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Identification of a Membrane Ig-Induced p38 Mitogen-Activated Protein Kinase Module That Regulates cAMP Response Element Binding Protein Phosphorylation and Transcriptional Activation in CH31 B Cell Lymphomas

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The cAMP response element (CRE) binding protein (CREB) is emerging as a key regulatory factor of gene transcription in B lymphocytes; however, the postreceptor pathways that regulate CREB activity and CRE-dependent gene transcription remain largely undefined. We investigated B cell Ag receptor (BCR)-mediated phosphorylation and activation of CREB in the surface IgM\(^*\) CH31 B cell lymphoma, which undergoes Ag-dependent cell death. The activity of p38 mitogen-activated protein kinase (MAPK) was increased in response to BCR ligation. Phosphorylation of CREB on serine 133, a modification that positively regulates its trans-activation, was concomitantly increased. Inhibition of p38 MAPK by pretreating CH31 B cells with the highly specific bicyclic imidazole inhibitor, SB203580, reduced BCR-induced CREB phosphorylation. BCR cross-linking also led to increased MAPK-activated protein kinase-2 activity, an enzyme that lies immediately downstream from p38 MAPK; MAPK-activated protein kinase-2 immune complexes phosphorylated a peptide substrate containing the CREB serine 133 phosphoacceptor motif. Given the role of CREB in regulating *junB* gene expression in mature B lymphocytes, we examined whether p38 MAPK activity was necessary for CRE-dependent *junB* transcription in CH31 B cells. BCR ligation led to increased *junB* mRNA levels, which were significantly reduced in CH31 B cells pretreated with SB203580. Activation of a CRE-dependent *junB* promoter/chloramphenicol acetyltransferase (CAT) reporter gene by the BCR was also blocked by SB203580. Similarly, inhibition of p38 MAPK in surface IgM\(^*\) WEHI-231 B cell lymphomas resulted in reduced BCR-induced *junB* mRNA expression and *junB* promoter activation. The results implicate a p38 MAPK pathway in BCR-mediated CREB phosphorylation and *junB* transcriptional activation in B cell lymphomas. The Journal of Immunology, 2000, 164: 2311–2319.

The B cell Ag receptor (BCR)\(^3\) regulates cell-cycle progression in a manner dependent on the developmental stage of B lymphocytes. For example, BCR ligation on mature B cells can lead to G\(_1\) progression and S phase commitment, whereas immature B lymphocytes undergo apoptosis (1–3). The mechanism(s) by which the BCR regulates cellular physiology and the cell cycle is not completely understood. The earliest consequence of BCR cross-linking is the activation of src-protein tyrosine kinases (4, 5). Subsequent signal transmission occurs along four known pathways that include a phosphatidylinositol 3-kinase/PI3K/GTPase/Raf/extracellular signal-regulated kinase (ERK) cascade, phospholipase C\(_\gamma\), and RhoGTPase (4–8). Although the biological function(s) of individual pathways remain unclear, it is recognized that signals must be transduced to the nucleus to alter gene expression. Some of the earliest known nuclear events include phosphorylation of Ets-1, induction of the immediate-early gene response, and synthesis of carbamoylphosphate synthetase-aspartate transcarbamylase-dihydroorotase and G\(_4\)cyclics (9–17).

It is now established that the 43-kDa phosphoprotein, cAMP response element (CRE) binding protein (CREB), is a major determinant in regulating gene transcription in B cells (18–20). For example, members of the CREB/activating transcription factor (ATF) family are involved in determining the activity of the 3\(^\prime\) enhancer in pre-B cell lines (18). CREB contributes to the activation of MHC class II promoters via X2 box sequences (21, 22). CREB also positively regulates bcl-2 gene transcription during B cell activation and rescue of immature B cells from apoptosis (23). It has been postulated that CREB/ATF family members may play a role in transcription dysregulation, given that several genes containing CRE are expressed aberrantly in malignant tumors and transformed cells (23, 24). A focal point of research at present is directed toward understanding the regulation of CREB trans-activation by B cell surface receptors. CREB is a member of the CREB/ATF bZip family of transcription factors that binds to nucleotide sequences homologous to the palindromic CRE, TGCAGTCA (25, 26). In nonlymphoid cells, surface receptors regulate trans-activation of CREB primarily through protein kinase A (PKA), pp90\(^{ERK}\), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), Ca\(_{2+}\)-dependent calmodulin kinase (CaMK) II, and CaMK IV pathways (25–28). Phosphorylation on serine 133 located within the kinase-inducible
domain does not affect its binding to the CRE site, but rather increases its association with adapter proteins, such as CREB-binding protein, leading to increased transcriptional activity from genes containing CRE (25, 26).

BCR cross-linking on mature B cells leads to increased phosphorylation of CREB on serine 133 (19, 20, 29, 30). Our laboratory demonstrated that BCR-stimulated transcription of the immediate-early response gene, junB, was mediated by a CRE-like sequence located between −194 and −42 bp (19, 30). This site is occupied by a protein heterodimer consisting of CREB/ATF-1. The relative importance of serine 133 phosphorylation to junB transcription was demonstrated in that a conserved serine-to-alanine substitution at amino acid 133 in CREB abrogated BCR-induced junB gene promoter activation (19). We recently provided evidence for a novel pathway controlling serine 133 phosphorylation in mature B cells that involves the opposing actions of PKA and an okadaic acid-sensitive serine/threonine protein phosphatase activity (30). In brief, CREB phosphorylation on serine 133 was achieved by a BCR-dependent decrease in the protein phosphatase activity without apparently affecting PKA-targeted serine 133 phosphorylation. Despite these observations, many of the intermediate steps that couple the BCR to CREB trans-activation remain poorly defined. In particular, it is not entirely clear to what extent signaling pathways activated by the BCR contribute to CREB phosphorylation. It is also recognized that little is known about the regulation of CREB activity in B cells other than mature B lymphocytes. We sought herein to identify intermediate signal transduction components that link the BCR to CREB phosphorylation and CRE-dependent transcription. Our experiments were conducted in surface IgM B CH31 and WEHI-231 B cell lymphomas, which have been used as models for immature B cell tolerance (1).

Materials and Methods

Cell culture and reagents

The surface IgM+ murine B cell lymphomas, CH31 and WEHI-231, were kindly provided by Dr. David W. Scott (Department of Immunology, American Red Cross, Rockville, MD) (31, 32). Dr. Richard Assofsky (National Institutes of Health, Bethesda, MD) provided the mature Bal17 lymphoma (33). Cells were maintained in log phase growth in a humidified atmosphere of 5% CO2. Mature splenic B lymphocytes from 8- to 12-wk-old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) as described (34). Mice were cared for and handled in accordance with National Institutes of Health Institutional Guidelines. F(ab')2 of goat anti-mouse IgM (anti-Ig) was obtained from Jackson Immunoresearch Laboratories (West Grove, PA). H-85 was purchased from Seikagaku (St. Louis, MO). Cells were cultured in RPMI 1640 containing 10 mM HEPES, pH 7.5, 2 mM L-glutamine, 5 mM 2-ME, and 10% FCS (BioWhittaker, Walkersville, MD). CH31 B cell lymphomas were grown in the presence of 1 mM sodium pyruvate and 1 mM sodium phosphate buffer (25, 26).

Total RNA was prepared using a MicroFastTrack kit (Invitrogen, Carlsbad, CA). Approximately 106 B cells were collected by centrifugation, washed in PBS, and lysed in a buffer containing 0.2 M Tris, pH 7.5, 0.2 M NaCl, 1.5 mM MgCl2, and 2% SDS. Insoluble material was removed by centrifugation at 4000 × g for 5 min, and then the lysate was adjusted to 0.5 M NaCl, and 40 μg/ml aprotinin, 1 mM PMSF, and 1 μg/ml leupeptin. The cell lysates were incubated for 18 h (4°C) with 1 μg anti-phospho(Thr35/Tyr36) p38 MAPK Ab (New England BioLabs)-bound protein A-Sepharose. The immune complexes were washed twice by centrifugation with 1 ml MAPK lysis buffer, twice with 1 ml kinase buffer (25 mM Tris, pH 7.5, 2.5 mM MgCl2, 2 mM DTT, 0.1 mM Na3VO4, and 10 mM MgCl2), and resuspended in 50 μl kinase buffer containing 100 μM ATP and 1 μM GST-ATF-2 fusion protein substrate. The kinase reactions were carried out at 30°C (30 min), and p38 MAPK-mediated phosphorylation of GST-A′Tβ' peptide substrate by anti-phospho(Thr35/Tyr36) ATF-2 Ab (New England BioLabs).

MAPKAP kinase-2 assay

CH31 B cells were solubilized in 25 μl MAP kinase lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM PMSF, and 1 μg/ml leupeptin). The cell lysates were incubated for 18 h (4°C) with 1 μg anti-phospho(Thr197) MAPKAP kinase-2 Ab (Upstate Biotechnology) plus protein A-Sepharose for 2 h. The MAPKAP kinase-2 immune complexes were collected by centrifugation, and the supernatant was incubated with 5 μg anti-MAPKAP kinase-2 Ab (Upstate Biotechnology) plus protein A-Sepharose for 2 h. The MAPKAP kinase-2 immune complexes were collected by centrifugation, washed six times with 1 ml lysis buffer, two times with 1 ml kinase buffer (25 mM HEPES, pH 7.4, 25 mM MgCl2, 0.5 mM Na3VO4, and 2 mM DTT), and then resuspended in 30 μl kinase buffer containing 25 μM ATP, 10 μCi [γ32P]ATP (6000 Ci/mmol; New England Nuclear, Boston, MA) and 5 μg recombinant heat shock protein (hsp) 25 substrate (StressGen Biotechnologies, Victoria, Canada). Kinase reactions were terminated after 30°C (30 min) by addition of 2× Laemmli sample buffer, separated by SDS-PAGE, and subjected to autoradiography.

p38 MAPK assay

CH31 B cells were sonicated for 5 s in 1 ml lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 20 mM Na3VO4, 1 mM NaF, 50 mM β-glycerophosphate, 1 μg/ml aprotinin and leupeptin, 1 mM PMSF, and 0.7 μg/ml pepstatin) and freeze-thawed in a dry-ice/water bath (35, 36). Cellular debris was removed by centrifugation, and the supernatant was incubated with 5 μg isotype-matched rabbit IgG plus protein A-Sepharose for 2 h (4°C). The immune complexes were recovered by centrifugation, and the supernatant was incubated with 5 μg anti-MAPKAP kinase-2 Ab (Upstate Biotechnology) plus protein A-Sepharose for 2 h. The MAPKAP kinase-2 immune complexes were collected by centrifugation, washed six times with 1 ml lysis buffer, two times with 1 ml kinase buffer (25 mM HEPES, pH 7.4, 25 mM MgCl2, 0.5 mM Na3VO4, and 2 mM DTT), and then resuspended in 30 μl kinase buffer containing 25 μM ATP, 10 μCi [γ32P]ATP (6000 Ci/mmol; New England Nuclear, Boston, MA) and 5 μg recombinant heat shock protein (hsp) 25 substrate (StressGen Biotechnologies, Victoria, Canada). Kinase reactions were terminated after 30°C (30 min) by addition of 2× Laemmli sample buffer, separated by SDS-PAGE, and subjected to autoradiography.
nuclear extracts corresponded to 5′-AGAGATTGCCTGACGTGAG AGCTAG-3′.

**DNA transfection and transient expression**

DNA transfection of B cell lymphomas was conducted as described by Sonenshein and coworkers (37); B cells (2 × 10⁷) were washed once in RPMI 1640 and resuspended in 1 ml of RPMI 1640 containing 20% FCS. Cells were then incubated on ice for 10 min and subsequently electroporated (240 V, 960 μF) in 250-μl aliquots containing 40 μg plasmid DNA using a Gene Pulser apparatus (Bio-Rad, Richmond, CA). B cells were then incubated sequentially on ice and at room temperature for 5 min, centrifuged, and resuspended at 2.5 × 10⁶ cells/ml in RPMI 1640 medium. B cells were stimulated with 15 μg/ml of anti-Ig for 8 h, washed in PBS, resuspended in 0.2 M Tris, pH 8.0, and subjected to four freeze-thaw cycles. The lysates were incubated at 68°C for 15 min, and insoluble material was removed by centrifugation at 15,000 × g for 15 min. CAT assays were performed using 250 μg of cellular protein as described by Chiles and Rodheinst (38). The plasmids were kindly provided by Dr. Michael Karin (University of California at San Diego, La Jolla, CA).

**Results**

**BCR engagement stimulates phosphorylation of endogenous CREB at Ser^{133} in CH31 B cells**

To monitor CREB phosphorylation in response to BCR cross-linking, whole-cell extracts were prepared from CH31 B cells and the level of CREB phosphorylation on serine 133 was measured following SDS-PAGE by immunoblotting with an anti-phospho(Ser^{133})CREB Ab that specifically recognizes CREB phosphorylated on serine 133 (39). CH31 B cells treated with anti-Ig exhibited an increased level of CREB phosphorylation (~4.3-fold) in comparison to control 

**Effects of kinase inhibitors on BCR-induced CREB serine 133 phosphorylation in CH31 B cells**

To identify protein kinase pathways that mediate BCR-induced CREB phosphorylation, we evaluated the effect of several kinase inhibitors on anti-Ig-induced CREB serine 133 phosphorylation, including SB203580 (an inhibitor of p38 MAPK), PD98059 (an inhibitor of MEK), and KN93 (an inhibitor of CaMK II) (40–42). Of note, the concentrations of inhibitors used in these experiments were removed by centrifugation at 15,000 × g for 15 min.

**Evidence that BCR-regulated protein kinases involved in CREB phosphorylation on serine 133 in CH31 B cells differ from mature B lymphocytes**

Previous reports have shown that BCR cross-linking in mature B lymphocytes induced rapid and transient phosphorylation of CREB on serine 133 (19, 20, 29). Additionally, anti-Ig stimulates p38 MAPK activity in B cells (42–46). Therefore, we sought to determine whether p38 MAPK activity was required for anti-Ig-induced CREB phosphorylation in splenic B lymphocytes and mature Bal17 B cells. Pretreatment of splenic B lymphocytes or Bal17 B cells with 20 μM SB203580 did not affect ERK1/2 and c-Jun NH₂-terminal kinase (JNK) activities, whereas anti-Ig-induced p38 MAPK activity was reduced to a level equal to unstimulated cells, suggesting that SB203580 was specific for the p38 MAPK (data not shown) (41).
that p38 MAPK mediates BCR-induced CREB phosphorylation in CH31 B cells, but is not inhibitory in splenic B lymphocytes or Bal17 B cells. A, Splenic B lymphocytes and Bal17 B cells were preincubated in the absence (−) or presence (+) of 20 μM SB203580 for 60 min and then cultured in medium alone (M) or stimulated with 10 μg/ml anti-Ig for 40 min. Whole-cell extracts were prepared and phospho(Ser133)CREB levels were measured by immunoblotting with anti-phospho(Ser133)CREB Ab as described in Materials and Methods. B, CH31 B cells and Bal17 B cells were preincubated in the presence or absence of 10 μM H-89 or 10 μM H-85. After 20 min, cells were cultured in medium (M) or in medium containing 10 μg/ml anti-Ig for 40 min. Whole-cell extracts were prepared and phospho(Ser133)CREB content was evaluated as described in A. pCREB denotes the position of phosphorylated CREB. Densitometric analysis of the pCREB bands is summarized below each lane.

The observation that SB203580 blocked BCR-induced CREB phosphorylation in CH31 B cells, but was not inhibitory in Bal17 B cells and splenic B lymphocytes stimulated with anti-Ig, suggests that the protein kinase pathway(s) required for BCR-induced CREB phosphorylation on serine 133 may differ in CH31 B cells in comparison to mature B cells. To test this further, we examined whether PKA activity was required for BCR-induced CREB phosphorylation in CH31 B cells. Our laboratory has identified a requirement for PKA in BCR-induced CREB phosphorylation in mature B cells (19). Anti-Ig-induced CREB serine 133 phosphorylation in CH31 B cells was not inhibited by preincubating cells with 10 μM H-89, a potent inhibitor of cAMP-dependent PKA activity (Fig. 2B, CH31 B cells) (47). In parallel experiments, anti-Ig-stimulated CREB serine 133 phosphorylation was not affected in CH31 B cells preincubated with 10 μM H-89, a control agent that is not selective in producing inhibition of PKA activity (Fig. 2B). By contrast, anti-Ig-stimulated CREB Ser133 phosphorylation in mature Bal17 B cells was effectively blocked by pretreatment with H-89, whereas H-85 was not inhibitory (Fig. 2B, Bal17 B cells), consistent with our previous reports in mature B lymphocytes (19, 30). The changes in phosphoCREB did not arise from differences in the total amount of cellular protein, based on immunoblotting with a polyclonal anti-actin Ab (data not shown). We interpret these data to mean that PKA activity is not required for BCR-induced CREB phosphorylation in CH31 B cell lymphomas.

**BCR cross-linking causes activation of p38 MAPK in CH31 B cells**

The experiments with pharmacological inhibitors demonstrated that p38 MAPK mediates BCR-induced CREB phosphorylation in CH31 B cells. Therefore, we examined further the regulation of p38 MAPK in response to BCR cross-linking. Western blot analysis of whole-cell extracts prepared from control and anti-Ig-treated CH31 B cells revealed the presence of constitutively expressed p38 MAPK (Fig. 3A, p38 Blot). Immunoblotting with a highly specific anti-phospho(Thr180/Tyr182) p38 MAPK Ab revealed that anti-Ig-stimulation increased phosphorylation of p38 CH31 B cells. Therefore, we examined further the regulation of p38 MAPK in response to BCR cross-linking. Western blot analysis of whole-cell extracts prepared from control and anti-Ig-treated CH31 B cells revealed the presence of constitutively expressed p38 MAPK (Fig. 3A, p38 Blot). Immunoblotting with a highly specific anti-phospho(Thr180/Tyr182) p38 MAPK Ab revealed that anti-Ig-stimulation increased phosphorylation of p38
MAPK on conserved Thr$^{180}$/Tyr$^{182}$ in the TGY activation motif (Fig. 3A, phospho-p38 Blot). Maximal phosphorylation of p38 MAPK was detected at the 40-min time point. Additional support for the activation of p38 MAPK in response to BCR cross-linking was obtained by measuring the phosphotransferase activity of p38 MAPK in immune complexes using a recombinant GST-ATF-2 fusion protein substrate (48). Control CH31 B cells exhibited a relatively small amount of GST-ATF-2 phosphorylation on threonine 71 (Fig. 3A, p38 Activity). Anti-Ig treatment of CH31 B cells led to an increased phosphorylation of GST-ATF-2 fusion protein substrate that was maximal at 40 min (14-fold above untreated B cells).

MAPKAP kinase-2 activity is increased in response to BCR cross-linking and mediates phosphorylation of the serine 133 phosphoacceptor site of CREB

To understand more fully how p38 MAPK regulates serine 133 site-specific phosphorylation of CREB, we evaluated the activity of MAPKAP kinase-2 in response to anti-Ig treatment of CH31 B cells. The decision to evaluate MAPKAP kinase-2 was prompted by recent reports demonstrating that MAPKAP kinase-2 is a substrate for p38 MAPK (36, 49). MAPKAP kinase-2 was immunoprecipitated from control and anti-Ig treated CH31 B cells, and the immune complexes were assayed for phosphorylation of recombinant hsp25 substrate. Untreated CH31 B cells exhibited detectable MAPKAP kinase-2 activity that was further increased following BCR ligation, with maximal levels observed at the 40-min time point (Fig. 3B). It is noteworthy that we consistently observed that the relative amount of phosphorylated hsp25 was somewhat lower at 60 and 90 min in comparison to control values. In parallel kinase assays, isotype matched rabbit IgG immune complexes recovered from cell lysates were devoid of hsp25 phosphotransferase activity (Fig. 3B).

The amino acid sequence defining the serine 133 phosphoacceptor site of CREB is homologous with the minimum consensus sequence (LXRXXSXX) required for efficient phosphorylation by MAPKAP kinase-2 (36, 39, 50). To test whether BCR-stimulated MAPKAP kinase-2 activity in CH31 B cells might be capable of phosphorylating the serine 133 phosphoacceptor site of CREB, MAPKAP kinase-2 was immunoprecipitated from whole-cell extracts and evaluated in vitro for phosphotransferase activity using the CREBtide substrate, a peptide corresponding to residues 127 to 136 of CREB and containing the conserved serine 133 phosphoacceptor site (30). MAPKAP kinase-2 immune complexes recovered from control CH31 B cells exhibited CREBtide phosphotransferase activity, consistent with the basal activity of MAPKAP kinase-2 (Fig. 3C). Phosphorylation of CREBtide substrate was increased in MAPKAP kinase-2 immune complexes from anti-Ig-stimulated CH31 B cells at 20 and 40 min, with CREBtide phosphorylation returning to control levels at the 90-min time point. Of note, MAPKAP kinase-2 immune complexes obtained from CH31 B cells pretreated with 20 μM SB203580 and then stimulated with anti-Ig for 40 min exhibited CREBtide phosphorylation equal to that of unstimulated CH31 B cells (data not shown).

**FIGURE 4.** BCR-induced junB mRNA levels are inhibited by SB203580. A, Nuclear extracts from control (M) and CH31 B cells stimulated with 10 μg/ml anti-Ig (Ig) for 40 min were examined by EMSA using a CRE probe as described in Material and Methods. Some nuclear extracts were preincubated with 0.5 μg/ml nonimmune rabbit IgG (NI), anti-CREB Ab (CREB), or anti-phospho(Ser$^{133}$)CREB Ab (pCREB) before EMSA. Lane P is migration of the CRE probe in absence of nuclear extract. Nuclear extracts from anti-Ig-stimulated CH31 B cells were examined for binding to the CRE probe in the absence (C) and presence of 50-fold (50X) and 10-fold (10X) molar excess of unlabeled CRE probe. Arrow indicates the position of the CRE nucleoprotein complexes. B, Poly(A$^+$) RNA was purified from CH31 or WEHI-231 B cells treated with 10 μg/ml anti-Ig for the indicated times. RNA was analyzed by Northern blotting for the expression of junB mRNAs as described in Materials and Methods. The blot was then stripped and reprobed for actin expression by a similar method. Poly(A$^+$) RNA was also purified from CH31, WEHI-231, or Bal17 B cells pretreated in the absence (−) or presence (+) of 20 μM SB203580 for 60 min and then stimulated with 10 μg/ml anti-Ig for 0 (−) or 40 (+) min. RNA was analyzed by Northern blotting for the expression of junB and actin mRNAs. Densitometric analysis of the bands is represented as relative optical units, standardized to each autoradiographic film.
with previous findings in mature B cells (19). The specificity of nuclear extract binding was confirmed insofar as DNA binding activity was competed by including in the binding assays excess unlabeled CRE probe (Fig. 4A). Moreover, CRE binding activity from both control and anti-Ig-treated CH31 B cells was inhibited by incubating nuclear extracts with 0.5 μg anti-CREB Ab (Fig. 4A, CREB), whereas parallel binding assays containing 0.5 μg of isotype-matched rabbit IgG were not inhibited (Fig. 4A, NI). Incubation of nuclear extracts isolated from control CH31 B cells with 0.5 μg anti-phospho(Ser133) CREB Ab resulted in inhibition of CRE binding activity (Fig. 4A, pCREB). Importantly, a relatively greater percentage of total CRE binding activity was inhibited by the anti-phospho(Ser133) CREB Ab in nuclear extracts prepared from anti-Ig-stimulated CH31 B cells.

Because CREB regulates junB gene expression in mature B cells, we sought to determine whether anti-Ig increased junB mRNA levels in CH31 B cells. Poly(A+) RNA was prepared from untreated or anti-Ig-treated CH31 B cells and probed for junB mRNA expression by Northern blot analysis. BCR cross-linking increased the steady-state level of junB mRNA (Fig. 4B). The increase in junB mRNA levels was not attributed to a greater amount of total poly(A+) RNA in the anti-Ig lanes, as confirmed by reprobing of the membrane with a radiolabeled actin cDNA probe (Fig. 4B). Pretreatment of the CH31 B cells with SB203580 significantly reduced the level of junB mRNA expression in response to BCR cross-linking (Fig. 4B). Collectively, these data suggest that p38 MAPK activity is required for BCR-induced junB mRNA levels. Cross-linking the BCR on surface IgM+ WEHI-231 B cell lymphomas also led to increased junB mRNA levels (Fig. 4B). Pretreatment of WEHI-231 B cells with SB203580 led to ~50% reduction in anti-Ig-stimulated junB mRNA levels, suggesting that active p38 MAPK is required, at least in part, for junB mRNA expression by the BCR (Fig. 4B). BCR cross-linking in WEHI-231 B cells resulted in the activation of p38 MAPK (42, 44) and phosphorylation of CREB on serine 133 (data not shown). In comparison to CH31 and WEHI-231 B cells, anti-Ig-stimulated junB mRNA expression in Bal17 B cells was not inhibited by SB203580 (Fig. 4B). Note, we have previously characterized the regulation of junB mRNA expression by the BCR (13). These results are consistent with the notion that BCR-induced junB mRNA expression in Bal17 B cells is not dependent upon active p38 MAPK.

**BCR cross-linking on CH31 and WEHI-231 B cells activates transcription of CRE-containing junB promoter constructs in a SB203580-sensitive manner**

In an earlier report, we demonstrated that a CAT reporter gene plasmid containing 194 bp of 5′-flanking junB gene sequences (plasmid denoted JB194CAT5) was stimulated following BCR cross-linking in mature B cells (19). Reporter gene activity from the junB promoter is dependent upon a CRE site located between −135 and −128 bp (19). Therefore, this plasmid affords evaluation of the BCR-coupled signaling pathways leading to CREB-mediated transcriptional activation. To determine whether p38 MAPK activity was required for BCR-induced transcriptional activation of the CRE-dependent junB promoter/CAT reporter construct, CH31 B cells were transiently transfected with the JB194CAT5 plasmid, followed by anti-Ig stimulation in the presence and absence of SB203580. BCR cross-linking increased CRE-dependent junB/CAT reporter gene expression (Fig. 5A). Pretreatment of CH31 B cells with SB203580 markedly inhibited anti-Ig-stimulated junB promoter/CAT reporter gene activity under the control of the CRE. Anti-Ig-stimulated junB promoter activation in transiently transfected WEHI-231 B cells was also reduced by pretreatment with SB203580 (Fig. 5A). These findings demonstrate that p38 MAPK activity is required for BCR-induced junB promoter activation in CH31 and WEHI-231 B cell lymphomas.

Based on these findings, we sought to determine whether activation of JB194CAT5 in a transfectable mature B cell line was...
dependent upon active p38 MAPK. The results in Fig. 5A demonstrate that anti-Ig-stimulated junB promoter/CAT reporter gene activity in transiently transfected Bal17 B cells. Pretreatment of a parallel population of Bal17 B cells with SB203580 did not inhibit anti-Ig-stimulated junB promoter/CAT activity. Taken together, these results suggest that p38 MAPK activity is not required for BCR-induced junB promoter activity in Bal17 B cells.

As a control for the specificity of SB203580 in blocking CRE-dependent transcription, experiments were conducted using chimeric plasmids containing three copies of a TRE sequence coupled to a HSV-tk promoter/CAT fusion gene (38). The TRE sequence binds members of the AP-1 family and in B cells has been shown to bind JunB/Fos and c-Jun/Fos heterodimers (13). AP-1 is activated by JNK family members (40, 48). CH31 B cells were transiently transfected with the 3XTRE/CAT plasmid, followed by phorbol diester plus ionomycin treatment to activate JNK. The results in Fig. 5B demonstrate that phorbol diester plus ionomycin increased TRE-dependent CAT reporter gene activity by ~15-fold above control B cells. In parallel CH31 B cells, pretreatment with SB203580 did not block phorbol diester/ionomycin-induced CAT reporter activity.

**Discussion**

We have shown in this study that ligation of the BCR on CH31 B cell lymphomas leads to increased p38 MAPK activity. The significance of this BCR-regulated activity is underscored by the requirement for active p38 MAPK in anti-Ig-induced CREB trans-activation via site-specific phosphorylation on serine 133, junB mRNA expression, and junB promoter activation. Support is also provided for a role of p38 MAPK in anti-Ig-induced junB mRNA expression and junB promoter activation in WEHI-231 B cells. This is the first report demonstrating a role for p38 MAPK in regulating CREB activity and CRE-dependent gene transcription in response to Ag receptor cross-linking.

We do not mean to imply that p38 MAPK is the only functional intracellular protein kinase that regulates CREB activity. In fact, several experiments support the participation of p38 MAPK in anti-Ig-induced CREB trans-activation of CREB downstream target of the p38 MAPK pathway in CH31 B cell lymphomas. This conclusion is based in part on previous findings in which CREB serine 133 phosphorylation in mature B lymphocytes was increased 10- to 15-fold as a result of SB203580 treatment. Moreover, we have shown that anti-Ig-stimulated CREB phosphorylation on serine 133 in a MAPKAP kinase-2-dependent manner. Our findings indicate that CREB is a downstream target of the p38 MAPK pathway in CH31 B cell lymphomas. This is supported by the observation that the p38 MAPK inhibitor, SB203580, blocked BCR-stimulated CREB phosphorylation on serine 133. Our experiments also implicate MAPKAP kinase-2, as directly contributing to BCR-induced CREB serine 133 phosphorylation. We base this latter conclusion on the finding that MAPKAP kinase-2 immune complexes from anti-Ig-treated CH31 B cells contributed to the phosphorylation of CREB on serine 133.

Several experiments support the participation of p38 MAPK in BCR signaling. Foremost, anti-Ig led to a transient increase in the phosphorylation of p38 MAPK on Thr180/Tyr182 of the conserved TGY activation motif, an event necessary for activation of its kinase/threonine kinase (40). BCR cross-linking also resulted in increased phosphorylation of recombinant GST-ATF-2 fusion protein substrate as directed by p38 MAPK immune complex. Consistent with these observations, we found that anti-Ig stimulated a small and transient increase in MAPKAP kinase-2 activity, a substrate that lies immediately downstream from p38 MAPK (36, 52).

The activation of p38 MAPK by the BCR in CH31 B cells agrees with recent studies in the immature WEHI-231 B cell lymphoma that showed a transient increase in p38 MAPK activity in response to anti-Ig (42, 44). Interestingly, anti-Ig also promotes activation of ERK2 and to a much lesser extent JNK in WEHI-231 B cells (44, 53, 54). A recent report by Parkerson and Parker (55) demonstrated that BCR and CD40 signals converge at MEK-1 to activate ERK. Though CH31 and WEHI-231 B cell lines are considered models for B cell tolerance (1), we were unable to detect substantial changes in the activities of JNK and ERK following BCR cross-linking in the CH31 B cell lymphoma (data not shown).

The mammalian p38 MAPK, an enzyme related to the HOG1 kinase from Saccharomyces cerevisiae, is activated by stress-inducing agents such as changes in osmotic strength, UV irradiation, and proinflammatory cytokines (40, 48, 56–58). p38 MAPK is also activated by CD40 and Fas in B and T cells (43, 59). The p38 MAPK family includes SB203580-inhibitable p38α and p38β isoforms and p38γ and p38δ isoforms that are activated by dual phosphorylation on TGY in kinase subdomain VIII by MAPK kinase (MKK) 3 and MKK6 (40, 41). Although the coupling of upstream MKKs, such as MKK3/6, to surface receptors remain to be completely defined, it is noteworthy that Clark and coworkers (60) recently showed, using Syk−/Lyn− double deficient DT40 B cells, that p38 MAPK was not efficiently activated following BCR ligation. By contrast, anti-Ig induced p38 MAPK activation in the corresponding Syk− or Lyn− deficient DT40 B cells. In a related study, Hashimoto et al. (61) demonstrated a requirement for Rac1 and phospholipase C γ2 in BCR-induced p38 MAPK responses in DT40 B cells.

The cellular substrates and gene targets of p38 MAPK are likewise incompletely defined. It is recognized that a conserved serine/proline motif located in several transcription factors (e.g., ATF-2, CHOP/GADD153, and Elk-1) is phosphorylated by p38 MAPK signaling pathways (40). p38 MAPK activity is required for the transcriptional activation of c-fos in response to UV irradiation, TNF-α-induced cytokine production, and IL-1β by LPS in monocytes (57, 62, 63). In the context of B lymphocytes, Craxton et al. (42) demonstrated that CD40-mediated NF-κB trans-activation is a target of p38 MAPK. Our findings indicate that CREB is a downstream target of the p38 MAPK pathway in CH31 B cell lymphomas. This is supported by the observation that the p38 MAPK inhibitor, SB203580, blocked BCR-stimulated CREB phosphorylation on serine 133. Our experiments also implicate MAPKAP kinase-2, as directly contributing to BCR-induced CREB serine 133 phosphorylation. We base this latter conclusion on the finding that MAPKAP kinase-2 immune complexes from anti-Ig-treated CH31 B cells exhibited increased phosphorylation of a peptide substrate containing the kinase-inducible domain serine 133 phosphoacceptor site of CREB. Our findings agree with an earlier report by Tan et al. (39) in human neuroblastoma SK-N-MC cells that fibroblast growth factor increased CREB phosphorylation on serine 133 in a MAPKAP kinase-2-dependent manner.

An important finding of this study concerns the diversity of intracellular protein kinases that regulate CREB trans-activation in B lymphocytes. Though we recognize the limited scope of these experiments, strong support is provided for the existence of distinct protein kinase pathways that mediate BCR-induced CREB phosphorylation on serine 133 in CH31 B cell lymphomas and mature B lymphocytes. This conclusion is based in part on previous findings in which CREB serine 133 phosphorylation in mature B cells stimulated with anti-Ig was blocked by PKA inhibitors.
(30). By contrast, experiments herein demonstrate that pretreatment of CH31 B cells with the PKA inhibitor, H-89, did not significantly reduce CREB phosphorylation on serine 133. Additionally, pretreatment of CH31 B cells with SB203580 prevented BCR-induced serine 133 phosphorylation of CREB, but did not affect the level of CREB phosphorylation stimulated by anti-Ig in the phenotypically mature BAL17 and splenic B lymphocytes. We interpret these findings to mean that p38 MAPK represents a critical pathway for the regulation of CREB trans-activation in CH31 B cells, whereas a PKA-dependent (SB203580 insensitive) pathway is required in mature B lymphocytes.

Perhaps most importantly, our data suggests that the p38 MAPK module serves to focus Ag-receptor signals to genes whose promoters contain CRE elements. EMSA experiments with anti-CREB and phosphoSer133 anti-CREBAbs demonstrated that phosphorylated CREB assembles into CRE-containing nucleoprotein complexes. The significance of this finding to CREB-regulated gene expression was assessed by Northern blot analysis of junB mRNA levels. We found that anti-Ig increased endogenous junB mRNA levels in both CH31 and WEHI-231 B cells. Importantly, the increase in junB mRNA levels was reduced by pretreatment of cells with SB203580, suggesting that p38 MAPK plays a role in regulating junB gene expression in response to BCR ligation. The regulation of junB promoter activity by p38 MAPK was assessed using junB promoter-CAT reporter fusion gene constructs in transient transfection assays. The decision to examine the junB gene promoter is based on our previous observation that junB transcriptional activation in response to BCR ligation is dependent on a CRE located 5’ to the transcriptional start site of the junB gene (19, 30). CREB binds the junB gene promoter CRE-like element, and serine 133 phosphorylation is necessary for BCR-stimulated junB promoter activation (19, 30). We found that anti-Ig-stimulated junB promoter/CAT reporter gene activity was significantly reduced by pretreating CH31 B cells with SB203580. In addition, transiently transfected WEHI-231 B cells exhibited anti-Ig-stimulated junB promoter/CAT reporter activity, which was sensitive to SB203580. By contrast, p38 MAPK activity does not appear to be required for anti-Ig-stimulated junB promoter activation in BAL17 B cells. These data point to a role for p38 MAPK in mediating BCR-induced junB promoter activation in both CH31 and WEHI-231 B cell lymphomas. Collectively, our data suggest a pathway for BCR-induced gene regulation that includes p38 MAPK→MAPKAP kinase-2→CREB→junB gene transcription.

An important question raised by these experiments concerns whether the requirement of p38 MAPK activity in BCR-regulated CREB activity is restricted to Ag-inhibited B cell lymphomas or reflects a developmental difference in BCR signaling between normal mature and immature B lymphocytes. Studies with primary immature B lymphocytes may provide insight into this important question. In addition, this study has not addressed the physiologic significance of p38 MAPK in Ag-induced apoptosis; however, p38 MAPK activity is required for BCR-induced apoptosis in the human B104 B cell line (46). Clark and coworkers (60) recently noted that in the DT40 cell line high doses of SB203580 were required to block BCR-mediated apoptosis. In data not shown, pretreatment of CH31 B cells with 20 μM SB203580 did not provide protection against BCR-induced apoptosis. We were unable to test higher concentrations of the inhibitor due to decreased cell viability in parallel CH31 B cell cultures pretreated with Me2SO solvent control. Thus, it is not known at present whether p38 MAPK may contribute to the induction of apoptosis in CH31 B cells following BCR cross-linking. Additional studies with dominant negative forms of p38 MAPK may provide insight into this important issue. Nevertheless, our findings are consistent with that of WEHI-231 B cells in which SB203580 failed to block anti-Ig-induced apoptosis (43).

In summary, experiments herein establish a role of the p38 MAPK module as a intracellular pathway of BCR-induced CREB serine 133 phosphorylation and CRE-mediated transcriptional activation in CH31 B cell lymphomas.

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