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Differential Roles for Extracellularly Regulated Kinase-Mitogen-Activated Protein Kinase in B Cell Antigen Receptor-Induced Apoptosis and CD40-Mediated Rescue of WEHI-231 Immature B Cells

Stephen B. Gauld, Derek Blair, Catriona A. Moss, Steven D. Reid, and Margaret M. Harnett

One of the major unresolved questions in B cell biology is how the B cell Ag receptor (BCR) differentially signals to transduce anergy, apoptosis, proliferation, or differentiation during B cell maturation. We now report that extracellularly regulated kinase-mitogen-activated protein kinase (Erk-MAP kinase) can play dual roles in the regulation of the cell fate of the immature B cell lymphoma, WEHI-231, depending on the kinetics and context of Erk-MAP kinase activation. First, we show that the BCR couples to an early (<2 h) Erk-MAP kinase signal which activates a phospholipase A2 pathway that we have previously shown to mediate collapse of mitochondrial membrane potential, resulting in depletion of cellular ATP and cathepsin B execution of apoptosis. Rescue of BCR-driven apoptosis by CD40 signaling desensitizes such early extracellularly regulated kinase (Erk) signaling and hence uncouples the BCR from the apoptotic mitochondrial phospholipase A2 pathway. A second role for Erk-MAP kinase in promoting the growth and proliferation of WEHI-231 immature B cells is evidenced by data showing that proliferating and CD40-stimulated WEHI-231 B cells exhibit a sustained cycling pattern (8–48 h) of Erk activation that correlates with cell growth and proliferation. This growth-promoting role for Erk signaling is supported by three key pieces of evidence: 1) signaling via the BCR, under conditions that induce growth arrest, completely abrogates sustained Erk activation; 2) CD40-mediated rescue from growth arrest correlates with restoration of cycling Erk activation; and 3) sustained inhibition of Erk prevents CD40-mediated rescue of BCR-driven growth arrest of WEHI-231 immature B cells. Erk-MAP kinase can therefore induce diverse biological responses in WEHI-231 cells depending on the context and kinetics of activation. The Journal of Immunology, 2002, 168: 3855–3864.

During the generation of a functional B lymphocyte repertoire, it is necessary to regulate the maturation of immature B cells to prevent the emergence of cells that bind self-Ag and which are therefore potentially autoreactive. Thus, in contrast to mature B lymphocytes which generally undergo a process of activation after Ag encounter, immature B lymphocytes undergo a process of negative selection that may involve deletion, anergy, or replacement of the self-reactive receptor with a non-self-reactive B cell Ag receptor (BCR). However, additional factors such as the microenvironment of the cell or costimulation by IL-4 or CD40 are also believed to play important roles in determining cell fate, the latter suggesting that these cells are responsive to some form of T cell-mediated rescue.

The murine B cell lymphoma cell line WEHI-231 is widely used as a model for immature B lymphocyte clonal deletion not least because it has a cell surface phenotype of an immature B lymphocyte (sIgM/H9001, sIgD/vlow, FcRlow, Faslow, and MHC class IIlow). Moreover, WEHI-231 B cells undergo growth arrest and apoptosis after BCR ligation (2–4) and can be rescued from BCR-mediated apoptosis by costimulation via CD40. The signaling mechanisms underlying such apoptosis and rescue remain to be precisely defined, but we have recently shown that the BCR couples to up-regulation of cytosolic phospholipase A2 (cPLA2) expression, induction of mitochondrial phospholipase A2 activity, arachidonic acid-mediated collapse of Δψm, and depletion of cellular ATP under conditions of apoptotic, but not proliferative, signaling via the BCR (5). Importantly, disruption of Δψm, ATP depletion, and apoptosis can be prevented by rescue signals via CD40 (5). In addition, it is clear that CD40-mediated induction of Bcl-xL plays a key role in protecting WEHI-231 cells from BCR-driven apoptosis (6–9). However, the key upstream regulators of these signaling events have not been defined given that many of the early signaling events following ligation of the BCR that result in either proliferation or apoptosis of B cells are similar. For example, although Erk-MAP kinase is generally considered to be a mitogenic signal, and the stress-activated kinases, Jun N-terminal kinase (Jnk) and p38 MAP kinase, have often been implicated in apoptotic signaling, all of these MAP kinases have been reported to be activated following BCR ligation in immature and mature B cells (10–13). Similarly, all of these MAP kinases have also been shown to be activated in B lymphocytes following CD40 ligation (11, 12, 14). Indeed, whereas BCR-mediated Erk signaling has been proposed to be associated with apoptotic signaling in WEHI-231 B cells (15), CD40 has been reported to preferentially activate Jnk and p38

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MAP kinase cascades (11). However, recent papers by Berberich et al. (14) and Sutherland et al. (11) have suggested that BCR and CD40 ligation may lead to different patterns of the type or kinetics of MAP kinase family activation depending on the maturation state of the cell suggesting that the overall balance of MAP kinase activation could determine B lymphocyte fate.

We now report that Erk-MAP kinase plays differential roles in BCR-induced apoptosis and CD40-mediated rescue of WEHI-231 immature B cells. For example, we find that the BCR couples to an early (∼2 h) Erk-MAP kinase signal which activates the mitochondrial phospholipase A2 pathway which results in apoptosis in WEHI-231 immature B cells. In contrast, CD40 signaling only marginally activates Erk at these early time points, and indeed costimulation with anti-Ig and anti-CD40 desensitizes this early Erk signaling and uncouples the apoptotic mitochondrial phospholipase A2 pathway. However, normal proliferating and CD40-stimulated WEHI-231 B cells exhibit a sustained cycling pattern of Erk activation over 48 h that correlates with cell cycle progression, cell growth, and proliferation. This proposal of an additional role for Erk in WEHI-231 B cell growth is supported by the findings that apoptotic signaling via the BCR completely abrogates such sustained Erk activation and that CD40-mediated rescue from apoptosis and growth arrest correlates with restoration of this late cycling pattern of Erk activation.

**Materials and Methods**

**Cells, reagents, and Abs**

The murine B cell lymphoma, WEHI-231 was cultured in RPMI 1640 containing 5% FCS, 1-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) (RPMM complete) at 37°C in 5% CO2. RPMI complete for WEHI-231 B cells was additionally supplemented with 2-ME (50 μM). All cell culture reagents were obtained from Life Technologies (Paisley, U.K.).

PD98059 and propidium iodide (PI) were obtained from Calbiochem (La Jolla, CA). U0126 was obtained from Promega (Southampton, U.K.). SB230580 was obtained from Alexis Biochemicals. [6-3H]Thymidine (5 Ci/mmol) was obtained from Amersham International (Aylesbury, U.K.). All other reagents were obtained from Sigma (Poole, U.K.).

Phospho-specific p44/p42 MAP kinase (Erk-MAP kinase/Erk), p44/42 MAP kinase (Erk), phospho-specific SAPK/Jnk (pJnk), SAPK/Jnk (Jnk), phospho-specific stress-activated protein kinase (pSEK), SEK, phospho-specific c-Jun (pJun), c-Jun (Jun), phospho-specific p38 MAP kinase (p38), p38 MAP kinase (p38), and anti-rabbit IgG-HRP Abs were obtained from New England Biolabs (Hitchin, U.K.). Purified monoclonal anti-IgM Abs (anti-mouse μ-chain) and anti-CD40 Abs were prepared from hybridomas (Becton-Dickinson), respectively, and tested for Lyt-2,3 negative B cells. The B7.6 and FGK45 hybridomas, respectively, were used as positive controls.

**DNA synthesis**

For measurement of DNA synthesis, WEHI-231 cells (10^4 cells/well) were cultured in triplicate in round-bottom microtiter plates in RPMI 1640 supplemented with glutamine (2 mM), sodium pyruvate (1 mM), 1% nonessential amino acids, 2-ME (50 μM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% FCS, in the presence of the appropriate agonist in a total volume of 200 μl. Cells were cultured at 37°C in a 5% (v/v) CO2 atmosphere at 95% humidity for 48 h. 3HThymidine (0.5 μCi/well) was added 4 h before harvesting of the cells with a automated cell harvester (Molecular Devices, Sunnyvale, CA). Incorporation label was estimated by liquid scintillation counting and is represented as dpm ± SEM.

**Cell stimulation and whole cell lystate preparation**

WEHI-231 cells (10^7 cells) were stimulated as required in RPMI 1640 supplemented with glutamine (2 mM), sodium pyruvate (1 mM), 1% nonessential amino acids, 2-ME (50 μM), penicillin (100 U/ml), streptomycin (100 mg/ml), and 5% FCS. In all experiments, except in the indicated panels of Fig. 4, stimulations described as medium contained 5% FCS. In Fig. 4, cells denoted as serum free were washed twice with RPMI 1640 and then cultured overnight at 37°C in serum-free medium (RPMM 1640 supplemented with 0.5 mg/ml BSA, 50 μg 2-ME, 1 μg pyruvate, and 2 mM glutamine) before being stimulated (17). Reactions were terminated by the addition of 2x ice-cold modified radiomunnoincipitation assay lysis buffer (50 mM Tris (pH 7.4), 150 mM sodium chloride, 2% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 1 mM EGTA) plus 10 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, chymostatin (10 μg/ml), leupeptin (10 μg/ml), antipain (10 μg/ml), and pepstatin A (10 μg/ml), and lysates were solubilized for 30 min on ice before centrifugation at 12,000 rpm for 15 min. The resulting supernatants (whole cell lysate) were stored at −20°C before being used for immunoprecipitation or Western blot analysis.

**Western blot analysis**

Equal protein loadings of lysates (determined by BSA protein assay (Pierce, Rockford, IL)) were resolved on 10% SDS-PAGE, followed by transference onto a polyvinylidine difluoride membrane and phospho-specific anti-Erk1 (pErk1) (both 1/5000), or with p38 MAPK, JNK, SEK, and, and c-Jun (1/2000) Abs for 2 h at 25°C or overnight at 4°C. Membranes were incubated with rabbit anti-pErk or anti-Erk (both 1/2000) or with anti-p38 MAPK, JNK, SEK, and Jnk (1/2000) Abs for 2 h at 25°C or overnight at 4°C followed by 1 h incubation at 25°C with a donkey anti-rabbit IgG-HRP Ab (1/5000 dilution in PBS, 0.1% Tween 20). Protein bands were visualized by incubation with the ECL system (Amersham International). Relative band densities were determined by the use of the Gel-Pro analysis program. The anti-phospho-Erk Abs used recognized p42 and p44 MAP kinase (Erk2 and Erk1, respectively) only when catalytically activated by phosphorylation at the T/EY motif corresponding to T202/Y204 on human Erk1. Likewise, the anti-Erk-3 Abs recognized both Erk2 and Erk1 as indicated in Fig. 2. However, in WEHI-231 B cells, phospho-Erk and Erk1 activity was generally barely detectable; thus, for consistency, all other figures are annotated simply as phospho-Erk or Erk. Similarly, although the anti-Jnk Abs can recognize both p46 and p54 Jnk, only phospho-p54Jnk was detectable; hence, these blots are labeled as Jnk or pJnk.

**In vitro ERK-MAP kinase kinase (MEK) assay**

Whole cell lysates were prepared from WEHI-231 cells (1 × 10^7 cells) stimulated with medium, anti-Ig (10 μg/ml), anti-CD40 (10 μg/ml), or anti-Ig and anti-CD40 (both 10 μg/ml) for the indicated time. MEK1/2-containing immune complexes were prepared from lysates (100 μg) with an anti-MEK1/2 Ab (New England Biolabs) and protein G-Sepharose beads. The MEK1/2 immune complexes were assayed for MEK activity using the MEK1 assay kit (TCS Biologicals, Botloph Claydon, U.K.), and 0.5 U human activated MEK1 was used as the positive control sample. Briefly, the immune complex samples, or human activated MEK1, were incubated with assay buffer (20 mM MOPS (pH 7.2), 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT), Mg^2+ATP mixture (75 mM magnesium chloride and 500 μM ATP in assay buffer ADB) and 1 μg of inactive GST-p42 MAP kinase at 30°C for 30 min. Samples were then analyzed by SDS-PAGE using 10% SDS gels. Western blotting was performed using anti-phospho-Erk1/2 (New England Biolabs) to detect phosphorylated GST-p42 MAP kinase (62 kDa). The amount of phospho-ERK-MAP kinase (MEK) kinase activity of MEK1/2 in the sample. Blots were stripped and reprobed with anti-MEK1/2 (New England Biolabs) to check the loading of the gel.

**Cell cycle arrest and analysis by laser scanning cytometry (LSC)**

Cells were pretreated for 24 or 40 h with aphidicolin (5 μg/ml) or oloclimycin (50 μM). The cells were then washed twice in medium before being analyzed by FACs or LSC as indicated in Fig. 10. Alternatively, the cells were then stimulated (resuspended at 2 × 10^6/ml for Western blotting or at 2 × 10^5/ml for LSC) with anti-Ig (10 μg/ml), anti-Ig (10 μg/ml) plus anti-CD40 (10 μg/ml), or medium alone for up to 48 h as indicated before processing for Western blot (see above) or LSC analysis. For LSC, the cells were attached to microscope slides by cytocentrifugation at 600 rpm for 4 min in a Shandon Cytospin centrifuge (Shandon, Pittsburgh, U.K.), then fixed in 4% formaldehyde in PBS for 10 min at room temperature, washed with PBS, and permeabilized with 2% FCS, 2 mM EDTA (pH 8.0), 0.01% w/v saponin for 5 min at room temperature. The slides were then washed three times with PBS and incubated with a blocking solution containing 10% goat serum, 1% BSA, and 0.02% sodium azide in PBS for 10 min. Subsequently, a 50-μl aliquot of a 1% BSA-PBS solution containing a 1/250 dilution of anti-phospho p44/42 MAP kinase Ab (Cell Signaling Technology, Beverly, MA) was placed on top of the site with the attached cells on the microscope slide and incubated for 30 min. The sites were then washed three times with 1% BSA-PBS and incubated with a 50-μl aliquot of a 1% BSA-PBS solution containing a 1/100 dilution of anti-rabbit FITC-conjugated secondary Ab in 5 μg/ml PI containing RNase A (200 μg/ml) for 30 min. Cells were washed a further three times in 1% BSA-PBS and
then allowed to dry in the dark before being mounted in Vectashield (Vector Laboratories, Burlingame, CA) without 4',6-diamidino-2-phenylindole and left in the dark at 4°C until analysis on the LSC using CompuCyte Software (CompuCyte, Cambridge, MA).

DNA content analysis by FACS analysis

Cells (5 × 10^5) were harvested at required time intervals. Cells were washed twice, resuspended in 100 µl PI stain (0.1% w/v sodium (tricitrate, 0.1% v/v Triton X-100, 50 µg/ml PI) and incubated at 4°C for 10 min and then at room temperature for at least 30 min. Cells were analyzed on a FACSCalibur (BD Biosciences, Mountain View, CA) using CellQuest software (BD Biosciences).

cPLA₂ assays

cPLA₂ activity was assessed by measurement of [³H]arachidonic acid release as described previously (18). This activity was blocked by the inhibitor arachidon trifluoromethyl ketone (selective for iPLA² and cPLA²) excluding a role for sPLA₂. Briefly, cells (10^⁶/ml) were prelabeled overnight with RPMI, 10% FCS medium containing 1 µCi/ml [³H]arachidonate. Before each experiment, cells were washed, resuspended in fresh isofrume-free medium, and cultured for a further hour at 37°C. The cells were then washed three times in HBSS, pH 7.4, containing 2% (w/v) BSA and 10 mM glucose, resuspended in this buffer at 10⁷ cells/ml, and equilibrated for 30 min at 37°C. Cells (10⁷/assay) were then stimulated with the appropriate agent for the indicated time at 37°C. Reactions were terminated by the addition of 1 ml ice-cold methanol and 15 µl glacial acetic acid followed by a further 0.5 ml methanol and 0.75 ml chloroform, and the cells were extracted for 30 min on ice. Phases were split by the addition of chloroform and water, and the chloroform phase was then dried under radioactivity.

Results

BCR activation of an early Erk-MAP kinase signal is associated with commitment to apoptosis of WEHI-231 B cells

The B cell lymphoma WEHI-231 is a widely used model system for investigating the signaling mechanisms underlying clonal selection of normal IgM⁺ IgD⁻ immature B cells. We and others have reported that ligation of slgM on the WEHI-231 immature B cell line induces growth arrest (suppression of DNA synthesis and cell cycle arrest) which is maximal at concentrations of anti-Ig between 0.1 and 1 µg/ml. Moreover, anti-Ig at concentrations ≥1 µg/ml induces apoptosis as indicated by disruption of mitochondrial potential, annexin V staining, DNA laddering, and subdiploid DNA content analysis (3, 5, 19). In contrast, treatment with anti-CD40 alone either weakly promotes or has little effect on DNA synthesis in WEHI-231 cells (5). Moreover, anti-CD40, at concentrations ≥1.0 µg/ml, is effective in completely rescuing slg-stimulated WEHI-231 cells from growth arrest (Fig. 1A and results not shown) and apoptosis (Fig. 1, B-D). This rescue from apoptosis is reflected by abrogation of BCR-mediated disruption of mitochondrial potential, annexin V staining, and DNA fragmentation (5).

It is widely established that the BCR couples to Erk-MAP kinase in B cells. To address whether Erk-MAP kinase is differentially activated under conditions of growth arrest, apoptosis, or rescue in WEHI-231 B cells, Western blot analysis of the dually phosphorylated, activated Erk was conducted on whole cell lysates derived from WEHI-231 B cells treated with increasing concentrations of anti-Ig. Treatment of WEHI-231 cells with concentrations of anti-Ig shown to promote apoptosis were capable of strong Erk2 activation and barely detectable Erk1 phosphorylation (Fig. 2A). Analysis of the kinetics of such Erk activation showed that anti-Ig (10 µg/ml) treatment resulted in the phosphorylation of Erk (pErk) as early as 1 min, with maximal Erk phosphorylation at 30 min (Fig. 2B). In contrast, anti-CD40 (10 µg/ml) was shown to induce little or no Erk phosphorylation over similar time ranges (Figs. 2C and 3A), findings consistent with previous studies (11, 12). Moreover, costimulation of WEHI-231 B cells with anti-Ig

FIGURE 1. BCR-mediated growth arrest and apoptosis of WEHI-231 cells is rescued by CD40 signaling. A, WEHI-231 cells (10⁵/well) were cultured in the presence of medium, various concentrations of anti-Ig, or anti-Ig (10 µg/ml) plus anti-CD40 (alg + aCD40; 10 µg/ml). Growth was assessed by measuring [³H]thymidine uptake at 48 h. Values are the means ± SD of triplicate wells. B–D, WEHI-231 B cells (5 × 10⁵/ml) were cultured with medium or anti-Ig (10 µg/ml) in the presence or absence of anti-CD40 (10 µg/ml). Levels of apoptosis were determined by PI staining and FACS analysis after 48 h. FL2-H, Fluorescence.

FIGURE 2. BCR-mediated growth arrest and apoptosis of WEHI-231 cells is associated with Erk-MAP kinase activation. A, WEHI-231 cells (1 × 10⁶/ml) were stimulated with 0–25 µg/ml anti-Ig for 30 min as indicated. B, WEHI-231 cells (1 × 10⁶/ml) were stimulated with medium (lanes 1 and 7) or anti-Ig (10 µg/ml; lanes 2–6) for 0–60 min as indicated. C, WEHI-231 cells (1 × 10⁶/ml) were stimulated with medium (lanes 1 and 7) or anti-Ig plus anti-CD40 (both at 10 µg/ml; lanes 2–6) for 0–60 min as indicated. Levels of pErk/Erk expression were determined by Western blotting (15 µg/lane). Densitometry indicates relative band density of phospho-p42 Erk-MAP kinase for anti-Ig-treated cells as: lane 1, 1; lane 2, 3; lane 3, 13; lane 4, 22; lane 5, 29; lane 6, 16, lane 7, 0.3. Densitometry indicates relative band density of phospho-p42 Erk-MAP kinase for anti-Ig plus anti-CD40-treated cells as: lane 1, 1; lane 2, 18; lane 3, 28; lane 4, 10; lane 5, 7; lane 6, 8, lane 7, 0.5.
BCR-signaling can induce Jnk and to a lesser extent p38 MAP kinase activation, particularly in cells cultured in serum-free medium. A and B, WEHI-231 cells (1 × 10⁶/ml) were stimulated with anti-Ig (10 μg/ml) for 0–48 h as indicated (lanes 1–7) before determining levels of pJnk/Jnk (A) or pp38/p38 (B) expression by Western blotting (25 μg/lane). WEHI-231 cells cultured in the presence (C and E) and absence (D and F) of 5% FCS were stimulated with medium (lanes 1 and 7) or anti-Ig (10 μg/ml; lanes 2–6) for 0–60 min as indicated (lanes 1–7) before determining levels of pJnk/Jnk (C and D) or pp38/p38 (E and F) expression by Western blotting (25 μg/lane).

In contrast, neither anti-Ig nor anti-CD40, either alone or in combination, stimulated p38 MAP kinase activity above basal levels over a 30-min time period (Fig. 3B). Likewise, little or no change in Jnk activity in terms of dually phosphorylated Jnk expression could be detected (Fig. 3C). Moreover, little or no activation (phosphorylation) of the upstream regulator of Jnk, SEK1, or its downstream effector, c-Jun, could be detected in response to either anti-Ig or anti-CD40 signals (Fig. 3C). However, anti-Ig was found to stimulate a weak, delayed transient activation of p54 Jnk, but not p38 MAP kinase, which was apparent between 1 and 2 h poststimulation before returning to basal levels (Fig. 4, A and B). Although at first sight these results may seem rather contradictory to previous studies reporting strong activation of Jnk and p38 MAP kinase via CD40 or the BCR in WEHI-231 B cells, these earlier studies were performed under serum-free conditions which may have primed BCR activation of the stress-activated kinases (11, 12). Indeed, we have analyzed Jnk and p38 activity under serum-free and serum-supplemented conditions, and these data show that the BCR is coupled to Jnk activity (maximal at 60 min) in serum-free but not serum-supplemented medium. Similarly, these data suggest that the BCR is also weakly coupled to p38 activity under serum-free conditions (Fig. 4).

Inhibition of slg-stimulated Erk-MAP kinase abrogates BCR-mediated apoptosis of WEHI-231 B cells

Taken together, the above results could suggest that the BCR coupling to early (within 1 h) Erk-MAP kinase signaling transduces apoptosis of WEHI-231 cells and that costimulation with CD40 may rescue such B cells from growth arrest and apoptosis, at least in part, by uncoupling the BCR from this Erk-MAP kinase-dependent apoptotic pathway. We therefore investigated whether pharmacological inhibitors of MEK (PD98059 and U0126), the upstream regulator of Erk-MAP kinases, would prevent BCR-mediated apoptosis of WEHI-231 cells.

To verify the ability of PD98059 and U0126 to inhibit the activity of Erk-MAP kinase in WEHI-231 cells, we tested whether
these reagents blocked anti-Ig-stimulated dual phosphorylation of Erk and hence activation in WEHI-231 cells. As shown in Fig. 5, A and B, PD98059 and U0126 were effective in the inhibition of Erk phosphorylation in both a time (pretreatment before BCR ligation) and dose-dependent manner. Optimal inhibition was achieved when the cells were pretreated with either inhibitor for between 90 min and 3 h, and under these conditions BCR-stimulated Erk-MAP kinase activity was completely suppressed (Fig. 5, A and B, and results not shown). Neither of these inhibitors, however, appeared to be effective in longer term cultures of WEHI-231 cells given that PD98059 and U0126 proved only partially capable of blocking Erk-MAP kinase activity over periods of 6–24 h (Fig. 5C), indicating that sustained inhibition of MEK over such time periods required multiple treatment of the cells with these reagents.

Having established that these inhibitors efficiently blocked BCR-mediated Erk-MAP kinase, we investigated the effects of these reagents on BCR-driven apoptosis as indicated by measurement of subdiploid DNA content. Pretreatment (2 h) of WEHI-231 cells with a single dose of U0126 (similar results were obtained using the MEK inhibitor PD98059) caused a dose-dependent decrease in BCR-induced apoptosis (maximum, 1–5 μM; Fig. 6 and results not shown). In contrast, the p38 MAP kinase inhibitor SB203580 had no effect on BCR-mediated apoptosis (data not shown), results consistent with our finding that anti-Ig did not significantly stimulate p38 MAP kinase under these conditions (Fig. 3B).

The above results suggest that BCR coupling to Erk-MAP kinase and its abrogation via CD40 signaling may play a major role in determining the commitment of WEHI-231 cells to apoptosis or survival. Consistent with this, we have recently shown that, in WEHI-231 cells, the BCR transduces apoptotic signals by stimulating the generation of mitochondrial arachidonic acid and resultant disruption of mitochondrial potential after mitochondrial translocation of cPLA2 (5), a signaling enzyme that is dependent on Erk-MAP kinase for activation (20). Interestingly, we also showed that costimulation with anti-CD40 abrogates BCR-coupling to cPLA2 activation and mitochondrial disruption (5). We now show (Fig. 7) that the MEK inhibitor, PD98059, also inhibits BCR-coupling to cPLA2 activity and mitochondrial disruption (5).
pling to cPLA₂, suggesting that this early Erk-MAP kinase signal couples the BCR to cPLA₂-dependent apoptosis.

**Anti-Ig treatment causes desensitization of sustained, cycling Erk activation observed in proliferating WEHI-231 B cells**

Although the MEK inhibitors PD98059 and U0126 blocked BCR-mediated apoptosis, these reagents did not relieve anti-Ig-induced arrest in the G₀-G₁ phase of the cell cycle (Fig. 6). These results suggested that Erk signaling, distinct from the early transient Erk response we have proposed to be associated with BCR-driven apoptosis, might play a role in the sustained proliferative response observed in untreated or CD40-rescued WEHI-231 B cells.

To test this, we investigated whether WEHI-231 B cells exhibited differential Erk signals during 48 h under conditions of normal growth, BCR-driven growth arrest, or CD40-mediated rescue (Fig. 8). We found that normal proliferating or anti-CD40-treated WEHI-231 cells exhibited strong dual phosphorylation of Erk, but not Jnk or p38, between 8 and 48 h, whereas protein levels of Erk, Jnk, and p38 expression remained constant (Fig. 8A and results not shown). This strong dual phosphorylation did not reflect a sustained elevated level of Erk activation but rather exhibited a cycling pattern of dual phosphorylation of Erk with peaks between 8–16 h and 32–48 h (Fig. 8D and results not shown) suggesting that cycling Erk activation was required for sustained progression of WEHI-231 cells throughout the cell cycle. In contrast, whereas apoptotic concentrations of anti-Ig induced a strong transient activation of Erk consistent with the apoptotic Erk signal described above, all Erk activity 2 h poststimulation was ablated (Fig. 8B).

Moreover, costimulation with anti-CD40 not only reduced the early transient Erk response but also restored (onset 8–24 h) and enhanced the late cycling pattern observed in unstimulated cells (Fig. 8C). That Erk activation within 8–24 h is critical for CD40-mediated rescue from BCR-driven growth arrest may be inferred from our finding that WEHI-231 B cells can still be rescued from anti-Ig-mediated growth arrest when anti-CD40 is added 8 h, but not 24 h, poststimulation via the BCR (Fig. 8E).

**Prolonged Erk-MAP kinase inhibition can block basal proliferation and CD40-mediated rescue.**

To investigate whether cycling Erk activity contributed to the survival and/or growth of WEHI-231 B cells, we tested the effect of sustained abrogation of MEK1/2, and hence Erk, activity, on WEHI-231 DNA synthesis and apoptosis. We found that sustained treatment of WEHI-231 B cells with U0126, PD98059 (results not shown), or U0126 plus PD98059 (Fig. 9A) not only appeared to enhance anti-Ig-mediated growth arrest but also almost completely blocked the DNA synthesis observed in unstimulated, anti-CD40-treated, or anti-Ig plus anti-CD40-treated cells (Fig. 9A and results not shown). To ensure that these results reflected the contribution of the late (≥8 h) cycling Erk pool, we investigated the effects of a single dose of PD98059 (Fig. 9C) or U0126 (Fig. 9D). These latter results confirmed our earlier cell cycle analysis (Fig. 6), which showed that blocking early Erk signals had only marginal effects on normal or CD40-rescued growth of WEHI-231 B cells. Taken together, these results suggest a role for late (≥8 h) Erk signaling both in the basal proliferation of WEHI-231 cells and in

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**FIGURE 9.** Sustained inhibition of MEK prevents CD40-mediated rescue of sIg-mediated growth arrest but does not prevent rescue from BCR-driven apoptosis. WEHI-231 cells (10⁵ cells/well) were preincubated for 3 h with DMSO vehicle or PD98059 plus U0126 (both 1 µM; A). Cells were then treated as indicated with 10 µg/ml anti-Ig (alg) or 10 µg/ml anti-CD40 (aCD40), or a combination of both. Cells treated with PD98059 and U0124 were treated with an additional dose (1 µM or DMSO vehicle for cells lacking inhibitors (Inh)) every 4 h during 28 h post-addition of anti-Ig and/or anti-CD40. Proliferation was assessed by measuring [³H]thymidine uptake at 48 h. Values are means ± SD of quadruplicate wells. B, WEHI-231 cells (5 × 10⁴/ml) were preincubated with PD98059 (1 µM) plus U0126 (1 µM) or DMSO vehicle for 3 h. Cells were then stimulated as indicated with 10 µg/ml anti-Ig or 10 µg/ml anti-CD40 or a combination of both. Cells originally treated with PD98059 plus U0126 were treated with additional doses (1 µM or DMSO vehicle for cells lacking inhibitors) every 4 h for a total of 28 h post-addition of anti-Ig and/or anti-CD40. Cells were then harvested after 48 h, and DNA content was analyzed by PI staining and FACS analysis as described in Materials and Methods. C, WEHI-231 cells (10⁵ cells/well) were preincubated for 3 h in PD98059 (1 and 5 µM) or DMSO vehicle before stimulation with anti-Ig (10 µg/ml), anti-CD40 (10 µg/ml), or a combination of both. Proliferation was assessed by measuring [³H]thymidine uptake at 48 h. Values are means ± SD of quadruplicate wells. D, WEHI-231 cells (10⁵ cells/well) were preincubated for 3 h in U0126 (1 or 5 µM) or DMSO vehicle before stimulation with medium, anti-Ig (10 µg/ml), or anti-Ig plus anti-CD40 (10 µg/ml). Proliferation was assessed by measuring [³H]thymidine uptake at 48 h. Values are means ± SD of quadruplicate wells.
the rescue of WEHI-231 cells from anti-Ig-mediated growth arrest by CD40.

To determine whether the inhibition of DNA synthesis resulting from sustained suppression of Erk activity reflected abrogation of Erk-dependent survival signals and resultant induction of apoptosis, the subdiploid DNA content of the cells exposed to PD95089 plus U0126 was analyzed (Fig. 9B). This analysis revealed that sustained blockage of MEK1/2 activity and growth of unstimulated WEHI-231 B cells led to apoptosis. The possibility that this apoptosis was due to inhibitor toxicity was ruled out by the finding that although sustained blockage of MEK1/2 activity in WEHI-231 cells treated with anti-CD40 or anti-Ig plus anti-CD40 induced profound growth arrest (Fig. 9A), it did not induce apoptosis in either case. These results therefore suggest that although signaling via CD40 induces signals which can compensate for ablation of Erk activity in terms of survival, additional signals including Erk are required to rescue anti-Ig-mediated growth arrest.

A role for cyclical Erk-MAP kinase activation in cell cycle progression of WEHI-231 B cells?

The MEK inhibitor data supported the proposal that cycling Erk activity contributed to the survival and/or growth of WEHI-231 B cells, presumably by promoting cell cycle transition. To investigate how cycling Erk activation correlates with cell cycle progression, we analyzed Erk signaling after arrest and release (removal of cell cycle blockers by washing) of cells from either the G1 or S phases of the cell cycle using the cell cycle blockers olomoucine (21, 22) and aphidicolin (23, 24), respectively (Fig. 10A). Although enriched populations of G1 and S phase cells were obtained, it was not possible to obtain fully synchronized populations as increasing either the concentrations of these cell cycle blockers or the length of preincubation with these reagents simply led to the induction of apoptosis, particularly with aphidicolin. Nevertheless, as with the asynchronous cells, subsequent treatment with anti-Ig alone induced inhibition of Erk activity within 16–48 h following removal of cell cycle blockers and subsequent release from either G1 or S phase arrest (Fig. 10B). In contrast, costimulation with anti-CD40 induces strong Erk activation during this time period (Fig. 10B). These results therefore supported our proposal that anti-Ig induced growth arrest of WEHI-231 cells by suppressing late Erk activity associated with cell cycle progression and that anti-CD40 rescued such cells by promoting Erk activity to drive mitosis. To address more directly the role of Erk activity in driving cell cycle progression, we next analyzed intracellular staining of phospho-Erk in conjunction with cell cycle analysis (using PI) by LSC. These latter data showed that the majority of cycling cells (i.e., those transiting G1, S phase, or G2-M) showed phospho-Erk staining (Fig. 10C and Table I). However, <50% of newly divided cells demonstrated phospho-Erk staining (Fig. 10C and Table I). Moreover, treatment with anti-Ig or olomoucine to induce growth arrest in G1 or G2 reduced the percentage of such cells expressing phospho-Erk to 30–35% (Table I and results not shown). These results, which indicate that BCR- or olomoucine-mediated growth arrest in G1 correlates with inhibition of Erk activity, suggest that suppression of late (≥2–4 h) Erk activation is also likely to be necessary for preventing transition through G1. Release of the olomoucine block followed by culture in medium alone showed synchronous transition of cells through G2-M within 48 h with >85% of such mitotic cells exhibiting Erk activation. Interestingly, stimulation of these cells with anti-Ig plus anti-CD40 resulted in asynchronous transition of the cells through cycle (all stages exhibiting >50% cells expressing phospho-Erk) within 48 h presumably due to the ability of anti-CD40 to promote growth and division (Table I).

Mechanism of BCR-mediated down-regulation of cycling Erk-MAP kinase activity

To address how BCR signaling abrogates cycling Erk-MAP kinase activation to achieve cell cycle arrest, we investigated the effect of anti-Ig on the activity of MEK, the upstream regulator of Erk. We found that although the cycling Erk activity was inhibited by BCR signaling, MEK activity (Fig. 11) was comparable in untreated and BCR-stimulated cells between 12 and 48 h as indicated both by MEK phosphorylation and by in vitro MEK kinase assays. These data therefore suggested that BCR signaling did not uncouple the upstream regulators of Erk-MAP kinase but rather induced negative feedback mechanisms that could override ongoing activation of Erk. Consistent with this, our data suggest that BCR signaling inhibits cycling Erk activity in WEHI-231 B cells by up-regulating expression of the MAP kinase phosphatase, Pac-1, and by promoting its association with Erk-MAP kinase (Fig. 11). Anti-CD40...
treatment does not appear to down-regulate Pac-1 expression per se but prevents Pac-1 association with Erk (Fig. 11 and results not shown). Use of this mechanism to regulate B cell proliferation is consistent with our previous findings that immunomodulatory products secreted by filarial nematodes can elicit B cell unresponsiveness by priming uncoupling of BCR-Erk-MAP kinase signaling by the MAP kinase phosphatase, Pac-1 (25).

**Discussion**

One of the major unresolved questions in B cell biology is how the BCR signals to differentially transduce anergy, apoptosis, proliferation, or differentiation during B cell maturation. The identification of key response-specific signals has been particularly difficult to address because many of the early signaling events such as phospholipase C, phosphatidylinositol 3-kinase, and MAP kinase activation appear to be recruited via the BCR under conditions leading either to proliferation or apoptosis. We now report that one such signal, Erk-MAP kinase can differentially signal to elicit either apoptosis or proliferation of the immature B cell lymphoma, WEHI-231 depending on the kinetics and context of Erk-MAP kinase activation. For example, we find that the BCR couples to an early (≈2 h) Erk-MAP kinase signal (Figs. 2, 3, 6, and 8) which activates a phospholipase A2 pathway (Fig. 7) that mediates arachidonic acid-mediated collapse of Δψ_m and depletion of cellular ATP, resulting in cathepsin B execution of apoptosis (5). Rescue of BCR-driven apoptosis by CD40 signaling desensitizes this early Erk signaling (Figs. 2 and 3) and uncouples the apoptotic mitochondrial phospholipase A2 pathway (Fig. 7). Consistent with this early Erk-MAP kinase signal playing a key role in the commitment of WEHI-231 cells to apoptosis is the finding that attenuation of the early Erk signal prevents BCR-mediated coupling to phospholipase A2 and resultant apoptosis (Figs. 6 and 7).

We have also identified a second role for Erk-MAP kinase in promoting the growth and proliferation of WEHI-231 immature B cells. Proliferating and CD40-stimulated WEHI-231B cells exhibit a sustained cycling pattern (8–48 h) of Erk activation (presumably in response to serum- or autocrine factors) which correlates with cell cycle progression, cell growth, and proliferation (Fig. 8). This distinct role for Erk signaling in the regulation of WEHI-231 B cell growth is supported by three key pieces of evidence: 1) apoptotic signaling via the BCR completely abrogates sustained Erk activation (Fig. 8); 2) CD40-mediated rescue from apoptosis and growth arrest correlates with restoration of this late cycling pattern of Erk activation (Fig. 8); and 3) sustained inhibition of the Erk activator MEK prevents CD40-mediated rescue of BCR-driven growth arrest of WEHI-231 immature B cells (Fig. 9). That such cycling Erk activation directly contributes to cell cycle progression is demonstrated by the finding that both BCR- and olomoucine-driven growth arrest in the G_0-G_1 phase of the cell cycle correlates with suppression of Erk activity (Fig. 10 and Table I). Such growth arrest therefore presumably reflects suppression of Erk-MAP kinase-inducible components of the cell cycle machinery (such

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**Table I. BCR- or olomoucine-driven growth arrest of WEHI-231 B cells in G_1 correlates with a decrease in phospho-Erk expression**

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>% in G_1 (% pErk)</th>
<th>% in S Phase (% pErk)</th>
<th>% in G_2-M (% pErk)</th>
<th>New Cells (% pErk)</th>
</tr>
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<tbody>
<tr>
<td>Media/media 48 h</td>
<td>46 (70.1)</td>
<td>30.8 (74.8)</td>
<td>11.6 (68.7)</td>
<td>9.2 (30.3)</td>
</tr>
<tr>
<td>Media/anti-Ig 48 h</td>
<td>68.9 (35.6)</td>
<td>12.8 (66.9)</td>
<td>3.1 (84.9)</td>
<td>11.8 (27.2)</td>
</tr>
<tr>
<td>Olomoucine/media 24 h</td>
<td>75.1 (35.9)</td>
<td>18.4 (57.2)</td>
<td>0.9 (92.9)</td>
<td>0.8 (46.3)</td>
</tr>
<tr>
<td>Olomoucine/media 48 h</td>
<td>0.2 (66.7)</td>
<td>6.3 (76.9)</td>
<td>72 (85.3)</td>
<td>13.1 (48)</td>
</tr>
<tr>
<td>Olomoucine/anti-Ig + anti-CD40 48 h</td>
<td>31.5 (52.2)</td>
<td>29 (81.2)</td>
<td>26.3 (79.8)</td>
<td>8 (40.6)</td>
</tr>
</tbody>
</table>

*WEHI-231 B cells were cultured in the absence (media) or presence of olomoucine (50 μM) for 48 h. The cells were washed twice in sterile medium before being resuspended at 2 × 10^7/ml and stimulated with anti-Ig (10 μg/ml), anti-Ig (10 μg/ml), plus anti-CD40 (10 μg/ml), or medium alone for up to 48 h as indicated. Cytozipsins were then fixed, permeabilized, and stained for PI and anti-phospho-Erk as described in Materials and Methods before analysis by LSC.

**FIGURE 11.** BCR-mediated down-regulation of Erk activity does not reflect suppression of MEK activation. WEHI-231 B cells were cultured for up to 48 h as indicated with medium (A) or anti-Ig (10 μg/ml; B) before preparing cell lysates. MEK-containing immune complexes were prepared, and in vitro MEK kinase assays were conducted using recombinant Erk-GST as substrate and analysis by Western blotting as described in Materials and Methods. A positive control for this assay is illustrated in lane +ve and reflected an in vitro kinase assay using recombinant activated MEK and the Erk-GST substrate. In addition, MEK kinase activity was supported by analysis of MEK activation by Western blotting of these immune complexes using anti-phospho-MEK Abs that recognize the active form of MEK1/2. Loading controls for Erk-GST and MEK expression are also shown. C. WEHI-231 B cells stimulated with anti-Ig (10 μg/ml) for up to 48 h were lysed and subjected to Western blot analysis of Pac-1 or Erk expression as indicated. D. Erk2-containing immune complexes derived from WEHI-231 B cells stimulated with anti-Ig (10 μg/ml) or anti-Ig (10 μg/ml) plus anti-CD40 (10 μg/ml) for up to 24 h as indicated were analyzed for Pac-1 or Erk2 expression by Western blotting.
as cyclin D) required for $G_1$-S phase transition. Alternatively, abrogation of Erk signaling may relieve down-regulation of $p27^{kip1}$-mediated inhibition of cell cycle progression (26–29). Our studies therefore demonstrate that Erk-MAP kinase can induce distinct biological responses in WEHI-231 B cells, depending on the kinetics of activation and the context of the downstream signaling machinery.

Interestingly, the onset (8–24 h) of the sustained Erk-MAP kinase signal restored by CD40-signaling correlates with the final time window in which anti-Ig-treated WEHI-231 B cells can be effectively rescued from growth arrest by anti-CD40 treatment (Fig. 8). This finite time frame for CD40-mediated rescue is reminiscent of the recently described progression of anti-Ig stimulated immature B lymphocytes through a series of “temporal windows” resulting, finally, in the induction of apoptosis (10). Such an orderly progression through initiation of an apoptotic program, followed by a delay in which the fate of the cell can be redirected is believed to allow external signals, such as those delivered through CD40, to determine the fate of the cell. Additionally, this temporal window is believed to allow self-reactive immature B lymphocytes time to initiate receptor editing so that they can alter their receptor specificity and re-enter the B lymphocyte pool before further maturation. However, failure to successfully rearrange their BCR or receive external help will ultimately result in deletion of these cells from the B lymphocyte pool (10). The ability of WEHI-231 cells to develop through similar temporal windows in which short term BCR-Erk-MAP kinase signaling similarly initiates a negative signal that, in the absence of an additional signal such as anti-CD40, ultimately leads to cell death suggests that maintenance of cycling Erk signaling could be a key element in the survival of immature cells during this selection process.

CD40 stimulation of WEHI-231 cells has been shown to upregulate the antiapoptotic protein Bcl-xL (30) which has been widely proposed to play a major role in the rescue of WEHI-231 cells from BCR-mediated apoptosis (4, 6, 7, 9). Although Erk signaling has previously been shown to promote up-regulation of Bcl-xL in other systems (31), it is likely that in WEHI-231 B cells CD40 up-regulates Bcl-xL and rescue from apoptosis by Erk-independent mechanisms given that we have found that CD40 can rescue WEHI-231 cells from BCR-mediated apoptosis even in the presence of sustained abrogation of MEK activity (Fig. 9). In contrast, sustained abrogation of MEK activity prevents CD40-mediated rescue of BCR-driven growth arrest of WEHI-231 B cells (Fig. 9). Taken together, these findings could provide a molecular mechanism to explain earlier reports that whereas overexpression of Bcl-xL could rescue WEHI-231 B cells from BCR-driven apoptosis but not growth arrest, CD40 signaling could abrogate such apoptosis and promote growth and proliferation (7, 9). Indeed, Erk-independent up-regulation of Bcl-xL by anti-CD40 could explain why unstimulated, but not CD40-stimulated WEHI-231 B cells undergo apoptosis in the sustained presence of MEK inhibitors. However, it is possible that such Erk-independent rescue from apoptosis may reflect the involvement of other antiapoptotic molecules that have been implicated in CD40-mediated rescue from BCR-induced apoptosis. In particular, a recent study has highlighted the role of another member of the Bcl-2 family, the antiapoptotic protein A1 (32). For example, overexpression of this protein was shown to render WEHI-231 cells resistant to BCR-mediated apoptosis, and CD40 stimulation was also shown to increase A1 RNA expression (32). However, the role of endogenous A1 in WEHI-231 cells remains to be explored, and the molecular mechanisms leading to its expression and activation to be elucidated.

In addition to our finding of differential roles for Erk-MAP kinase in mediating both apoptotic and proliferative responses in WEHI-231 cells, dual roles for Erk-MAP kinase in the induction of negative and positive selection have also recently been reported for thymocytes (33). Similarly, although Erk activation has been widely established to be crucial for T cell activation, recent studies have shown Erk to play a critical role in cytokine unresponsiveness (anergy) and activation-induced cell death (34–37). How Erk differentially signals to regulate these distinct physiological responses is not known, but in the T cell systems, sustained hyperactivation of Erk appears to be associated with anergy and apoptosis. In contrast, in our WEHI-231 system, rescue and growth signals appear to be associated with sustained, albeit cycling, Erk activity. An additional factor may be the ability of Erk to act in both cytosolic and nuclear compartments and hence target differential downstream effector elements. Indeed, similarly to previously published findings (38, 39), we have preliminary results to suggest that whereas CD40 stimulation enhances nuclear Erk-MAP kinase activity, BCR stimulation results predominantly in cytoplasmic Erk-MAP kinase activity in WEHI-231 B cells. That CD40 signaling promotes nuclear Erk-MAP kinase activity may fit with current models suggesting that CD40 rescue signals promote transcription factor activation and new protein synthesis (40). In contrast, a cytoplasmic location for BCR-mediated Erk-MAP kinase activity is necessary for activation of cPLA2 and initiation of the mitochondrial apoptotic pathway in WEHI-231 B cells. Although early BCR-coupled Erk activity in mature B cells also seems to be predominantly located in the cytosol, these signals are not apoptotic, presumably because cPLA2 is not expressed or activated in the cells (18). Thus, Erk-MAP kinase signaling may induce diverse biological responses in B cells depending on the kinetics and amplitude of activation, the subcellular localization of the pool of Erk used, and the maturation stage-dependent context of the downstream signaling machinery recruited.

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References


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