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Role of *Egr-1* Gene Expression in B Cell Receptor-induced Apoptosis in an Immature B Cell Lymphoma*

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Ligation of B cell receptor (BCR) on BKS-2, an immature B cell lymphoma by anti-IgM antibodies (Ab) caused apoptosis. Here we report that signaling through B cell receptor in wild type BKS-2 cells down-regulated the expression of *Egr-1*, a zinc finger-containing transcription factor. A reduction in the level of *Egr-1* mRNA could be demonstrated as early as 30 min after the ligation of BCR on BKS-2 cells. Immunocytochemical and Western blot analysis revealed that the expression of EGR-1 protein was also inhibited by anti-IgM treatment. Antisense oligonucleotides to *Egr-1* caused growth inhibition and apoptosis in BKS-2 cells, suggesting that expression of *Egr-1* is important for the survival of these B lymphoma cells. In contrast to wild type BKS-2 cells, the mutant 1.B5 cell line, which is refractory to B cell receptor-mediated growth-inhibitory signals, showed an increased expression of *Egr-1* upon treatment with anti-IgM. These results implicate a role for *Egr-1* in blocking B cell receptor-mediated apoptosis in immature B cells.

During B lymphocyte development in the bone marrow, a large number of B cells that are specific to a broad array of antigens are generated. This repertoire of B cells also includes a considerable proportion of B cells that recognize self-antigens. Elimination of such self-reactive B cells appears to occur at the immature B cell stage of development and is believed to be initiated as a result of interaction between B cell receptor (BCR)¹ and self-antigens (1–3). Apoptosis represents one of the major mechanisms by which self-reactive B cells are eliminated during B cell development (4). In contrast to immature B cells, engagement of the BCR induces mature B cells to proliferate and differentiate into memory cells and antibody-producing cells (5).

The diverse responses initiated by the BCR in the B cells of different maturation stages do not appear to be due to differences in the architecture of the BCR itself (1–5). Consistent with this, signaling through the BCR induces a number of early biochemical events, such as the activation of protein-tyrosine kinases and protein kinase C isoenzymes, which have been

found to be nearly identical in immature and mature B cells (5). Recent studies found that the later downstream events that affect G₁/S transition, such as the activities of cyclin E and cdk2 were increased in mature but not immature B cells (6). Unlike these late events, thus far, very few early biochemical changes that are unique to growth *versus* apoptosis response of B cells have been identified. Expression of *Egr-1*, an immediate early gene, has been reported to be increased in mature B cells triggered via BCR to proliferate (7). Therefore, we investigated the importance of this gene for growth and apoptosis response of immature B cells using a lymphoma model.

The immediate early gene, *Egr-1* encodes a nuclear protein that is a prototypic member of a family of transcription factors (8, 9). Expression of *Egr-1* is rapidly induced in many cell types by a variety of mitogens during G₀-G₁ transition (10–14). *Egr-1* is also known as NGFI-A (13), Krox 24 (12), Zif 268 (10), TIS8 (15), and it activates transcription by high affinity binding to the regulatory element GCG(G/T)GGGCG in a zinc-dependent manner (8, 16, 17). The GC-rich consensus target sequence of *Egr-1* has been identified in: the promoter regions of transcription factors such as *junD* and *nur77* (16, 18); growth factors such as platelet-derived growth factor, insulin-like growth factor-II, basic fibroblast growth factor, epidermal growth factor receptor (19, 20); thymidine kinase, an enzyme that is crucial in DNA biosynthesis (9); and cell cycle regulators such as the retinoblastoma susceptibility gene, Rb (21), cyclin D1 (22), tumor necrosis factor- α (23), and interleukin-2 (24). Recently, EGR-1 has been shown to regulate transcription of ICAM-I and CD44 molecules in B lymphocytes (25, 26).

Signaling through BCR induced a rapid *Egr-1* expression in mature splenic B cells, which was found to be a protein kinase C-dependent event (7, 19, 27). Studies with *Egr-1*-specific antisense oligonucleotides have demonstrated that induction of *Egr-1* is necessary for antigen receptor-mediated activation of B and T lymphocytes (7, 28). However, in contrast to mature B cells, anti-IgM stimulation failed to induce *Egr-1* expression in immature B cells (7, 29, 30). Thus, the major difference observed between mature and immature B cells in response to antigen receptor-mediated signaling appears to be the variation in expression of *Egr-1*. Given the fact that EGR-1 trans-regulates several molecules involved in cell cycle and growth (8–26, 30), its expression may have a critical role in the determination of positive *versus* negative growth responses in mature and immature B cells triggered with anti-IgM. Therefore, studies were undertaken to examine the role of *Egr-1* expression in anti-IgM-induced negative growth response in BKS-2, an immature B lymphoma cell line (31–35). We have shown previously that BKS-2 cells undergo growth inhibition and apoptosis in response to signals transduced by BCR cross-linking as well as by ionomycin (31, 32). This growth inhibition

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¹ The abbreviations used are: BCR, B cell receptor; Ab, antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

in BKS-2 cells was abrogated by T helper cell contact-mediated signals (32). Rapamycin, a potent immunosuppressive drug, has also been shown to cause apoptosis in BKS-2 cells (33). In the present study, we report that BKS-2 cells constitutively express high levels of *Egr-1* mRNA, which are down-regulated upon signaling through BCR. Blocking *Egr-1* expression by antisense oligonucleotides caused apoptosis in BKS-2 cells. Furthermore, we demonstrate that *Egr-1* mRNA expression is up-regulated in 1.B5, a mutant BKS-2 cell line that is refractory to BCR-mediated growth inhibitory signals.

MATERIALS AND METHODS

Cell Lines—Isolation and characterization of BKS-2 lymphoma has been described elsewhere (35). This lymphoma originated in a CBA/N mouse transferred with spleen cells from aged CBA/Ca mice. The characteristics of BKS-2 cells are similar to immature B cells in that they express high levels of IgM but have very little or no IgD. BKS-2 cells are monoclonal in origin and are grown in CBA/N mice or SCID mice as splenic tumor by serial intravenous injections. These cells attain maximal growth ($4-6 \times 10^8$) in about 7–10 days and are collected for experimental use at this stage. BKS-2 cells from CBA/N or SCID hosts behave similarly (31–33), and cells from the former were used in most experiments. Depletion of host residual T cells was performed as described previously (31).

1.B5 is one of the several clones of ethylmethanesulfonic acid-mutagenized BKS-2 cells selected by their resistance to anti-IgM-induced growth inhibition. Briefly, 40×10^6 BKS-2 lymphoma cells were treated with 300 μ g/ml ethylmethanesulfonate for 24 h. Then, the lymphoma cells were washed and recultured for 2–3 days in IF-12 medium until there were visible signs of growth and were subjected to selective conditions by requiring them to grow in the presence of PMA or anti- μ . The anti- μ -resistant cells were cloned by limiting dilution in 96-well plates using T cell-derived supernatants to augment growth under low cell density conditions. The expression of surface IgM and several other B cell surface molecules such as class I, class II, FcR, CD19, CD22, CD45RB, CD5, and CD72 were similar in the mutant 1.B5 and the wild type BKS-2 cells.² Like the wild type BKS-2, the mutant 1.B5 cells were also susceptible to growth inhibition by rapamycin as well as ionomycin.

Normal Splenic B Lymphocytes—Spleen cells were obtained from 8–10-week-old DBA/2 mice (Jackson Laboratory, Bar Harbor, ME). T cells were depleted by treatment with a mixture of anti-T cell antibodies and complement as described above. Depletion of T cells was verified by flow cytometry and by 99% depletion of response to the T cell mitogen, concanavalin A.

Reagents—The characteristics of the monoclonal rat anti-mouse μ chain Ab, AK11, have been described previously (34, 36). This hybridoma cell line obtained from Dr. R. Noelle (Dartmouth Medical School, Hanover, NH), was grown *in vitro*, and the culture supernatant was affinity-purified by passing through a column containing mouse IgM protein (HPCM2, a hybridoma from BALB/c origin) coupled to agarose beads (Bio-Rad) (34). AK11 monoclonal Ab was coupled to CNBr-Sepharose 4B (Pharmacia) and was resuspended at a concentration of 10% in 0.85% saline (34). This suspension was used in cultures at a final concentration of 0.5% or 1.0%. S4B6, a rat monoclonal Ab (IgG2_a) specific for IL-2, was used as a control Ab. In experiments aimed at blocking *Egr-1* expression, we used a phosphorothioate-capped antisense *Egr-1* oligodeoxynucleotide (5'-G_sC_sGGGGTGCAGGGGCACAsT-3') and a control phosphorothioate-capped nonsense oligodeoxynucleotide (5'-CsG_sCCGACCACCGCGAGTsCsA-3') purchased from the Regional DNA Synthesis Laboratory, Calgary, Alberta, Canada. The phosphorothioate oligomers 1d and 3Db containing CpG motifs described by Krieg *et al.* (37) were also obtained from the Regional DNA Synthesis Laboratory. The CD72 and the G_s-specific oligomers were obtained from Genosys Biotechnologies (The Woodlands, TX).

Northern (RNA) Analysis—Isolation of total RNA by the guanidium isothiocyanate-phenol-chloroform method and Northern analysis were performed as described previously (38, 39). The probe for *Egr-1* was prepared with a 3.1-kilobase cDNA fragment from pCMV-EGR-1 (40). Prehybridization and hybridization were done overnight at 62 °C, and the blots were washed to a final stringency of $0.1 \times$ SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) at 62 °C before autoradio-

graphy as described previously (41). Equal loading of RNA was verified by probing blots with cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Immunocytochemistry—EGR-1-positive BKS-2 cells were detected by immunocytochemistry as described (41). Briefly, cells were exposed to control or anti-IgM Ab for 24 or 48 h as indicated and subjected to immunocytochemistry with anti-EGR-1 Ab (Santa Cruz Biotechnologies, Santa Cruz, CA). Reactions with biotinylated anti-rabbit immunoglobulin Ab and avidin-biotin-peroxidase complexes and staining with diaminobenzidine-H₂O₂ were sequentially performed as described in the Vectastain Elite ABC kit instruction manual (Vector Laboratories, Burlingame, CA). To enable black and white photography of images, counterstaining with methyl green was omitted.

Western Blot Analysis—BKS-2 cells (1×10^7 /ml) were cultured with anti-IgM Ab, AK11 (10 μ g/ml) or control Ab (S4B6) for 24 h. After stimulation, cells were pelleted, washed, and solubilized in 100 μ l of lysis buffer (1% Triton X-100, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml leupeptin, and 2 μ g/ml aprotinin) on ice for 30 min. The detergent-insoluble materials were removed by centrifugation at $12,000 \times g$ for 20 min at 4 °C. Protein extracts were precleared with protein A-Sepharose beads for 1 h at 4 °C, following which anti-EGR-1 Ab (Santa Cruz) was added and further incubated for 6 h at 4 °C. The immune complexes were collected on protein A-Sepharose beads by an additional 4 h incubation. The beads were separated by centrifugation and washed extensively with 1.5 M NaCl and followed by 10 mM Tris-HCl, pH 7.5, before resuspending in SDS-PAGE sample buffer. Proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel. The resolved proteins were transferred onto Immobilon-P nitrocellulose membranes (Millipore, Bedford, MA) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol, pH 9.0, at 90 V for 1 h. Prestained protein standards (Bio-Rad) were included to indicate the molecular weight range and to verify transfer of proteins. After blocking in 5% milk powder in TBST (50 mM Tris, 200 mM NaCl, pH 7.4, 0.05% Tween 20) for 2 h, the nitrocellulose membranes were incubated with anti-EGR-1 Ab for overnight followed by horseradish peroxidase-conjugated goat anti-rabbit Ab (Santa Cruz, CA) for additional 2 h. The blots were then developed with an enhanced chemiluminescence kit (NEN Life Science Products) according to manufacturer's instructions.

Proliferation Assay—Proliferation of cells was assessed by tritiated thymidine uptake. Lymphoma cells (2×10^4 /well) or splenic B cells (2×10^5) were cultured in 96-well flat bottom microtiter plates (Costar, Cambridge, MA) in 0.2 ml of 1:1 mixture of Iscove's and Ham's F12 (IF-12) medium containing supplements and 5% heat-inactivated FCS as described previously (31). Cultures were incubated at 37 °C with 5% CO₂ for a total of 48 h and were pulsed with 1 μ Ci of [³H]thymidine (specific activity 2 Ci/mmol; NEN Life Science Products) during the last 4-h culture period. The cultures were harvested onto glass fiber filters using a Packard 96-well harvester, and [³H]thymidine incorporation was determined by a Matrix-96 β counter (Packard, Downers Grove, IL).

Apoptotic Staining—BKS-2 cells were stained for apoptosis as described previously (42, 43). Briefly, cells treated with and without anti-IgM (5 μ g/ml) for 24 or 48 h were resuspended at 1×10^6 /ml in PBS. Hoechst 33342 was added at 5 μ g/ml, and the cells were incubated in dark for 30 min at 37 °C. Excess of dye was removed by centrifugation, and cells were incubated with Merocyanine 540 (MC 540) (2 μ g/ml) for 20 min at room temperature in dark. After washing the excess of dye in PBS, cells were analyzed by flow cytometry using a FACSTAR flow cytometer (Becton Dickinson, CA). Gates were set to analyze cell cycle and apoptosis stages. Hoechst 33342 is a DNA-specific dye that measures the amount of DNA, while MC540 binds to the inner membrane phospholipids that become exposed during early stages of apoptosis. This method of staining distinguishes five different stages of cells. They are R1: viable resting cells, 2n DNA and MC540 dull; R2: viable cycling cells, >2n and MC540 dull; R3: resting cells undergoing apoptosis, 2n and MC540 bright; R4: cycling cells undergoing apoptosis, >2n and MC540 bright; and R5: cells that moved into later stages of apoptosis as they become Hoechst 33342 dull.

RESULTS

Signaling through BCR Inhibits *Egr-1* mRNA Expression in BKS-2 Cells—We have shown previously that signaling through BCR using anti-IgM Ab causes apoptosis in BKS-2 lymphoma cells (31). To examine whether this signal alters the expression of *Egr-1*, Northern analysis was performed using total RNA isolated from BKS-2 cells stimulated with anti-IgM

² Muthukkumar, S., Venkataraman, C., Woods, T., and Bondada, S., *Mol. Immunol.*, in press.

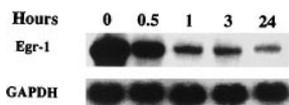


FIG. 1. Anti-IgM stimulation inhibits *Egr-1* mRNA expression in BKS-2 cells. BKS-2 cells were stimulated with anti-IgM (10 μ g/ml) for various intervals of time as indicated. Then the cells were processed for Northern analysis with *Egr-1* or GAPDH cDNA probe. The same blot was later analyzed for the expression of GAPDH. The times of exposure to x-ray film for the *Egr-1* blot and GAPDH blot were 24 and 6 h, respectively. Results from one of four experiments are shown.

Ab for various periods of time. As shown in Fig. 1, a high level of constitutive expression of *Egr-1* was observed in BKS-2 cells (lane 1). A strong reduction in the level of *Egr-1* mRNA was noticed as early as 30 min after exposure to anti-IgM Ab (lane 2). A continued reduction in the level of *Egr-1* message was evident in cells treated with anti-IgM Ab for 1 and 3 h. At 24 h after anti-IgM treatment, *Egr-1* expression was barely detectable in BKS-2 cells (lane 5). Probing for GAPDH message demonstrated equal loading of RNA in all five lanes. These results suggest a correlation between down-regulation of *Egr-1* gene expression and the pathway leading to apoptosis in response to signaling via BCR in BKS-2 cells.

Stimulation of BKS-2 Cells with Anti-IgM Ab Inhibits EGR-1 Protein Expression—To elucidate whether expression of EGR-1 protein is correlated with the reduction of its mRNA in anti-IgM-treated cells, we performed immunocytochemical analysis as a method to test the level of EGR-1 protein expression. Additionally, we initiated studies with *Egr-1*-specific antisense oligonucleotides to directly evaluate the relation between the reduction in *Egr-1* levels and anti- μ -induced growth inhibition. Immunocytochemical analysis was performed to determine the effectiveness of the oligonucleotide treatment on the protein expression. The BKS-2 cells were treated with control Ig, anti-IgM Ab, nonsense, or antisense oligomers to *Egr-1* for 24 h. Then the cells were stained with anti-EGR-1 Ab as described under "Materials and Methods." As shown in Fig. 2 (A and C), most of the BKS-2 cells were EGR-1-positive after nonsense oligomer or control antibody treatment. On the other hand, a large number of BKS-2 cells that were stimulated with anti-IgM Ab failed to express EGR-1 (Fig. 2D), demonstrating that the reduction in *Egr-1* message seen in Fig. 1 is accompanied by a decrease in the protein. The treatment with antisense oligomers for 24 h was also effective in reducing the number of BKS-2 cells expressing the EGR-1 protein (Fig. 2B).

To further confirm the down-regulation of EGR-1 protein in response to signaling through BCR, we also performed Western blot analysis on protein extracts from BKS-2 cells treated with anti-IgM or control Ig. As shown in Fig. 3, control Ig-treated BKS-2 cells expressed a high level of EGR-1 protein, which corresponds to the approximate molecular weight of 82 kDa (lane 1). In contrast, a strong reduction in the levels of EGR-1 protein was observed in the cells that were stimulated with anti-IgM (lane 2). Densitometry analysis showed a more than 3-fold reduction in the level of EGR-1 protein in the cells that are stimulated with anti-IgM. The integrated optical density values for EGR-1 protein in control Ig and anti-IgM-treated BKS-2 cells were 7.83 and 2.07, respectively. The molecular weight of EGR-1 protein is consistent with that reported in other cell types (8). The band seen below the EGR-1 protein is Ig heavy chain, and its intensity was not altered by either control Ig or anti-IgM treatment. Similar intensities of heavy chain band in both lanes also point to the equal loading of proteins. Together, these results clearly indicate the down-regulation of EGR-1 protein in BKS-2 cells upon signaling through BCR.

Antisense Egr-1 Oligomer Causes Growth Inhibition in

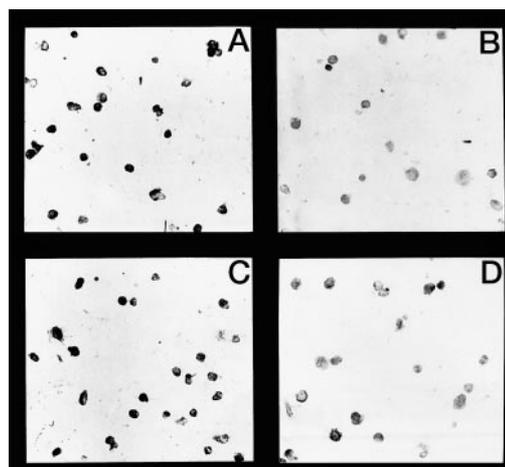


FIG. 2. *Egr-1* antisense oligomer and anti-IgM treatment inhibits EGR-1 protein expression in BKS-2 cells. BKS-2 cells (1×10^6 /ml) were cultured with either 10 μ M nonsense or antisense oligomers for 24 h. Other groups of cells were stimulated with 10 μ g/ml control Ig or anti-IgM Ab. The cells were then subjected to immunocytochemistry using anti-EGR-1 Ab, as described under "Materials and Methods." The methyl green counterstaining was not performed in these experiments so as to enable black-and-white photography of these cells. EGR-1-positive cells showed a dark brown nuclear staining with diaminobenzidine- H_2O_2 substrate. A and B show cells treated with nonsense and antisense oligomers to *Egr-1*, respectively. Cells stimulated with control Ig (C) and anti-IgM (D) are also shown. Similar results were obtained in four other independent experiments.

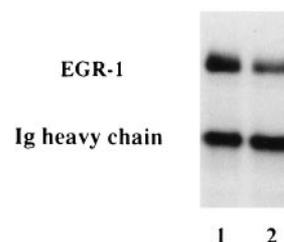


FIG. 3. Stimulation with anti-IgM Ab inhibits EGR-1 protein expression. BKS-2 cells (10×10^6) were stimulated with 10 μ g/ml anti-IgM or control Ig for 24 h. Whole cell protein extracts were prepared and were then subjected to Western blot analysis for EGR-1 as described under "Materials and Methods." Lane 1 shows cells treated with control Ig, and lane 2 shows cells stimulated with anti-IgM. The integrated optical density values for EGR-1 protein in control Ig and anti-IgM-treated BKS-2 cells were 7.83 and 2.07, respectively. Equal loading of protein can be visualized by similar intensities of Ig heavy chain band in both lanes. Results from a representative experiment are shown.

BKS-2 Cells—To identify whether or not the anti-IgM-induced down-regulation of *Egr-1* expression is a probable cause of growth inhibition in BKS-2 cells, we treated them with various concentrations of nonsense and antisense *Egr-1* oligomers for 48 h. The growth response was then assessed by [3H]thymidine incorporation. Antisense oligomer at 10 μ M caused a strong growth inhibition in BKS-2 cells (Fig. 4). A complete growth inhibition was observed when the cells were treated with antisense *Egr-1* oligomer at 25 μ M concentration. On the other hand, same concentrations of nonsense oligomer did not inhibit the cell growth. Similar results were seen when the cells were exposed to nonsense or antisense *Egr-1* oligomer for 24 h (data not shown). The lack of growth inhibition in nonsense oligomer-treated cultures (prepared in a manner similar to that of antisense) suggests that the growth inhibitory effect caused by antisense oligomer is not due to toxicity. These results demonstrate that *Egr-1* expression is critical for the survival of BKS-2 immature B lymphoma cells.

Since oligonucleotides can have unexpected toxic effects, a

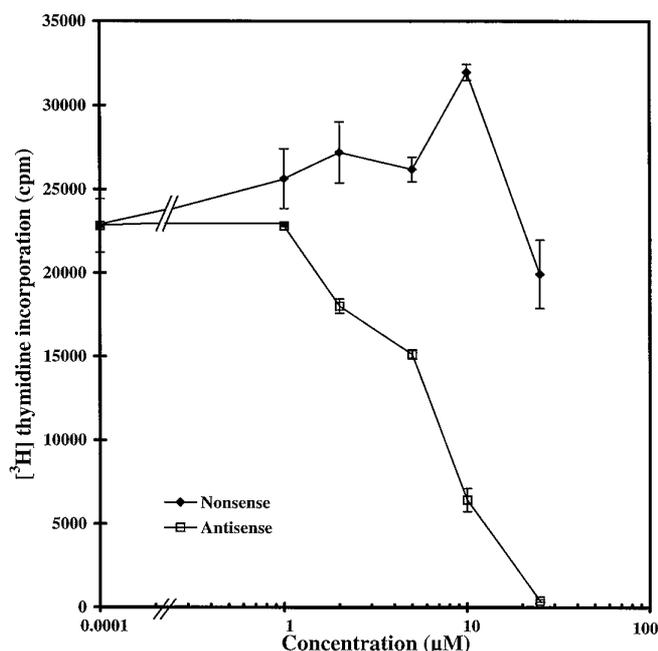


FIG. 4. Antisense *Egr-1* oligomer induces growth inhibition in BKS-2 cells. BKS-2 cells (2×10^4 /well) were cultured with the indicated concentrations of nonsense or antisense *Egr-1* oligomers for 48 h. Cells were pulsed with [3 H]thymidine during the last 4 h culture. Data points indicate mean counts/min \pm S.E. of triplicate cultures from a representative experiment. The first point indicating 0.0001 μ M is really with no oligomer, but was given this value since log scale does not allow a zero value. This experiment was repeated five times with similar outcomes.

number of control studies were performed to be certain that the effects of the antisense *Egr-1* oligomers were specific. First, we tested if the antisense and nonsense oligomers were mitogenic to normal B lymphocytes. As shown in Fig. 5A, these oligonucleotides did not induce nonspecific growth in splenic B lymphocytes, whereas in the same experiment two CpG-containing oligomers (1d and 3Db), which were known to be mitogenic for normal B lymphocytes (37), induced a strong proliferation response. Second, both 1d and 3Db oligomers did not inhibit the growth of BKS-2 cells but mildly enhanced the growth response (Fig. 5A). Consistent with a slight positive effect on BKS-2 growth, these two oligomers also enhanced *Egr-1* expression in BKS-2 cells at 30 and 60 min after stimulation, which declined subsequently (Fig. 5B). Third, antisense and nonsense oligomers specific to CD72, a molecule expressed on BKS-2 cells, or to the α -subunit of the trimeric G protein also did not inhibit the growth of BKS-2 cells.³

Antisense *Egr-1* Oligomer Induces Apoptosis in BKS-2 Cells—Experiments were carried out to examine whether antisense *Egr-1* oligomer treatment blocks only cell cycle progression or if it indeed causes cell death by apoptosis. In this experiment, BKS-2 cells were cultured with either antisense or nonsense oligomers for 24 h and then the cells were stained for apoptosis using Hoechst 33342 and Merocyanine 540 as described under “Materials and Methods.” The results shown in Fig. 6B indicated that majority of BKS-2 cells (81% of the cells were in gates R3, R4, and R5 that detect MC540 high cells) treated with antisense oligomer undergo apoptosis. On the other hand, most of the nonsense oligomer-treated cells were in gate R2 (47%), which detects cycling cells that are viable (Fig. 6A), and this is similar to control Ig-treated BKS-2 cells (R2 = 45%; Fig. 6C). Induction of apoptosis caused by anti-IgM Ab

³ V. Ramiya, S. Muthukkumar, and S. Bondada, unpublished observations.

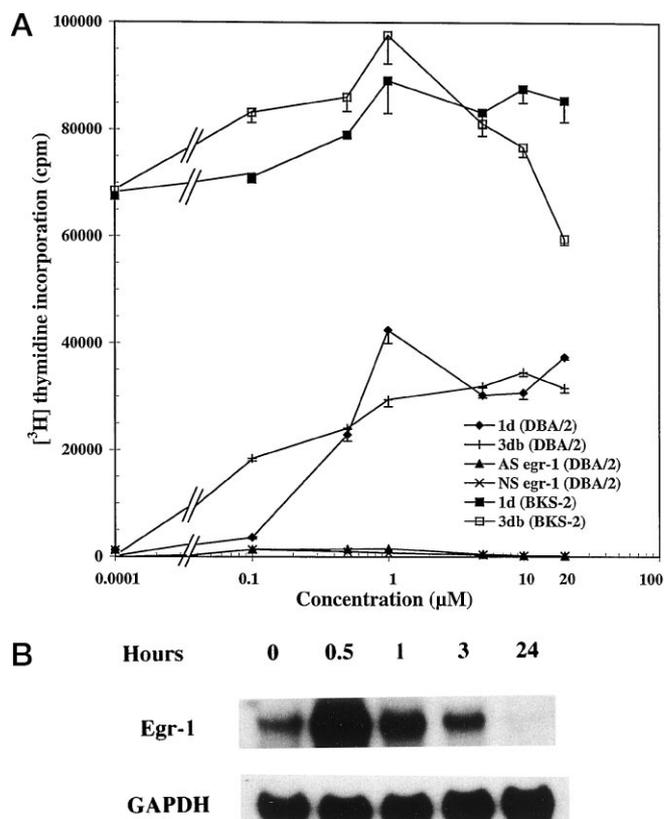


FIG. 5. Effects of CpG-containing oligomers on BKS-2 and normal B cell growth. A, normal DBA/2 mouse derived splenic B cells (2×10^5) and BKS-2 (2×10^4 /well) were cultured with varying amounts of the CpG-containing oligomers 1D and 3Db as described under “Materials and Methods.” The normal B cells were also cultured with indicated concentrations of antisense and nonsense *Egr-1*-specific oligomers. Growth was monitored by [3 H]thymidine incorporation at 48 h as described before. B, the 3Db oligomer induces *Egr-1* expression in BKS-2 cells. The BKS-2 cells were cultured with 1.0 μ M 3Db oligomer for various time periods as indicated. The cells were then harvested, and RNA was isolated and analyzed by Northern blot using either the *Egr-1* or the GAPDH cDNA probe. The first point indicating 0.0001 μ M is really with no oligomer, but was given this value since log scale does not allow a zero value.

treatment in BKS-2 cells was shown in Fig. 6D, and the number of cells undergoing apoptosis in this group (78% in gates R3, R4, and R5) was comparable to the proportion of cell death caused by antisense oligomer. These results imply the significance of expression of *Egr-1* in the survival of BKS-2 cells.

BKS-2 Mutant Cell Line 1.B5 Is Resistant to Anti-IgM-induced Growth Inhibition—We have isolated several independent mutants of BKS-2 that are resistant to anti-IgM-induced growth inhibition.² One such mutant, namely 1.B5, was used in this study. Ligation of BCR by anti-IgM Ab causes growth inhibition and subsequent apoptotic cell death in BKS-2 cells (31). In contrast to the parental BKS-2 cells, 1.B5 mutant cells are completely resistant to the growth inhibitory effect of anti-IgM, as measured by [3 H]thymidine incorporation (Table I). Even immobilized anti-IgM Ab, which are known to cause strong growth inhibition in wild type BKS-2 cells due to effective cross-linking (32, 34), failed to affect the growth of 1.B5 cells demonstrating that 1.B5 cells are completely resistant to the growth inhibitory signal delivered via BCR.

Up-regulation of *Egr-1* mRNA Expression in a BKS-2 Mutant Cell Line, 1.B5—To examine whether anti-IgM-resistant phenotype of 1.B5 cells correlates with the expression of *Egr-1*, we performed Northern analysis using total RNA isolated from 1.B5 cells stimulated with anti-IgM for various time intervals. In contrast to wild type BKS-2 cells, 1.B5 mutants exhibit a

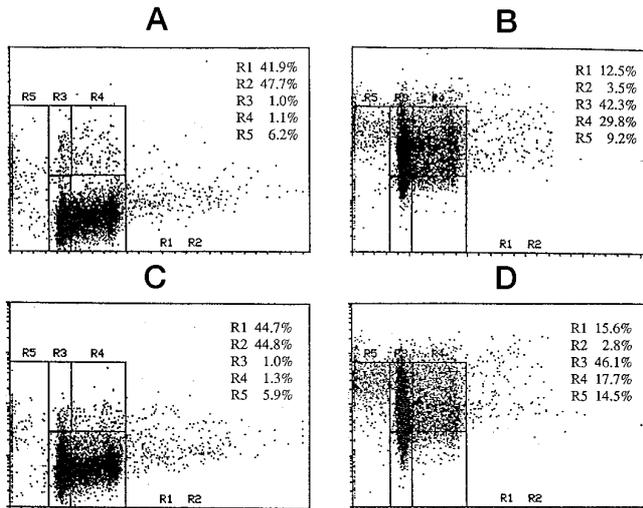


FIG. 6. Flow-cytometric analysis of apoptosis induced by anti-sense *Egr-1* oligomer in BKS-2 cells. BKS-2 cells (1×10^6 /ml) were cultured with $10 \mu\text{M}$ nonsense or antisense *Egr-1* oligomers for 24 h. Other groups of cells were stimulated with $10 \mu\text{g/ml}$ anti-IgM Ab or control Ig. Cells were then stained with Hoechst and Merocyanin dyes and analyzed by flow-cytometry as described under "Materials and Methods." The results are shown as nonsense oligomer-treated cells (A), antisense *Egr-1* oligomer-treated cells (B), control Ig-stimulated cells (C), and anti-IgM-treated cells (D). This method of staining distinguishes five different stages of cells and they are denoted as R1: viable resting cells, $2n$ DNA and MC540 dull; R2: viable cycling cells, $>2n$ and MC540 dull; R3: resting cells undergoing apoptosis, $2n$ and MC540 bright; R4: cycling cells undergoing apoptosis, $>2n$ and MC540 bright; and R5: cells that moved into later stages of apoptosis become Hoechst 33342 dull, as their DNA is fragmented. The fraction of apoptotic cells in any sample is represented by the sum of percentages of cells in each of the gates R3, R4 and R5. The results from one of two similar experiments are shown.

TABLE I

BKS-2-derived 1.B5 mutant cell line is resistant to anti-IgM-induced growth inhibition

BKS-2 and 1.B5 cells (2×10^4) were cultured for 48 h, and [^3H]thymidine incorporation was measured in the last 4 h of culture. Control Ig and soluble anti-IgM were used at $5 \mu\text{g/ml}$, and Sepharose-coupled anti-IgM (1 mg of Ab/ml of beads) was used at 1% final concentration.

Stimulation	[^3H]Thymidine incorporation	
	BKS-2	1-B5
	<i>cpm</i> \pm <i>S.E.</i>	
None	42,090 \pm 362	62,093 \pm 1,357
Control Ig	43,732 \pm 965	65,221 \pm 2,018
Soluble anti-IgM	3,735 \pm 244	66,399 \pm 1,176
Immobilized anti-IgM	1,073 \pm 51	56,444 \pm 183

reduced level of constitutive expression of *Egr-1* mRNA (Fig. 7). Thus densitometry analysis of the *Egr-1* and GAPDH bands in Figs. 1 and 6 showed that the ratio of *Egr-1* to GAPDH in the untreated wild type BKS-2 cells (lane 1 in Fig. 1) was 1.4:1.0 and 0.26:1.0 in the mutant 1.B5 cells (lane 1 in Fig. 7). Interestingly, a marked increase in the expression *Egr-1* message was observed in 1.B5 cells as early as 30 min after stimulation with anti-IgM (lane 2). Although a gradual decline in the message was noticed in 1.B5 cells treated with anti-IgM for 1 and 3 h (lanes 3 and 4), the levels were significantly higher than the untreated cells (lane 1). However, at 24 h after anti-IgM stimulation, the *Egr-1* message expression returned to the basal level (lane 5) as in untreated cells. These results indicate a strong correlation between *Egr-1* expression and the nature of signal (positive versus negative) transduced through antigen receptor on B lymphocytes. Thus, the continued proliferation of the 1.B5 cells in the presence of anti-IgM is likely to be due to the sustained expression of *Egr-1* gene.

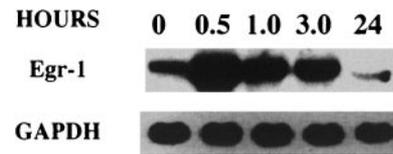


FIG. 7. Anti-IgM stimulation causes up-regulation of *Egr-1* expression in 1.B5 cells. 1.B5 mutant cells were stimulated with anti-IgM ($10 \mu\text{g/ml}$) for various intervals of time as indicated. Then the cells were processed for Northern analysis with *Egr-1* or GAPDH cDNA probe. The same blot was later analyzed for the expression of GAPDH. The times of exposure to x-ray film for the *Egr-1* blot and GAPDH blot were 24 h and 6 h, respectively. Results from one of four independent experiments are shown.

DISCUSSION

In this report, BKS-2 lymphoma cells were used as a model to study the role of *Egr-1* in the induction of apoptosis caused by BCR-mediated signals. The BKS-2 cells undergo apoptosis upon signaling through BCR (31–35), a cellular response that mimics the process of deletion of self-reactive immature B cells during negative selection. Our data showed that the BKS-2 cells expressed *Egr-1* constitutively and that signaling through BCR inhibited *Egr-1* expression in BKS-2 cells both at message and protein levels. This reduction in *Egr-1* expression appeared to be causally related to the subsequent induction of apoptosis response to BCR cross-linking because specific blocking of *Egr-1* expression in these cells using antisense oligomer also caused apoptosis. The effects of the *Egr-1* antisense oligomers were specific, since several other oligonucleotides (a nonsense oligomer, two pairs of oligomers specific to two other cellular proteins and two CpG-containing oligomers proven to be mitogenic to B cells) failed to inhibit the growth of BKS-2 cells like the *Egr-1* antisense oligomer. These data strongly indicate that *Egr-1* expression is crucial for the survival of BKS-2 cells.

The association of *Egr-1* down-regulation with apoptotic signal is consistent with the observation that, in many cell types, this gene is induced by growth stimulatory agents (10–14). Similar growth-associated induction of *Egr-1* has also been reported in mature B lymphocytes after stimulation through BCR (27). Although down-regulation of *Egr-1* expression has not been shown to be involved in apoptosis of any cell types previously, two of us have recently reported that a melanoma cell line, A375-C6, stably transfected with a dominant negative construct of *Egr-1* is highly sensitive to interleukin-1-induced growth arrest (41). Similarly, transfection of human epithelial tumor cell line, SQ-20B with *Egr-1* dominant negative construct prevented the onset of S phase and reduced the survival of these cells exposed to ionizing radiation (44). Studies on mouse fibroblast NIH 3T3 cells also have indicated that *Egr-1* may confer resistance to growth inhibition caused by ultraviolet radiation (45). Even though the mechanistic basis of protective function of *Egr-1* in these cells is not known, it has been postulated that EGR-1 protein may regulate genes such as metallothionein-IIa and *Ras*, which have protective functions and whose promoters also contain EGR-1 binding sites (46, 47). In particular, *Ras* has been shown to have a critical role in transmission of growth signals for various cell types including mature B cells (5, 25, 30). It is provocative that expression of dominant negative *Ras* inhibits *Egr-1* expression in normal B cells, while *Ras* promoter contains an EGR-1-specific binding site (25). In contrast, expression of dominant negative *ras* did not affect the negative selection of thymocytes (48). This issue has not yet been examined for B cell apoptosis but if the same rules apply, then *Egr-1* expression may be independent of *Ras* in immature B cells. Alternatively, *ras* may be important for B cell apoptotic response by its ability to influence *Egr-1* expression. Thus far, no studies exist that directly examine the role of

EGR-1 protein on *ras* gene expression or the requirement for *ras* in anti-IgM-induced B cell apoptosis.

In contrast to its role as a gene required for cell growth, recently *Egr-1* was found to be one of the factors required for thapsigargin and ionizing radiation-induced apoptosis in A375-C6 melanoma cell line (49, 50). In this cell line, blockade of *Egr-1* expression with a dominant negative construct or antisense probes inhibited apoptosis induced by calcium elevation or ionizing radiation. It is conceivable that in A375-C6 cells *Egr-1* up-regulates *nur77* or a related gene that has been shown to be required for apoptosis in certain cell types (18). Dual roles for *Egr-1* resemble those for *c-myc*, which also protects as well as promotes apoptosis (51). In particular, B cell lines have been shown to be growth-inhibited when *c-myc* levels fall below background levels, and the growth is restored by constitutive expression of *c-myc* (51). It is conceivable that the growth inhibition and apoptosis of BKS-2 cells in response to reduction in *Egr-1* levels is due to an inhibition of *c-myc* expression.

Egr-1 is neither constitutively expressed nor up-regulated by BCR crosslinking either in neonatal B cells or in WEHI-231-like immature B cell lymphoma cells (7, 29) in which the response to anti-IgM stimulation is growth inhibition and apoptosis. However, the lipopolysaccharide-mediated protection of WEHI-231 cells from anti-IgM-induced apoptosis is associated with *Egr-1* induction (52). Thus, the apparent relation between *Egr-1* expression and B cell growth can be demonstrated with both B cell lymphoma cell lines and normal B cells of different maturation stages. Hence, the differential regulation of *Egr-1* expression in mature and immature B lymphocytes is likely to be due to a difference in early signaling events between these cells.

The mutant cell line, 1.B5, was refractory to anti-IgM-induced growth inhibitory signals and showed a lower level of constitutive expression of *Egr-1* (Figs. 1 and 7) in contrast to the wild type BKS-2 cells. Interestingly, these mutant BKS-2 cells behaved like normal mature B cells in that signaling through BCR caused a strong induction of *Egr-1* message (27). Anti-IgM-induced early signaling events are nearly identical in the immature B cell lines, WEHI-231 and BKS-2 cells² that undergo apoptosis and in the normal mature B lymphocytes that respond by entering into cell cycle (5). In both immature B cell lines and mature B cells, cross-linking of BCR induces activation of Src family (Blk, Lyn) as well as non-Src family protein-tyrosine kinases (5). In both cell types, expression of *c-myc* is initially elevated but the subsequent down-regulation is unique to each cell type (51). Studies presented here suggest that the regulation of *Egr-1* gene dramatically differs between the mature and immature B cells, with the former exhibiting an up-regulation while the latter respond by down-regulation of *Egr-1*. At present, both protein kinase C pathway and the Ras-MAP kinase cascade have been shown to induce elevation of *Egr-1* gene (7, 30). The wild type and mutant BKS-2 cells along with normal mature B cells provide a unique model system to define the early signaling events that lead to differential regulation of *Egr-1* gene. Our preliminary studies with BKS-2 and the mutant 1.B5 cells suggest that activation of Blk and Lyn protein-tyrosine kinases may not be required for up-regulation of *Egr-1* expression.²

Although some of the recent reports clarify the role of *Egr-1* in BCR-mediated activation of mature B cells (25, 26), its role in the negative growth response is poorly understood. It is possible that *Egr-1*-mediated control of the negative growth response in BKS-2 cells might be exerted at the transcriptional level by regulating the expression of certain key growth response genes. Indeed, EGR-1 has been shown to be a positive

activator of transcription by binding to GC-rich consensus DNA sequences of many target genes involved in positive regulation of cell growth, but the effect of *Egr-1* on the expression of the survival genes belonging to the *Bcl-2-Bcl-x_L* family has not yet been investigated (53). We have observed that rapamycin-induced apoptosis of BKS-2 cells (33) is associated with down-regulation *Egr-1* as well as *Bcl-2* gene products.⁴ Therefore, we are currently evaluating the effect of anti-IgM and antisense *Egr-1* oligomers on the expression of Bcl-2 gene family in BKS-2 cells.

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Role of *Egr-1* Gene Expression in B Cell Receptor-induced Apoptosis in an Immature B Cell Lymphoma

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