Impaired Proliferation and Survival of Activated B Cells in Transgenic Mice That Express a Dominant-negative cAMP-response Element-binding Protein Transcription Factor in B Cells*

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The cAMP-response element-binding protein (CREB) is activated by phosphorylation on serine 133 and mediates the proliferative response to a number of different signals. A mutant CREB with a serine to alanine substitution at position 133 (CREBM1) functions as a dominant-negative inhibitor. Transgenic mice that express the dominant-negative CREB protein in B lymphocytes were developed as a means to study the effects of the inhibition of CREB function on B-cell proliferation and survival. We have shown previously that CREB up-regulates Bcl-2 expression in B cells in response to activation signals. B cells from CREBM1 transgenic mice expressed lower levels of Bcl-2 with and without stimulation. Proliferation of B cells from the transgenic mice was impaired in part by lack of induction of activator protein 1 (AP1) transcription factors. B cells from the transgenic mice were more susceptible to induction of apoptosis with several different agents, consistent with the decreased expression of Bcl-2. These studies demonstrate that B-cell activation requires phosphorylation of CREB for the proliferative response and to protect against activation-induced apoptosis.

The cAMP-response element-binding protein (CREB) is a basic leucine zipper transcription factor that binds to the CRE as both a homodimer and a heterodimer with other members of the CREB/ATF and AP1 families. The CREB/ATF family mediates the transcriptional response to a number of different signals including growth factors, Ca²⁺, peptide hormones, and neurotransmitters. CREB is phosphorylated on serine 133 in response to these signals by several different serine/threonine kinases. Recent studies have demonstrated that CREB functions as a survival factor and protects cells against apoptosis (1–3). CREB is phosphorylated during T-cell activation and is required for cell cycle progression and survival (3, 4). CREB—

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The abbreviations used are: CREB, cAMP response element-binding protein; CREBM1, mutated CREB with a serine to alanine substitution at position 133; NTg, nontransgenic; Tg, transgenic; ATF, activating transcription factor; AP, activator protein; PMA, phorbol 12-myristate 13-acetate; CD, cluster of differentiation; Ig, immunoglobulin; XTT, 2,3-bis-(2-methoxy)-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

mice die shortly after birth from pulmonary atelectasis (5), whereas inactivation of the ATF1 gene does not cause any clear phenotypic abnormalities (1). The lack of a strong phenotype in some of the CREB family knock-out mice is thought to be the result of functional compensation by other family members (6). Embryos that are deficient for both CREB and ATF1 die before implantation because of developmental arrest (1). Transgenic mice that express dominant-negative forms of CREB have been developed to study the role of CREB in different cell types. A mutant CREB that contains a serine to alanine substitution (CREBM1) at position 133 functions as a dominant-negative repressor of CREB-dependent gene expression (7, 8). Transgenic mice that express CREBM1 in cells of the anterior pituitary demonstrate a dwarf phenotype with atrophied pituitary glands that are deficient in somatotroph cells (9). Expression of CREBM1 in the thyroid gland results in decreased thyroid differentiation and function (10). The CD2 promoter has been used to express CREBM1 in thymocytes of transgenic mice. Differentiation of T cells was normal, but proliferative defects were found with decreased interleukin-2 production of T cells from the transgenic mice (4). Transgenic mice with CREBM1 expression regulated by the lch promoter have defective T lymphocyte helper function with increased susceptibility of T lymphocyte helper cells to activation-induced cell death. Decreased levels of Bcl-2 were observed in these cells, and the susceptibility to activation-induced cell death was reversed by overexpression of Bcl-2 (3). Expression of CREBM1 in cardiac myocytes resulted in cardiac dilatation with attenuated contractile responses to β-adrenergic agonists (11).

We have shown previously that CREB regulates bcl-2 expression during B-cell activation and during rescue of immature B cells from apoptosis (12). Apoptosis plays an important role in the development of mature B cells. Widespread apoptosis is observed in the germinal centers, and engagement of surface immunoglobulin (Ig) results in rescue from apoptosis. Increased expression of Bcl-2 is observed in the germinal center cells that are prevented from undergoing apoptosis (13, 14). Our studies have shown that the CRE in the bcl-2 promoter mediates the increase in Bcl-2 expression after surface Ig cross-linking or phorbol ester treatment in mature and immature B cells (12). CREB is also involved in the deregulated expression of the translocated bcl-2 gene in t(14; 18) lymphomas (15). To examine the role of CREB proteins in activation and survival of B lymphocytes and in the regulation of Bcl-2 expression, transgenic mice were made that express CREBM1 under the control of the Ig heavy chain intron enhancer. We show that B-cell differentiation proceeds normally, but defects exist in B-cell activation and survival. In addition, Bcl-2 expression is decreased in B cells that express CREBM1.
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EXPERIMENTAL PROCEDURES

Production of Transgenic (Tg) Mice—CREBM1, which contains an alani ne at position 135, was inserted into the E\(_b\)SV plasmid. This construct contained the Ig heavy chain intron enhancer and the SV40 polyadenylation site. The construct was linearized by digestion with NotI and injected into the pronucleus of fertilized single-cell eggs of FVB mice, which were then surgically transferred into the oviducts of pseudopregnant females. Germline transmission was documented by PCR-based screening and Southern blotting. The primers used for PCR were GCCCGGAAATTCAGTCGCG from the CREB sequence and GGCCCGGAATTCAGTCGCG from the SV40 sequence. A 550-bp fragment was amplified from CREBM1 mice. Southern blotting with a 400-bp probe that included a sequence from the 3’ end of the CREB gene and the 5’ end of the SV40 sequence was used to confirm the identity of the PCR products.

Northern Blot Analysis—Total RNA was purified from several organs of the CREBM1 and nontransgenic (NTg) mice using the Tri-Reagent (Molecular Research Corp.). 20 \( \mu \)g of total RNA was electrophoresed through 1% denaturing (formaldehyde) agarose gels and transferred to nitrocellulose membranes. Hybridization was performed with the complete RPMI 1640 medium with 10% fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM l-glutamine, and 10\( ^{-5} \) M 2-mercaptoethanol in 48- or 96-well tissue culture plates in the presence or absence of various chemicals as described in the text.

Lymphocyte Preparation and Culture—Cells from the spleen, bone marrow, and thymus were harvested from 9- to 15-week-old normal FVB NTg and Tg mice. Lymphocytes were purified from total splenocytes, bone marrow cells, and thymocytes with Ficoll-Paque (Amersham Biosciences) and were used directly for flow cytometry analysis. For B-cell activation and functional studies, lymphocytes from the spleen were further purified by negative selection using mouse B-cell recovery columns (Collegeville Biotech). The average purity of the B cells was 89-94% as determined by flow cytometry. Purified splenic B cells were cultured in complete RPMI 1640 medium with 10% fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM l-glutamine, and 10\( ^{-5} \) M 2-mercaptoethanol in 48- or 96-well tissue culture plates in the presence or absence of various chemicals as described in the text.

Western Blotting—Purified splenic B cells (4 \( \times \) 10\(^6\) cells/ml) were stimulated with PMA (15 ng/ml), PMA (15 ng/ml) and ionomycin (0.5 \( \mu \)g/ml), or anti-IgM (15 \( \mu \)g/ml) for the indicated times. Protein lysates from unstimulated or stimulated B cells were subjected to Western blotting using antibodies against Ser-133-phosphorylated CREB (pCREB), CREB (phosphorylated and unphosphorylated), Bcl-2 (Upstate Biotech), c-Fos, FosB, and JunB (Santa Cruz Biotechnology) at a 1:1000 dilution. Immunoblots that contained cell extracts from 4 \( \times \) 10\(^6\) freshly purified splenic B cells were probed for ATF-1, ATF-2, ATF-3, CREB-2, and CRE-M (Santa Cruz Biotechnology). After treatment of B cells with 1 \( \mu \)g/ml ionomycin and preparation of lysates, immunoblots were probed with antibodies for Bcl-2, Bax, and Bcl-X\(_r\) (Santa Cruz Biotechnology). For each experiment, an equal amount of protein was loaded in each lane, separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with a specific primary antibody. Membranes were washed and incubated with secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies and developed with an enhanced chemiluminescent system (Amersham Biosciences) according to the vendor’s instructions. All of the blots were reprobed with an antibody to actin (Sigma) to control for variations in protein loading.

Flow Cytometry—Total bone marrow cells were stained with phycoerythrin-conjugated anti-B220 and fluorescein isothiocyanate-conjugated anti-CD4 and anti-CD8. Splenocytes were stained with fluorescein isothiocyanate-conjugated anti-IgD and phycoerythrin-conjugated anti-CD4 and fluorescein isothiocyanate-conjugated anti-CD8. All antibodies were purchased from Pharmingen. Fluorescence was analyzed using the FACsCaliber flow cytometer (BD Biosciences). Ten thousand events were collected and analyzed by CellQuest software (BD Biosciences).

Cell Cycle Analysis—2.5 \( \times \) 10\(^6\) cells were washed twice in cold phosphate-buffered saline. Cells pellets were resuspended in ice-cold 75% ethanol with slow vortexing, fixed overnight, and then resuspended in 0.1 ml of propidium iodide staining solution (50 \( \mu \)g/ml, BD Pharmingen) and 14 \( \mu \)g/ml RNase A and incubated at 4°C in the dark for 2 h. 2 \( \times \) 10\(^6\) gated events were collected on a FACsCaliber cytometer and analyzed using CellQuest software.

Proliferation Assay—Purified splenic B cells (3 \( \times \) 10\(^4\)) were cultured in triplicate in 100 \( \mu \)l of medium in 96-well tissue culture plates. Cells were stimulated with medium alone, anti-CD40 (5 \( \mu \)g/ml), anti-IgM (15 \( \mu \)g/ml), PMA (15 ng/ml) and ionomycin (0.5 \( \mu \)g/ml), or lipopolysaccharide (5 \( \mu \)g/ml) for 48 h. Cell proliferation was measured by a colorimetric assay using XTT (Roche Molecular Biochemicals).

RESULTS

Production and Molecular Characterization of CREBM1 Transgenic Mice—CREBM1 contains a substitution of alanine for serine at amino acid number 133 (9). This protein cannot be phosphorylated in response to activation signals and functions as a dominant-negative inhibitor of CREB/ATF family transcription factors. To limit expression to lymphocytes, the Ig heavy chain intron enhancer was used. The construct is shown in Fig. 1A.

Three independently derived CREBM1 founders were produced. PCR followed by probing with a fragment from the vector confirmed the presence of the transgene (Fig. 1B) for two of the lines. Expression of the mutant CREB gene was demonstrated by Northern analysis of RNA from spleen (Fig. 1C). A low level of expression in the thymus was also observed (Fig. 1C). Previous studies have shown that the Ig heavy chain intron enhancer is active in both T and B cells. Other tissues did not show expression of the transgene (the liver and kidney are shown in Fig. 1C). Protein immunoblotting demonstrated that resting B cells from the transgenic mice contained higher levels of total CREB compared with B cells from NTg mice (Fig. 1D). In addition, less phosphorylated CREB was present in the B cells of the transgenic mice than in the B cells of the NTg littermate controls (Fig. 1D). The levels of other members of the CREB family were unchanged in the B cells of the transgenic mice (Fig. 1E).

Differentiation of B Cells Is Normal in CREBM1 Tg Mice—To examine whether expression of the dominant-negative CREB interfered with B-cell differentiation, fluorescence-activated cell sorter analysis was performed on total bone marrow cells and splenocytes from NTg and Tg mice. As shown in Fig. 2A, no significant differences were found in the number of lymphocytes between the two different lines of mice. The numbers of pro-B and pre-B cells (B220\(^{hi}\), IgM\(^{-}\)), immature B cells (B220\(^{lo}\), IgM\(^{+}\)), and mature B cells (B220\(^{lo}\), IgM\(^{+}\)) in the bone marrow were not statistically different between the NTg and Tg mice (Fig. 2A). Examination of the spleen revealed equal numbers of lymphocytes and no difference in the numbers of immature B cells (IgM\(^{+}\), IgD\(^{-}\)) and mature B cells (IgM\(^{+}\), IgD\(^{lo}\), IgD\(^{hi}\)) between NTg and Tg mice (Fig. 2B). Thus, expression of the dominant-negative CREB does not significantly disrupt differentiation of B lymphocytes. Histologic examination of the lymph nodes and spleen revealed no clear abnormalities. No differences were observed in the numbers of the different T-cell subsets between the Tg and NTg mice when thymocytes were analyzed (data not shown). This finding agrees with the results obtained when CREBM1 was expressed with the CD2 promoter/enhancer (4).

Phosphorylation of CREB Is Inhibited in CREBM1 B Cells—Activation of several signaling cascades results in rapid phosphorylation and activation of CREB in B cells. Lower levels of phosphorylated CREB were observed in resting CREBM1 B cells as noted above, but we wished to examine whether phosphorylation of CREB occurred after appropriate stimulation of the B cells. Phosphorylation of CREB in response to three different stimuli, PMA, PMA and ionomycin, and anti-IgM, was analyzed. Increased phosphorylation of CREB was detected at 1 min and reached the maximum level in 5–30 min in each case with the B cells from NTg and CREBM1 mice (Fig. 3A). The phosphorylation of CREB at its maximal level was reduced by...
**FIG. 1.** Characterization of CREBM1 Tg mice. A, schematic representation of the CREBM1 transgene. The mutation at position 133 is indicated. Primers 1 and 2 were used for PCR amplification, and the 400-bp probe was used for the Southern and Northern analyses. B, PCR amplification of tail DNA from two independently derived Tg (+) lines. The PCR product was hybridized with the probe indicated in A to confirm the identity of the PCR products. Lane C indicates a positive control for the PCR product. C, Northern blot analysis of transgene expression in tissues (S, spleen; T, thymus; L, liver; K, kidney) from one of the Tg lines and a NTg mouse. The probe hybridizes to both the transgene and the endogenous CREB gene. The size of the transcript from the transgene is 2.5 kb. Size markers are shown in lane M. The blot was hybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to control for variation in RNA loading. D, Western blot analysis of CREB and phospho-CREB (pCREB) expression in B cells from NTg and Tg mice. Equal protein loading was verified by probing with an antibody to actin. E, Western blot analysis of other CREB family members, ATF-1, ATF-2, ATF-3, CREB-2, and CREM-1, in B cells from NTg and Tg mice.

**FIG. 2.** Differentiation of B cells is normal in CREBM1 mice. A, flow cytometric analysis of bone marrow from Tg and NTg mice. Bone marrow cells were stained with antibodies to B220 and IgM. Pro-B and pre-B cells are observed in box a, mature B cells in box b, and immature B cells in box C. B, flow cytometric analysis of splenocytes, which were stained with antibodies to IgM and IgD. Mature B cells are found in box a and immature B cells in box b. The analyses were repeated at least three times, and representative plots are shown. FSC, forward scatter; SSC, side scatter.

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This treatment bypasses B-cell membrane receptors and provides an intrinsic measure of the proliferative capacity of the B cells. Proliferation of CREBM1 B cells was decreased by 43% compared with proliferation of NTg B cells after treatment with PMA and ionomycin (Fig. 4A). Cell cycle analysis confirmed these effects of PMA and ionomycin and revealed a decreased number of cells in the S and G2/M phases with an increase in the number of cells in the G1 phase in the CREBM1 B cells compared with the NTg B cells (Fig. 4B). Increased apoptosis was observed in the CREBM1 B cells compared with NTg B cells after PMA and ionomycin treatment. The CREBM1 B cells displayed a 25% decrease in viability with activation; however, no significant difference in viability between the NTg and the CREBM1 B cells could be detected without stimulation (Fig. 4C).

**Induction of AP1 Transcription Factors Is Decreased in CREBM1 B Cells—**Fos and Jun family members are involved in proliferation, and several of these genes are regulated by CREB. CRE sites are located in the promoter regions of the JunB, c-fos, and fosB genes. The protein levels of these transcription factors were measured after treatment of splenic B cells with PMA and ionomycin. The expression of JunB was similar in B cells from wild type and CREBM1 mice, and little change was noted with activation. In contrast, the levels of c-Fos and FosB were reduced by 45% and 86%, respectively, in the CREBM1 B cells compared with the NTg B cells after treatment with PMA and ionomycin (Fig. 4D).

**Apoptotic Stimuli Result in Increased Cell Death of CREBM1 B Cells—**Phosphorylation of CREB is involved in the rescue of B cells from apoptosis, and one of the CREB target genes is bcl-2. The response of B cells from the CREBM1 Tg mice to different apoptotic stimuli was investigated. Apoptotic cells were detected with annexin V and propidium iodide staining after 24 h of treatment. As shown in Fig. 5A, the number of viable CREBM1 B cells was decreased compared with the number of viable NTg B cells after treatment with ionomycin, vincristine, or etoposide at a range of concentrations. Western analysis was performed on the ionomycin-treated B cells to examine the levels of several Bcl-2 family members. Bcl-2 levels decreased by −20% in B cells from the NTg mice and by 50% in the B cells from the CREBM1 mice (Fig. 5B). The unstimulated CREBM1 B cells expressed −3-fold less Bcl-2 than the NTg B cells, and they showed a greater drop in Bcl-2 with ionomycin treatment. The net result is a 5-fold lower level of expression of Bcl-2 in the CREBM1 B cells after ionomycin treatment compared with the level in NTg B cells after ionomycin treatment. No significant differences were found in the levels of Bax or Bcl-XL in the B cells of the CREBM1 mice compared with the NTg mice (Fig. 5B).

**DISCUSSION**

Our studies showed no defect in differentiation of B cells that express a dominant-negative CREB. This finding is similar to studies of the dominant-negative CREB in T cells (3, 4). Defective proliferation of B cells was observed in response to several different signals. Again, these findings are consistent with the effects of CREBM1 in T-cell proliferation. Proliferative signals induce a number of changes in gene expression in B cells, and bcl-2 expression is increased at the mRNA level (16, 17). Several transcription factors are activated, including NF-κB, CREB, and AP1. CREB has been shown to regulate the transcription of several genes that are involved in proliferation, including fos, jun, and proliferating cell nuclear antigen (PCNA) (18–20). Unphosphorylated CREB represses transcription of both the fos and jun promoters. When serine 133 is phosphorylated, the repression is relieved and transcription is observed. It is likely that dominant-negative CREBM1 inhibits...
the transcription of some of the genes involved in the proliferation of B cells. The levels of c-Fos and FosB after activation were dramatically reduced in CREBM1 B lymphocytes. Bcl-2 levels were also reduced, and although Bcl-2 is not directly involved in proliferation, its increased expression serves to prevent activation-induced cell death.

B-cell development is normal in bcl-2 knock-out mice. However, these mice are not able to maintain B-lymphocyte homeostasis because of massive apoptosis within a few weeks after birth (21, 22). These findings demonstrate that Bcl-2 is required for normal B-cell function and survival. Apoptosis of germinal center B lymphocytes is observed during normal B-cell development, and activation signals cause the up-regulation of Bcl-2 expression and result in protection of the lymphocytes from apoptosis (13, 14). We have shown previously that IgM cross-linking in mature B-cell lines induces the expression of Bcl-2. CREB is rapidly phosphorylated, and the CRE in the bcl-2 promoter is required for the increased expression of Bcl-2 in response to anti-IgM or PMA (12). Our studies with the dominant-negative CREBM1 confirm and extend these observations by demonstrating that similar signaling pathways are involved in the up-regulation of Bcl-2 expression in normal B lymphocytes.

The role of Bcl-2 in the prevention of apoptosis in response to different signals in numerous cell types has been well documented. Bcl-2 expression protects lymphocytes from apoptosis induced by many different chemotherapeutic agents (23, 24). Down-regulation of Bcl-2 levels by antisense oligonucleotides...
increases the susceptibility of lymphocytes to chemotherapeutic agent-induced apoptosis (25). We investigated the sensitivity to induction of apoptosis of the CREBM1 B cells because they express decreased levels of Bcl-2 and are defective in the ability to up-regulate Bcl-2 expression. As predicted, the B cells from CREBM1 mice showed increased susceptibility to induction of apoptosis by several different agents including chemotherapeutic drugs. The mechanism that results in decreased Bcl-2 levels with ionomycin treatment is not clear. We observed a somewhat more pronounced decrease in Bcl-2 levels in the B cells from the CREBM1 mice, so it is possible that CREB transcription factors play a role in maintenance of Bcl-2 expression in this situation.

These studies on the expression of the dominant-negative CREBM1 in B cells clearly demonstrate the role of CREB in the regulation of Bcl-2 levels in B lymphocytes. Our previous studies in B-cell lines showed that the bcl-2 promoter was up-regulated by phosphorylated CREB proteins (12). The B cells from the CREBM1 Tg mice displayed defects in proliferation, although some proliferation was observed. We also found that CREB could be phosphorylated in response to activation signals in the CREBM1 B cells, although at a decreased level compared with that of NTg B cells. This finding most likely reflects the fact that the dominant-negative CREBM1 is not expressed at a level sufficient to interfere with the function of all of the endogenous CREB proteins. Bcl-2 levels are decreased by 3-fold in resting lymphocytes from CREBM1 mice, and the CREBM1 B cells are more susceptible to induction of apoptosis.

In addition to CREB, we have shown that NF-κB family members regulate Bcl-2 expression through the CRE site (26). NF-κB family members are induced by several of the same signals that activate CREB proteins. They may be responsible for the continued expression of Bcl-2 in the CREBM1 B cells and for the proliferation that is observed in response to activation signals. Further studies will be required to evaluate the contribution of each of these transcription factors to the regulation of Bcl-2 expression in B cells in response to activation and apoptotic signals.

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Fig. 5. Increased apoptotic susceptibility of CREBM1 B cells. A, purified splenic B cells were treated with ionomycin (0.1, 1, and 3 μg/ml), vincristine (0.1, 0.5, and 2 μg/ml), or etoposide (0.1, 1, 3 μM) for 24 h. Viability was determined by flow cytometry after annexin V and propidium iodide staining. Cell viability was normalized to spontaneous cell death in untreated controls, which was similar in Tg and NTg B cells. Duplicate samples of each treatment in eight independent experiments were assayed. The percentage of viable cells is shown as the mean with the standard deviation. B, Western analysis of Bcl-2 family members in ionomycin-treated splenic B cells from CREBM1 (Tg) and control (NTg) mice. Purified splenic B cells were untreated (Uns) or treated with 1 μg/ml ionomycin (I) for 24 h. Immunobots were probed with antibodies against Bcl-2, Bax, and Bcl-XL.
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