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Inhibition of the MEK/ERK Signaling Pathway Blocks a Subset of B Cell Responses to Antigen

James D. Richards,2 Shaival H. Davé,3 Chih-Hao G. Chou, Alusha A. Mamchak, and Anthony L. DeFranco4

Signal transduction initiated by B cell Ag receptor (BCR) cross-linking plays an important role in the development and activation of B cells. Therefore, considerable effort has gone into determining the biochemical signaling events initiated by the BCR and delineating which events participate in specific biological responses to Ag. We used two inhibitors of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) 1 and MEK2, PD98059, and U0126, to assess the role the Ras-mitogen-activated protein kinase pathway plays in several BCR-induced responses. PD98059 or U0126 treatment substantially inhibited the BCR-induced activation of the extracellular signal-regulated kinase (ERK) forms of mitogen-activated protein kinase in the immature B cell line WEHI-231, in immature splenic B cells, and in mature splenic B cells. However, MEK-ERK inhibition did not block BCR-induced growth arrest or apoptosis of WEHI-231 cells or apoptosis of immature splenic B cells, indicating that the MEK-ERK pathway is not required for these events. In contrast, PD98059 and U0126 treatment did inhibit the up-regulation of specific BCR-induced proteins, including the transcription factor Egr-1 in WEHI-231 and mature splenic B cells, and the CD44 adhesion molecule and CD69 activation marker in mature splenic B cells. Moreover, both inhibitors suppressed BCR-induced proliferation of mature splenic B cells, in the absence and in the presence of IL-4. Therefore, activation of the MEK-ERK pathway is necessary for a subset of B cell responses to Ag. The Journal of Immunology, 2001, 166: 3855–3864.

B cell Ag receptor (BCR)5 signaling plays important roles in the development, survival, and activation of B lymphocytes. The first result of BCR engagement is the activation of Src family and Syk protein tyrosine kinases. These kinases trigger a complex network of signaling pathways downstream of the receptor, including the activation of phospholipase C (PLC)-γ1 and -γ2, phosphatidylinositol (PI) 3 kinase, the Ras-Raf-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase pathway, and phosphorylation of the Vav proto-oncogene product (1–3). The resulting signals quickly reach the nucleus and alter gene expression. The ultimate effects on the B cell are profound and vary depending on the maturation state of the cell and on the additional signals the cell receives. Ag contact with immature B cells typically causes them to enter into an anergic state or undergo apoptosis, responses that promote immunological tolerance to self-Ags. In contrast, mature B cells contacting Ag enter G1 phase of the cell cycle and up-regulate many proteins involved in adhesion and Ag presentation to Th cells. Strong BCR stimulation can also induce B cell proliferation (3). Th cells enhance this proliferation and induce B cell differentiation into the Ab-secreting plasma cell state by providing cell-cell contact signals via CD40 and by releasing cytokines, such as IL-4 and IL-5 (3).

Both genetic and pharmacologic approaches have been used to determine how individual signaling pathways participate in these biological responses to Ag. For example, wortmannin, which inhibits PI 3 kinase, has been shown to block BCR-induced apoptosis in a human immature B cell line (4). In addition, gene disruption experiments demonstrate that PI 3 kinase participates in B cell development and proliferation as well (5, 6). Experiments with pharmacologic agents that mimic the second messengers resulting from PLC-γ activation, and with mutant cell lines defective in this pathway, have demonstrated its importance for BCR-induced growth arrest and apoptosis of B cell lines (7–10) and proliferation of mature splenic B cells (11, 12). Moreover, mice and cell lines with targeted mutations in the signaling proteins Vav (13, 14) and HS1 (15, 16) both exhibit decreased B cell responses to Ag.

By comparison, relatively little is known about the role(s) of the Ras-Raf-MEK-ERK MAP kinase pathway in B cell responses to Ag. When in its active GTP-bound state, Ras activates the serine/threonine protein kinase Raf, which phosphorylates and activates the protein kinases MEK1 and MEK2. The MEK1/2 kinases in turn phosphorylate and activate the classical p44 and p42 MAP kinases, also called ERK 1 and 2, respectively (17). This pathway has been found to be important in many cell types for receptor-induced biological responses, such as proliferation, growth arrest, and differentiation (17–22). In B cells, Ras-ERK MAP kinase activation correlates with BCR-induced growth arrest and apoptosis in immature B cells, and cell-cycle entry and activation in mature

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5 Abbreviations used in this paper: BCR, B cell Ag receptor; PLC, phospholipase C; PI, phosphatidylinositol; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; BrdUrd, 5-bromo-2′-deoxyuridine; JNK, c-Jun N-terminal kinase; SRE, serum response element.
MEK-ERK CASCADE IN B CELL RESPONSES TO ANTIGEN

B cells (3, 23, 24). We used two structurally and mechanistically distinct MEK1/2 inhibitors, PD98059 and U0126 (25–27), to investigate the role that this pathway plays in these BCR-induced events. Both inhibitors suppressed BCR-induced activation of the ERK MAP kinases in all B cells examined. These inhibitors also suppressed up-regulation of the early response gene product Egr1 in mature splenic B cells and in the WEHI-231 immature B cell line and partially blocked the induction of the CD44 adhesion protein and CD69 activation marker in mature splenic B cells. In addition, both inhibitors blocked BCR-induced proliferation of mature splenic B cells. In contrast, MEK-ERK inhibition failed to suppress BCR-induced growth arrest or apoptosis of WEHI-231 cells and did not inhibit BCR-induced apoptosis in primary immature splenic B cells. Thus, the Ras-ERK MAP kinase pathway is important for mediating some, but not all, specific B cell responses to Ag.

Materials and Methods

Abs and other reagents

Affinity-purified goat-anti-mouse IgM was obtained from Jackson ImmunolResearch (West Grove, PA). The anti-IgD mAb HA6/1 was generously provided by Fred Finkelman, University of Cincinnati, as a mouse ascites fluid. Mouse rLrL4 was from Genzyme (Cambridge, MA). Hybridomas HO13.4 (anti-Thy1), 53.6.172 (anti-CD8), and GK1.5 (anti-CD4) were obtained from American Type Culture Collection (Manassas, VA). Anti-ERK2 (C-14), anti-c-Jun terminal kinase (JNK) (C-17), and anti-Egr-1 (C-19) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ACTIVE MAP kinase polyclonal Ab (pTeP) was purchased from Promega (Madison, WI), PE- and FITC-conjugated-RA3–6B2 (anti-B220), FITC-conjugated GL-1 (anti-CD86), FITC-conjugated anti-CD44, FITC-conjugated anti-CD69, and anti-CD16/CD32 were purchased from PharMingen (San Diego, CA). 5-Bromo-2′-deoxyuridine (BrdUrd) was purchased from Sigma (St. Louis, MO). Anti-BrdUrd-FITC was purchased from Becton Dickinson (San Jose, CA). Avidin-TriColor was obtained from CalTag (South San Francisco, CA). [3H]Thymidine was purchased from NEN (Boston, MA). PD98059 and the GST-Elk-1 fusion protein were purchased from New England Biolabs (Beverly, MA). U0126 was purchased from Calbiochem (La Jolla, CA). The GST-Jun(1–79) fusion protein was prepared as described (28). PD98059 and U0126 were dissolved in DMSO and stored at −20°C until used.

Cell culture

Splenic B cells were cultured in RPMI 1640 medium supplemented with 10% FCS (Life Technologies, Rockville, MD), 2 mM pyruvate, 20 mM glutamine, 50 µM 2-ME, and penicillin/streptomycin. WEHI-231 cells were cultured as previously described (2). In all experiments, PD98059, U0126, or DMSO carrier was added to the splenic B cells or WEHI-231 cells 1 h before stimulation, unless otherwise indicated. Cells were stimulated and cultured in medium that always contained PD98059, U0126, or DMSO carrier.

Isolation and apoptosis of immature splenic B cells

Three-week-old BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were given 500-rad whole-body irradiation 13.5 days before sacrifice (29). A single cell suspension of the spleen cells in either ice-cold RPMI 1640 or DMEM with 10% FCS was treated with an equal volume of 17 mM Tris-HCl and 1.44% ammonium chloride for 5 min on ice to lyse erythrocytes. The T cells and IgD+ B cells were lysed by incubation in a cocktail containing anti-CD4, anti-CD8, anti-Thy1.2, and HSA/1 anti-IgD ascites on ice for 30 min, followed by complement lysis at 37°C for 30 min. The isolated immature splenocytes were IgM+IgD−, IgG+IgA−, and negative for Gr1, CD3, and Mac-1 as shown by flow cytometry. The immature splenic B cells at a density of 2–4 × 10^7/ml were incubated for 30 min to 1 h with 20 µM PD98059 and then stimulated with various concentrations of anti-IgM in the presence or absence of 20 µM PD98059 for 18 h. The apoptosis of immature splenic cells was quantified by annexin V/PI staining (Oncogene Research Products, Cambridge, MA) following the manufacturer’s protocol.

Isolation and stimulation of mature splenic B lymphocytes

Female (C57BL/6 × DBA2) F1 (BDF1) mice from Simonsen Laboratories (Gilroy, CA) or Charles River Breeding Laboratories (Hollister, CA) were sacrificed at 2–4 mo of age. Small resting B cells were isolated from the spleen as described (30). Cells were collected from the 60%–70% Percoll interphase, washed, and rested in normal medium at 37°C for 30 min to 2 h before treatment with DMSO or inhibitors. The purity of the splenic B cell preparations was typically at least 96% B220^+^, as verified by flow cytometry.

Splenic B cells (1 × 10^7 cells/ml) were stimulated with goat anti-mouse IgM and/or IL-4 (50 U/ml) for the indicated times and washed twice with ice-cold PBS containing 1 m NaVO_3 before lysis.

ERK2 and JNK immunocomplex protein kinase assays

Cells were stimulated for 4 min (ERK2 assays) or for 15 min (JNK assays), and subsequently lysed as previously described (31). Analysis of the kinetics of activation of these MAP kinases indicated that strong inductions were reproducibly seen at these times. Anti-JNK and anti-ERK2 immunoprecipitations were performed as described (28). Immunocomplex protein kinase assays were performed on the immunoprecipitates as described (30), with GST-Elk-1 as the substrate for ERK2 and GST-Jun(1–79) as the substrate for JNK. Reaction products were resolved by SDS-PAGE, transferred to nitrocellulose, and detected by autoradiography. Quantitation of kinase activity was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). To calculate inhibition of kinase activity by the inhibitors, we used the formula: Percent inhibition = 1 − ((stimulation untreated) / (stimulation untreated)) × 100. Blots were reprobed with anti-ERK2 Abs (1:1000) to ensure that all reactions contained equal amounts of ERK2.

Egr-1 and anti-phosphoMAP kinase immunoblotting

For examining Egr-1 up-regulation, cells (5 × 10^6 cells/lanes) were stimulated for 1 h and lysed in SDS-PAGE sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. The anti-Egr-1 Ab was used at 1:500 for detection. General SDS-PAGE and immunoblotting procedures were described as described (2). For anti-phosphoMAP kinase immunoblotting time course experiments, 1 × 10^6 splenic B cells/ml or 2.5 × 10^6 WEHI-231 cells in 0.5 ml were stimulated with 10 µg/ml goat anti-mouse IgM for the indicated times, washed, and lysed in SDS-PAGE sample buffer. Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-ACTIVE MAP kinase Ab (pTeP) (diluted 1:1000). For anti-IgM dose-response experiments, cells were stimulated with the indicated concentrations of anti-IgM for 4 min before washing, lysis, SDS-PAGE and immunoblots.

Growth arrest and apoptosis of WEHI-231 cells

For growth arrest, WEHI-231 cells were plated in triplicate at 2 × 10^6 cells/ml in 96-well plates and stimulated with goat-anti-mouse IgM for 28 h. Cells were pulsed with [3H]TdR (1 µCi/well) for the last 4 h, harvested onto glass-fiber filters (Wallac), and the amount of incorporated [3H]TdR was determined using a Betaplate reader (Wallac). For apoptosis, WEHI-231 cells were plated at 1.5 × 10^6 cells/ml in 12-well plates, stimulated, and cultured for 48 h. Cell survival was measured by ability to exclude 1 µg/ml propidium iodide, as determined by flow cytometry, or by annexin V staining following protocols supplied by the manufacturer.

Proliferation and survival of mature splenic B cells

Proliferation of murine splenic B cells was measured either by incorporation of [3H]thymidine or incorporation of BrdUrd. For [3H]TdR incorporation assays, splenic B cells were plated in triplicate in 96-well plates (2.5 × 10^6 cells/ml), in the presence of anti-IgM ± 50 U/ml IL-4, and cultured for 45 h. Cells were pulsed with [3H]TdR for the last 5 h of culture, and incorporation was determined as described above. In vitro BrdUrd-labeling experiments were performed as described (30). Briefly, RBC-depleted splenocytes were plated at 5 × 10^5 cells/ml, stimulated, and cultured for 48 h. BrdUrd (10 µM) was added during the final 24 h. Cells were fixed, permeabilized with 0.5% Tween 20 in PBS, the DNA denatured, and the cells subsequently were stained with anti-B220 and anti-BrdUrd and analyzed by flow cytometry. The data are presented as the percentage of B220^+^ cells that are BrdUrd^+^. For survival experiments, RBC-depleted splenocytes or purified splenic B cells were cultured for 48 h. B220^−^ cells were then tested by flow cytometry for their ability to exclude 1 µg/ml propidium iodide.
Up-regulation of cell surface molecules

RBC-depleted splenocytes were cultured at 5 \times 10^4 or 1 \times 10^6 cells/ml. Cells were stimulated with goat anti-mouse whole Abs or F(ab)\(^2\), fragments for 18 h. Cells were harvested, Fc receptors blocked with anti-CD16/CD32, and subsequently costained with anti-B220 and anti-B7-2 (CD86), anti-CD44, or anti-CD69 and analyzed by flow cytometry. Only live (as determined by propidium iodide exclusion or by forward- and side-scatter profiles) B220\(^+\) cells were analyzed.

Results

**PD98059 and U0126 inhibit activation of the ERK MAP kinases in WEHI-231 and purified splenic B cells**

In vitro protein kinase assays were performed to determine whether PD98059 inhibited the BCR-induced activation of the ERK2 form of MAP kinase, as expected. As previously reported (23, 32, 33), stimulation of the WEHI-231 immature B cell line with anti-IgM Abs substantially increased the activity of ERK2 (Fig. 1A). This activation was inhibited in a dose-dependent manner by PD98059 at a 4-min time point (Fig. 1A). PD98059 also inhibited ERK2 activation over a 24-h time course (see below and data not shown). At low concentrations of stimulating Ab, ERK2 activation was always greatly inhibited by 5–20 \(\mu\)M PD98059 and in some experiments completely blocked. Inhibition by 10–20 \(\mu\)M PD98059 was substantial but not absolute at higher doses of stimulating Ab, consistent with previous results in other systems (26). Likewise, 5–10 \(\mu\)M PD98059 suppressed ERK2 activation in purified murine splenic B cells stimulated with anti-IgM (Fig. 1B) and anti-IgM plus IL-4 (Fig. 1C). IL-4 did not reproducibly increase or decrease BCR-induced ERK2 activation at a 4-min time point (Fig. 1C) and it did not alter the magnitude and kinetics of BCR-induced ERK2 activation over a 2-h time course (data not shown). Moreover, PD98059 was equally as effective at blocking BCR-induced ERK2 activation in the presence or absence of IL-4 (compare Fig. 1, H and I). In contrast, PD98059 in this dose range did not decrease or increase the BCR-induced activation of JNK, another MAP kinase protein that is downstream of the MEK family member MKK4 (34), in purified splenic B cells (Fig. 1D) or in WEHI-231 B cells (data not shown). PD98059 also did not block the BCR-induced activation of the tyrosine kinase Syk or the appearance of tyrosine phosphoproteins in the lysates of BCR-stimulated B cells (data not shown). Therefore, PD98059 seemed to inhibit MEK1/2, and, thus, ERK specifically in B cells, as it does in other cell types up to concentrations of at least 50 \(\mu\)M (25, 26, 35, 36).

The ERK kinases must be dually phosphorylated by MEK1/2 on specific threonine and tyrosine residues in the activation loop to become activated (37). Thus, immunoblotting with Abs that recognize the dually phosphorylated forms of ERK1 and ERK2 is a useful assay for both MEK1/2 action and ERK activity. Inhibition of ERK1/2 phosphorylation by 20 \(\mu\)M PD98059 and by a second, structurally and mechanistically distinct MEK1/2 inhibitor, 10 \(\mu\)M U0126, therefore, was examined by anti-phosphoMAP kinase immunoblotting. Both inhibitors blocked ERK phosphorylation over a 4-h time course in purified splenic B cells (Fig. 1E) and in WEHI-231 B cells (Fig. 1F). Similar to the above protein kinase assays, the inhibition of ERK phosphorylation provided by PD98059 was significant but incomplete. (However, note that by the end of the time course, ERK phosphorylation was inhibited back down to levels approximating baseline.) In contrast to PD98059, U0126 blocked BCR-induced phosphorylation of ERK completely or almost completely at all time points in both cell types. Both inhibitors also blocked ERK phosphorylation over a wide range of stimulating Ab in purified B cells (Fig. 1G) and in WEHI-231 cells (data not shown). As with the time course assays, U0126 inhibited ERK phosphorylation more completely than did PD98059.

**PD98059 inhibits the BCR-induced up-regulation of Egr-1, CD44, and CD69**

BCR-stimulated B cells up-regulate the expression of many transcription factors (38, 39). This up-regulation leads to changes in the expression of proteins important for cell-cycle regulation, cell-cell adhesion, and activation of T cells. One BCR-induced transcription factor is Egr-1, which is important for the BCR-induced expression of the adhesion proteins, CD44 and ICAM-1 (40, 41). In addition, putative Egr-1 binding sites exist in the promoter of the gene encoding the human CD69 activation marker (42). BCR-induced transcription of a reporter gene linked to the egr-1 promoter can be blocked by dominant negative forms of Ras and Raf (39). Therefore, we wished to determine whether BCR-induced Egr-1, CD44, and CD69 protein expression required MEK activity. PD98059 treatment (20 \(\mu\)M) greatly suppressed anti-IgM-induced up-regulation of Egr-1 in mature splenic B cells (Fig. 2A) and in WEHI-231 B lymphoma cells (Fig. 2B). In addition, both PD98059 and U0126 partially inhibited the BCR-induced up-regulation of CD44 and CD69 in mature splenic B cells (Fig. 2, C–E). In contrast, PD98059 and U0126 did not inhibit BCR-induced expression of B7-2 (CD86) (Fig. 2F), a protein that provides an important costimulatory signal for Th cells (43).

**MEK inhibitors do not inhibit BCR-induced growth arrest or apoptosis in WEHI-231 B cells or apoptosis in immature splenic B cells**

BCR stimulation induces WEHI-231 B cells to arrest in the G1 phase of the cell cycle and then undergo apoptosis (7–9, 44, 45). To determine whether the MEK-ERK pathway is important for these events, we pretreated WEHI-231 cells with varying amounts of PD98059 or U0126 before stimulation with anti-IgM Abs. No inhibition of BCR-induced growth arrest was seen, as assessed by incorporation of \(^{3}H\)thymidine (Fig. 3 , A–B). However, 20 \(\mu\)M PD98059 and 10 \(\mu\)M U0126 did reproducibly decrease basal proliferation of WEHI-231 cells, suggesting that the MEK-ERK pathway may be required for the proliferation of this cell line. PD98059 and U0126 also failed to inhibit the BCR-induced apoptosis of these cells, as determined by propidium iodide exclusion (Fig. 3, C and D), and by annexin V staining (data not shown). These data suggest that the MEK-ERK pathway is not required for anti-IgM-mediated growth arrest or apoptosis in WEHI-231 cells.

Immature B cells from spleen or bone marrow also undergo apoptosis in response to Ag receptor stimulation (46). To determine whether inhibition of MEK would affect these events, we used autoreconstitution of sublethally irradiated mice (29) as a method for isolating sufficient numbers of immature B cells. The isolated immature B cells were incubated in culture with or without 20 \(\mu\)M PD98059 and with various concentrations of polyclonal anti-IgM. Although there was a substantial spontaneous apoptosis of these immature B cells after 18 h of incubation in culture, the fraction of cells that had initiated apoptosis, as judged by staining with annexin V, increased appreciably in response to low concentrations of anti-IgM (Fig. 4A). Similar results were obtained when apoptosis was judged by failure to exclude propidium iodide, representing a late step in apoptosis (data not shown). PD98059 treatment did not decrease the fraction of immature B cells that were apoptotic upon stimulation with anti-IgM, whereas it did partially inhibit ERK2 activation (by 50–72% in the experiment shown in...
Fig. 4B), as seen in the other B cell populations tested. It should be noted that PD98059 treatment by itself often increased the fraction of cells that had initiated apoptosis. In some experiments, this effect was small, or, as in the example shown in Fig. 4A, moderate. In other experiments, the fraction of immature B cells initiating apoptosis was similar in the cultures treated with PD98059 alone, anti-IgM alone, and the cultures containing both agents. It may be that stimulation of the Ras-ERK MAP kinase pathway by some element of the culture conditions (serum, for example) provides a survival signal, such that inhibition of this pathway leads to greater apoptosis even in the absence of BCR stimulation.
PD98059 and U0126 inhibit proliferation of BCR-stimulated splenic B cells

In contrast to its effect on immature B cells, BCR engagement causes mature B cells to enter the G1 phase of the cell cycle and to proliferate. Addition of T cell-derived cytokines such as IL-4 enhances this proliferative response (47–49). We wished to determine whether the MEK-ERK pathway is important for BCR-induced proliferation of murine splenic B cells. Anti-IgM treatment induced B cell proliferation in a [3H]thymidine incorporation assay, and PD98059 blocked this proliferation in a dose-dependent manner (Fig. 1A). In agreement with earlier reports (47–49), the addition of IL-4 greatly enhanced the proliferation of the anti-IgM-stimulated B cells (Fig. 5B, note different scale on x-axis). PD98059 was also able to suppress B cell proliferation in the presence of IL-4, but to a lesser degree than when cells were stimulated with anti-IgM alone. IL-4 increases [3H]thymidine incorporation in this assay both by acting as a B cell survival factor and by increasing the percentage of B cells that enter the cell cycle (47–49). We investigated whether a block in MEK-ERK activity would inhibit one or both of these effects. As shown in Fig. 5C, the percentage of B cells that entered S phase in response to anti-IgM plus IL-4 was significantly diminished by treatment with either PD98059 or U0126. U0126 proved to be the more potent inhibitor, because 10 μM U0126 was sufficient to block proliferation essentially completely. In contrast, neither inhibitor substantially altered B cell survival in the presence of IL-4 (Fig. 5D).

Discussion

We wished to study the role(s) that the Ras-Raf-MEK-MAP kinase pathway plays in B cell responses to Ag. To do this, we used two pharmacologic inhibitors of MEK1 and MEK2, PD98059 and U0126, to inhibit activation of this pathway in WEHI-231 B lymphoma cells, in immature splenic B cells, and in mature splenic B cells. PD98059 inhibits MEK1/2 specifically and does not block activation of the closely related MEK family members MKK3 or SEK1/MKK4, which are upstream of JNK and p38 MAP kinases, and it does not inhibit numerous other serine/threonine, tyrosine, or lipid kinases (Fig. 1D and data not shown) (25, 26, 35, 36). U0126 also specifically inhibits MEK1/2, without blocking the activation of JNK, p38 MAP kinase, or several other protein kinases (27, 50, 51). In vitro immunocomplex kinase assays demonstrated that PD98059 was able to substantially inhibit BCR-induced ERK2 activity in a dose-dependent manner (Fig. 1, A–C). Likewise, anti-phosphoMAP kinase blots determined that U0126 inhibited ERK1/2 activity over a wide range of times and doses of stimulation (Fig. 1, E–G). Treatment with PD98059 also inhibited the BCR-induced expression of the early response gene product Egr-1, demonstrating the importance of the MEK-ERK kinase pathway for the expression of this transcription factor. This result is consistent with previous studies demonstrating the requirement of promoter serum response elements (SREs) for egr-1 expression (52), and the ability of dominant negative mutants of Ras and Raf to block egr-1 promoter-driven reporter gene expression in response to BCR cross-linking (39). Anti-IgM-induced proliferation of splenic B cells was also substantially inhibited by PD98059 and U0126, in the absence and presence of IL-4. Thus, MEK1/2 activity and presumably ERK activity play important roles in BCR-induced proliferation. In contrast, this pathway is not required for the BCR-induced expression of B7-2 (CD86) in mature splenic B cells, for growth arrest and apoptosis of the immature WEHI-231 B cell line, or for...
BCR-induced apoptosis of immature splenic B cells. In addition to demonstrating that some BCR-induced effects are not dependent on the MEK-ERK signaling pathway, these results demonstrated that PD98059 and U0126 did not exert significant toxicity or non-specific inhibition of B cell functions. Moreover, because these two structurally and mechanistically distinct inhibitors gave very similar results, this strongly suggests that they are indeed working via the MEK-ERK MAP kinase pathway and not via some other unknown actions.

Our results indicating that activation of the Ras-ERK MAP kinase pathway is important for proliferation of splenic B cells are consistent with results obtained in other cell types, such as fibroblasts and PC12 cells, where this pathway also contributes to cellular proliferation (17–22). The mechanism by which ERK promotes BCR-mediated proliferation is not yet defined, although it is likely to involve changes in gene expression; BCR stimulation rapidly induces changes in expression of several important cell cycle regulators, such as cyclins, cyclin-dependent kinases, and cell-cycle inhibitors (53–55). The ERK kinases phosphorylate and activate several transcription factors, including Elk-1 and SAP-1a, which help enhance transcription of genes whose promoters contain SREs (34). Indeed, the egr-1 early response gene contains several SREs and is rapidly induced upon BCR cross-linking, and ERK activity was required for Egr-1 expression (Fig. 2).

In addition to the Ras-ERK MAP kinase pathway, HS1, Vav, and the PLC-γ pathway are also important for BCR-mediated proliferation (11–15). Just as ERK kinases activate Elk-1 and Sap-1a, each of the above signaling pathways are likely to activate other pre-existing transcription factors. For example, the PLC-γ pathway activates the serine/threonine phosphatase calcineurin, which dephosphorylates and activates NFAT (56). Together, these pre-existing but newly activated transcription factors induce expression of early response genes, many of which, like egr-1, encode transcription factors themselves. These secondary transcription factors likely control the expression of many other genes important for events such as proliferation and interaction with Th cells (3). Indeed, Egr-1 has been implicated in the BCR-induced expression of CD44 and ICAM-1 (see below). Different signaling pathways, then, could regulate the expression of different cell cycle genes by virtue of the transcription factors they activate. Some cell-cycle genes may require the activation of transcription factors controlled by one signaling pathway and others by multiple pathways.

The induction of proliferation by the BCR is known to be dramatically enhanced by IL-4. We have found that this enhancement is not paralleled by an increase in ERK activation (Fig. 1C and data not shown). Thus, IL-4 appears to promote B cell proliferation by its action on other signaling pathways, perhaps those involving IRS-1, IRS-2, or Stat-6 (57–61). Interestingly, high concentrations of PD98059 only partially inhibited proliferation in response to anti-IgM and IL-4, whereas it completely or almost completely inhibited proliferation to similar or even higher doses of anti-IgM alone. This difference in proliferative inhibition occurred despite...
the fact that ERK activation was suppressed equally well in B cells stimulated in the absence or presence of IL-4 (Fig. 1, H and I). Thus, it appears that BCR-induced B cell proliferation has a decreased requirement for MEK/ERK signaling events in the presence of IL-4. However, this proliferation of B cells was still dependent on a low level of ERK activation, because it was completely blocked by the more effective MEK1/2 inhibitor, U0126. MEK-ERK activity appears to be required for cell-cycle entry (Fig. 5C) rather than for survival (Fig. 5D).

BCR-stimulated mature B cells also up-regulate adhesion molecules and a variety of other cell surface proteins, some of which are important for promoting interaction with Th cells. MEK-ERK inhibition failed to block up-regulation of B7-2 (CD86) (Fig. 2F), a protein that provides a costimulatory signal to T cells through its interaction with CD28 (43). In contrast, BCR-induced up-regulation of the transcription factor Egr-1 was suppressed by PD98059, as mentioned above. Egr-1 expression has been implicated in the up-regulation of the adhesion proteins CD44 and ICAM-1 (40, 41). CD44 has been proposed to facilitate B cell migration to and retention in secondary lymphoid follicles, where B cells interact with T cells (40), and ICAM-1 expression helps mediate adhesion between Ag-presenting B cells and Th cells during T cell activation, and subsequent stimulation of the B cells (3, 62). Up-regulation of CD44 expression was partially blocked by PD98059 (Fig. 2C) and U0126 (Fig. 2D), consistent with the hypothesis that Egr-1 plays a significant role in this induction. These data provide evidence that in addition to its role in BCR-induced proliferation, the Ras-ERK MAP kinase pathway is important for the up-regulation of specific proteins involved in cell-cell interactions and B cell trafficking. In addition, putative Egr-1 binding sites are found in the human CD69 promoter (42), and the Ras pathway is involved in its up-regulation in T cells (63, 64). Our data demonstrate that CD69 up-regulation is MEK1/2-dependent in murine B cells (Fig. 2E) and are consistent with a role for Egr-1 in its up-regulation.

These data are also consistent with recently published results that PD98059 inhibits the BCR-induced up-regulation of Egr-1 mRNA (65). However, in contrast to Egr-1, this group found that BCR-induced induction of Egr-2 mRNA was not significantly blocked by PD98059. The persistence of Egr-2 expression in PD98059-treated B cells may explain why CD44 and CD69 up-regulation is only partially inhibited by PD98059 and U0126.

Whereas in most situations the Ras-ERK MAP kinase pathway seems to be involved in promoting cell growth or differentiation, this pathway has been implicated in growth arrest in murine fibroblasts (20–22), and ERK activity also has been reported to be important for apoptosis in some instances (24, 66, 67). However, we observed no inhibition in BCR-induced growth arrest or apoptosis in WEHI-231 B cells treated with PD98059 or with U0126, which blocks BCR-induced MEK-ERK activity almost completely (Fig. 3). Moreover, 20 μM PD98059 also failed to inhibit BCR-induced apoptosis in immature splenic B cells (Fig. 4A), although this same concentration of PD98059 dramatically blocked BCR-induced proliferation and Egr-1 and CD69 up-regulation in mature splenic B cells.

In contrast to our results, Lee and Koretzky (24) found that overexpression of MKP-1, a phosphatase that can dephosphorylate and inactivate ERK1/2, did block apoptosis of WEHI-231 cells. This conclusion was based on experiments examining apoptosis at early times when <20% of cells had undergone apoptosis, so it is possible that MKP-1 overexpression delayed but did not prevent apoptosis. However, it should be noted that we did not observe inhibition of apoptosis by PD98059 even at relatively early times when apoptosis had occurred in a minority of cells (data not shown). Alternatively, it is possible that MKP-1 overexpression exerted its effects by dephosphorylating other MAP kinases, such as JNK or p38 MAP kinase. MKP-1 has been demonstrated to dephosphorylate and inhibit the activity of JNK and p38 in many cell types, including lymphocytes (68–71), and BCR cross-linking has been found to activate JNK and p38 (Fig. 1D) (23, 33, 72–75). Moreover, Graves and colleagues (74) have presented data indicating that JNK and p38 (73, 74) MAP kinases are involved in BCR-induced apoptosis in human B cell lines, although contrasting data have been presented that p38 is not required for apoptosis in WEHI-231 cells (75). If MKP-1 overexpression inactivates JNK and/or p38 in B cells, as does in other cell types, this could explain the block in BCR-induced apoptosis reported by Lee and Koretzky (24). Indeed, MKP-1 blocks UV-induced apoptosis in U937 human leukemia cells, apparently by inhibiting JNK activity (70). In contrast to MKP-1 overexpression, PD98059 and U0126 appear to inhibit ERK activity specifically without inhibiting JNK or p38 (Fig. 1) (25, 26, 35, 36, 50, 51). Moreover, neither inhibitor blocked BCR-induced apoptosis in our experiments, even when BCR-induced ERK activity was blocked almost completely (Figs. 3 and 4). Thus, whereas JNK and/or p38 activity may be required for BCR-induced apoptosis of immature B cells, we conclude that ERK activity likely is not required.
The data presented here demonstrate that the Ras-ERK MAP kinase pathway plays an important role in a subset of B cell responses to Ag. BCR-induced proliferation and up-regulation of specific proteins important for B cell function are dependent on activation of this pathway. In contrast, anti-IgM-induced growth arrest and apoptosis do not appear to require MEK-ERK activity. BCR cross-linking activates many signaling pathways in both immature and mature B cells; however, it appears that particular biological responses to Ag require only some of these events.

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References

FIGURE 5. PD98059 and U0126 inhibit BCR-induced proliferation in the absence and presence of IL-4. A and B, Murine splenic B cells were treated with PD98059 or DMSO and stimulated with anti-IgM (A) or anti-IgM plus 50 U/ml IL-4 (B) for 48 h. Cells were pulsed with [3H]thymidine for the last 5 h. [3H]thymidine incorporation from a representative experiment, performed on the same preparation of B cells, is shown (note different scales on x-axis). Error bars indicate SEM. C, RBC-depleted splenocytes (5 × 10^5/ml) were treated with PD98059, U0126, or DMSO and stimulated with anti-IgM plus 50 U/ml IL-4 for 48 h. BrdUrd (10 μM) was added for the final 24 h. Shown is the percentage of B220+ B cells that incorporated BrdUrd, indicative of entry into S phase of the cell cycle. An average of three experiments is shown. Error bars indicate SEM. D, The ability of 20 μM PD98059 or 10 μM U0126 to inhibit IL-4-mediated survival was assessed. RBC-depleted splenocytes were treated with PD98059, U0126, or DMSO and stimulated with anti-IgM plus 50 U/ml IL-4 for 45 h. Survival was measured by the ability to exclude propidium iodide. A representative experiment is shown.


75. Salmon, R. A., I. N. Foltz, P. R. Young, and J. W. Schrader. 1997. The p38 mitogen-activated protein kinase is activated by ligation of the T or B lymphocyte antigen receptors, Fas or CD40, but suppression of kinase activity does not inhibit apoptosis induced by antigen receptors. *J. Immunol.* 159:5809.