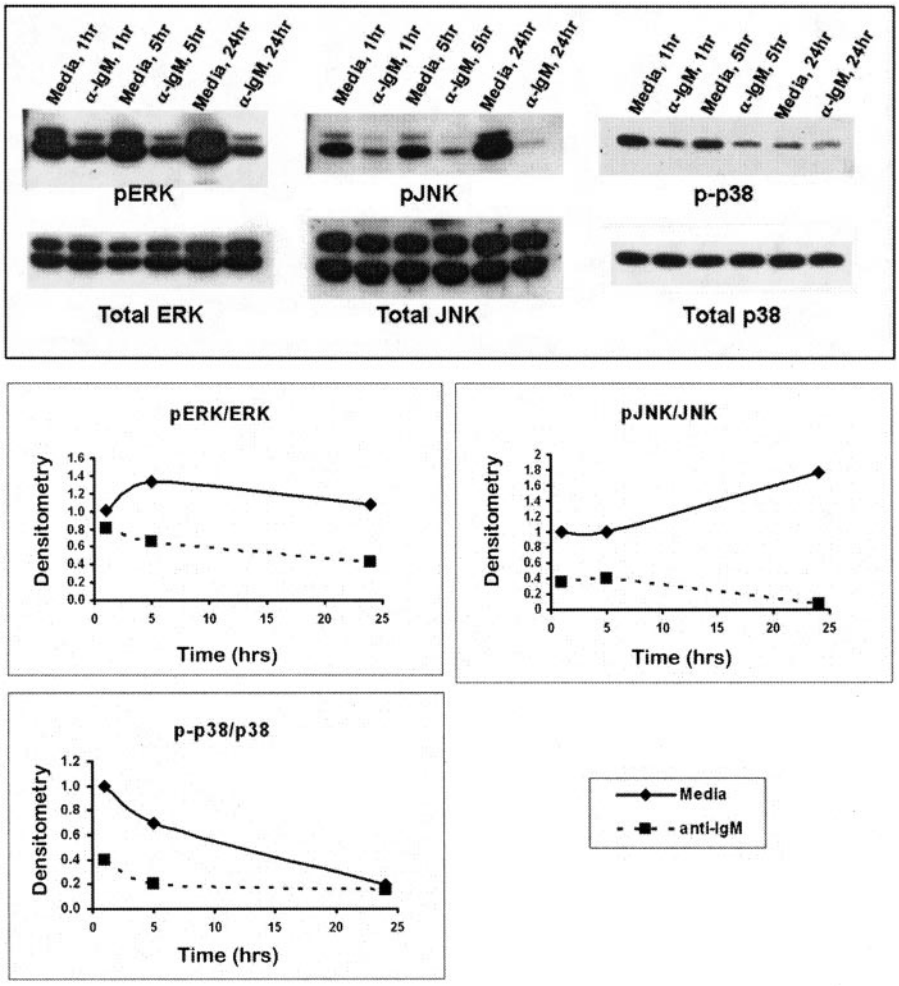


FIGURE 9. **JNK and ERK activities are down-regulated by BCR cross-linking.** BKS-2 cells ($4 \times 10^6$) were cultured in the absence or presence of 5 $\mu$g/ml anti-IgM antibody for 1, 5, and 24 h. Cells were harvested and lysed. Protein lysates were analyzed by Western blotting using anti-phosphorylated (*p*) ERK, JNK, and p38 MAPK antibodies. The same blot was stripped and reprobed for total JNK, ERK, and p38 MAPK as a loading control. The phospho-JNK/JNK, phospho-ERK/ERK, and phospho-p38 MAPK/p38 MAPK ratios are plotted *versus* time and shown below the actual Western blots. Data are representative of three independent experiments.

that inhibition of Egr-1 leads to inhibition of BKS-2 cell growth using either an antisense ODN for Egr-1 or retrovirus-mediated expression of a dominant-negative inhibitor of Egr-1. Moreover, we found that PMA-induced overexpression of Egr-1 or ectopic expression of Egr-1 cDNA by a plasmid vector can overcome anti-IgM antibody-induced growth inhibition. These data collectively support the proposal that Egr-1 acts as a growth stimulator for B lymphoma cells and that reduction of Egr-1 levels (antisense ODN) or function (dominant-negative approach) leads to growth inhibition. Consistent with our data, Egr-1 has been found to play an important role in many transformed cells, including human prostate cancer (12), Nb2 lymphoma (49), and body cavity BC-2 lymphoma (13) cells.

Several studies using different cell lines reported that the up-regulation of Egr-1 by various stimuli, including estrogen, lipopolysaccharide, erythropoietin, T cell receptor, and BCR, is mediated through the Ras/Raf-1/MEK/ERK pathway (19, 24–27). In many lymphoma cells, including BKS-2 cells, MAPK activities are constitutively active (31). This prompted us to study the role of three MAPKs (ERK, JNK, and p38) in maintaining constitutive *egr-1* expression in BKS-2 cells. We found that the constitutive expression of *egr-1* in this lymphoma cell line is dependent mainly on ERK activity and partially dependent on JNK activity. Because ERK and JNK phosphorylate downstream targets such as c-Myc, Elk-1, Stat1/3 (signal transducer and activator of transcription), c-Jun, and activating transcription factor-2, which are transcription factors, we studied the transcriptional regulation of *egr-1* in BKS-2 cells. The 903-bp *egr-1* promoter region has a number of putative DNA elements (Fig. 7*A*). Using different truncations of the *egr-1* promoter linked to the firefly luciferase gene, we found that constitutive *egr-1* transcription is dependent mainly on the five SREs, with SRE5 having the most important role. McMahon and Monroe (34) reported the preferential usage of SRE3 and SRE4 along with adjacent Ets motifs in BCR-mediated up-regulation of *egr-1* transcription in normal B cells. It appears that all five SREs contribute to constitutive *egr-1* transcription in B lymphoma cells. Transient expression of a 903-bp *egr-1* promoter-driven luciferase construct and treatment with MAPK inhibitors demonstrated that ERK and JNK regulate Egr-1 at the transcriptional level. Moreover, the ERK-mediated transcriptional activation of *egr-1* is mediated through the five SREs (Fig. 8*B*). In normal B cells, a ternary complex composed of a homodimer of serum response factors and a member of the Ets family of transcription factors called the ternary complex factor mediates induction of *egr-1* transcription (34). We postulate that constitutively active ERK or JNK can phosphorylate the ternary complex factor (such as Elk-1) and the serum response factor, which form a ternary complex and bind to the five SREs, mainly SRE5, on the *egr-1* promoter to activate its transcription. Our data also show that p38 MAPK negatively regulated Egr-1 at the protein level. Recently, it was shown that erythropoietin-induced expression of Egr-1 is inhibited by MEK1/2 inhibitors but enhanced by a p38 MAPK inhibitor in murine erythroleukemia cells (25). It was shown that inhibition of p38 MAPK by SB203580 has a stimulatory effect on the ERK1/2 MAPK pathway. In B lymphoma cells, the p38 MAPK inhibitor slightly increases *egr-1* transcription. The up-regulation of Egr-1 protein by the p38 MAPK inhibitor may be partly through an increase in *egr-1* transcription in B lym-

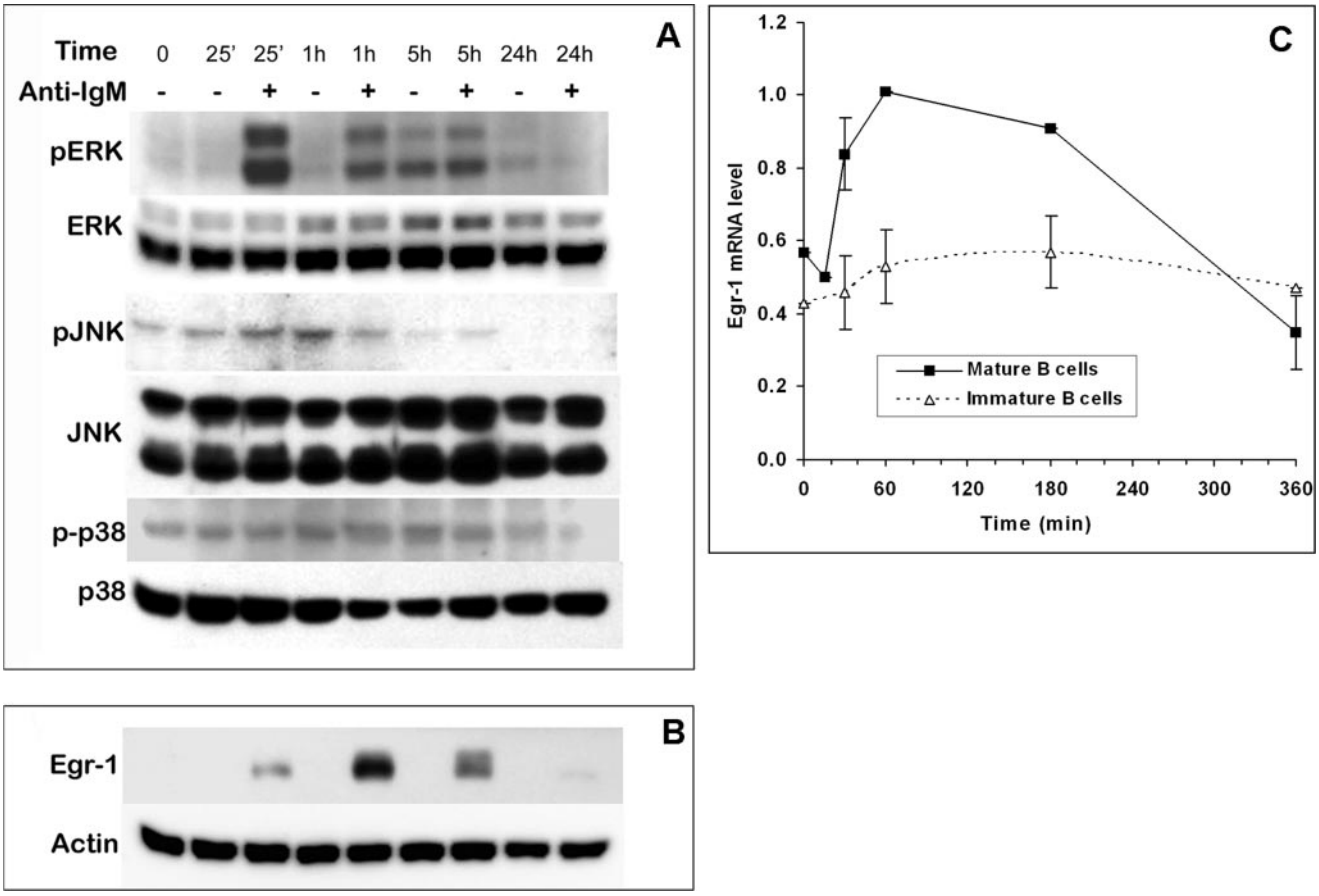


FIGURE 10. **Regulation of MAPK activation and *egr-1* expression by BCR cross-linking in immature and mature B cells.** *A*, BCR-induced MAPK activation in immature B cells. Immature B cells ($3 \times 10^6$) were cultured in the absence or presence of 20 $\mu$g/ml anti-IgM antibody for 25 min and 1, 5, and 24 h. Cells were harvested and lysed. Protein lysates were analyzed by Western blotting using anti-phosphorylated (*p*) JNK, ERK, and p38 MAPK antibodies. The same blot was stripped and reprobed for total JNK, ERK, and p38 MAPK as a loading control. *B*, BCR-induced *egr-1* expression in immature B cells. The same lysates as described for *A* were analyzed by Western blotting using anti-Egr-1 antibody. The same blot was stripped again and reprobed for actin as a loading control. *C*, BCR cross-linking induces lower Egr-1 mRNA levels in immature than in mature B cells. Splenic immature and mature B cells were isolated from neonatal and adult mice, respectively. Cells ($1 \times 10^6$) were cultured in the absence or presence of 50 $\mu$g/ml anti-IgM antibody for different time periods, and total RNA was subsequently isolated. Egr-1 and $\beta$-actin mRNAs were quantified by real-time PCR as described under "Experimental Procedures." Data are representative of two independent experiments.

phoma cells, although other post-transcriptional regulatory mechanisms may also exist.

Different BCR-mediated responses in mature and immature B cells have been studied for a long time, and several mechanisms have been proposed to account for BCR-mediated apoptosis in immature B cells, including defective BCR translocation into lipid rafts, lower levels of B cell coreceptors such as CD19 and CD22, inability to activate protein kinase C, and defective activation of adapter molecules such as Gab1/2 (50–52). In particular, several studies have indicated that BCR-induced ERK1/2 activity plays an important role in both the survival and apoptosis of immature B cells. Lee and Koretzky (53) reported that ERK2 is involved in mediating anti-IgM antibody-induced apoptosis of WEHI-231 cells. Koncz *et al.* (52) reported that BCR cross-linking induces only transient ERK1/2 phosphorylation in immature B cells but sustained ERK1/2 phosphorylation in mature B cells. Gauld *et al.* (29) reported that, in the immature B cell line WEHI-231, BCR-mediated early activation of ERK (≤2 h) activates the phospholipase $A_2$ pathway and leads to apoptosis, whereas the sustained cycling pattern of activation of ERK (8–48 h) leads to cell growth and proliferation. In this study, we found that BCR cross-linking down-regulates all three MAPK activities, particularly the ERK and JNK activities. Consistent with the report of Gauld *et al.*, this suggests that BCR signaling inhibits the sustained activation of ERK to inhibit cell growth and proliferation. Moreover, BCR signaling also inhibits JNK activity, which was also shown to be essential for B cell growth and survival in our recent study (31). The data reported here suggest that the growth inhibition induced by BCR signaling could be due to synergistic inhibition of both ERK and JNK activities. This may also explain why growth inhibition induced by ERK inhibitors alone is not as potent as that induced by BCR ligation (Fig. 2*A versus* Fig. 6). Because the constitutive activities of ERK and JNK are higher than that of p38 MAPK and because p38 MAPK undergoes spontaneous down-regulation with time (Fig. 9), p38 MAPK may not play as significant a role as JNK and ERK, although it is also down-regulated by BCR signaling. King *et al.* (51)proposed the imbalance theory, which suggests that BCR signaling can induce similar levels of calcium response in both mature and immature B cells, but fails to activate protein kinase C in immature B cells, leading to apoptosis. Because protein kinase C is the

upstream enzyme of the three MAPKs, BCR signaling in immature B lymphoma cells may induce the down-regulation of protein kinase C activity, which then leads to the down-regulation of the three MAPK activities. Consistent with this theory, the BCR-induced growth inhibition is reversed by PMA, a protein kinase C activator. Alternatively, the inhibition of MAPK activities could be due to BCR-induced up-regulation of MAPK phosphatase activities (29, 54).

In contrast to immature B lymphoma cells, constitutive MAPK activation was almost undetectable in resting immature B cells. Upon BCR cross-linking, there was a transient increase in ERK activity in immature B cells, in agreement with a previous report (52), but it was different from the sustained ERK activation observed in mature B cells. Interestingly, the level of Egr-1 was also transiently up-regulated with delayed kinetics, in contrast to the higher and/or prolonged expression of Egr-1 in mature B cells (4–12 h at the protein level according to Seyfert *et al.* (55)) or lymphoma cells (Fig. 2*B*). These results are consistent with the concept that a threshold level of Egr-1 may be necessary for B cells to enter the cell cycle upon BCR cross-linking and that such levels are reached in unstimulated B lymphoma cells or BCR-stimulated adult B cells, but not in BCR-stimulated immature B cells or B lymphoma cells (rapid down-regulation). The later two scenarios show no or reduced proliferation upon BCR cross-linking (normal immature B cells (37, 51) and immature lymphoma cells (Fig. 2*A*)). Normal immature B cells are quiescent, which may explain the lack of constitutive MAPK activation, unlike lymphoma cells, which are in an active cell cycle.

Thus, unlike most systems in which Egr-1 is up-regulated by hormones, lipopolysaccharide, or BCR cross-linking, immature BKS-2 lymphoma cells represent a unique system in which BCR signaling down-regulates Egr-1 mRNA and protein. This down-regulation of Egr-1 mRNA appears to be at the transcriptional level. Because constitutive *egr-1* expression is dependent mainly on ERK activity and partially on JNK activity and because BCR signaling down-regulates ERK and JNK activities, the BCR-mediated down-regulation of *egr-1* could be mediated through the down-regulation of both ERK and JNK activities, which are essential for *egr-1* transcription.

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# The Role of MAPKs in B Cell Receptor-induced Down-regulation of Egr-1 in Immature B Lymphoma Cells

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