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# Differential Coupling of Membrane Ig and CD40 to the Extracellularly Regulated Kinase Signaling Pathway<sup>1</sup>

Jeffrey M. Purkerson<sup>2\*</sup> and David C. Parker<sup>2\*</sup>

**Coupling of membrane Ig (mIg) and CD40 to the extracellularly regulated kinase (ERK) signal transduction pathway was examined in the WEHI-231 B lymphoma and normal mouse B cells. Cross-linking mIg induces ERK activation in both WEHI-231 and normal B cells. In contrast, CD40 cross-linking failed to induce ERK activation in WEHI-231, but signals through CD40 were more effective than mIg as a stimulus for ERK activation in normal B cells. However, several lines of evidence suggest that CD40 and the B cell Ag regulate ERK through distinct pathways that converge at the level of MEK-1, mitogen-activated protein kinase kinase. Abs to mIg or CD40 induced MEK-1 activation with different kinetics. Cross-linking of mIg, but not CD40, induced tyrosine phosphorylation of the SHC adapter molecule that couples receptors to Ras-dependent signaling pathways. Finally, agents that elevate cAMP, causing protein kinase A-mediated inhibition of Raf-1, inhibited activation of ERK in response to mIg cross-linking, but had no effect on ERK activation in response to anti-CD40 or Jun N-terminal kinase activation by signals through either receptor. Thus, CD40 uses an unidentified protein kinase A-insensitive MEK kinase, rather than Raf-1, to regulate ERK activity. *The Journal of Immunology*, 1998, 160: 2121–2129.**

**T**he B cell Ag receptor (BCR)<sup>3</sup> and CD40 elicit overlapping yet distinct functional responses in B lymphocytes. In resting B cells the BCR is comprised of the membrane-bound forms of IgM and IgD (mIg) and functions to capture and internalize whole protein Ags for processing and subsequent presentation of peptides in the context of MHC class II (1). In addition, mIg is a signal transducing receptor that regulates B cell activation (2). CD40 is the principle receptor through which T cells deliver contact-dependent help for B lymphocytes, and therefore it plays a pivotal role in collaboration between T and B cells in an immune response (3). Either cross-linking of mIg or ligation of CD40 promotes B cell function as APC by inducing expression of the costimulatory molecules B7-1 and B7-2 (4–6) and by up-regulation of expression of MHC class II molecules (J. M. Purkerson et al., unpublished observation). Signaling through either mIg or CD40 promotes cell cycle progression that is facilitated by IL-4, and either receptor can regulate the expression of IL-5R, thereby enabling B cells to respond to cytokine signals that promote B cell maturation (7). However, activation of B cells by signaling through mIg vs CD40 results in the expression of different cell surface molecules that characterize distinct B cell subsets. Cross-linking of mIg, but not CD40, induces expression of CD5; conversely, cross-linking of CD40, but not mIg, up-regulates CD23

expression on B lymphocytes (8). CD5 expression defines a subset of the B-1 cell lineage, while high level expression of CD23, the low affinity receptor for IgE, is a characteristic of the B-2, or conventional B cell, subset (9–11). Furthermore, although mIg cross-linking enables isotype switching to IgG1 that is regulated by IL-5 (12, 13), signals provided by CD40 are required to induce switch recombination to the  $\epsilon$  heavy chain locus and to induce synthesis of IgE (14, 15).

Structurally, CD40 and mIg are very different receptors that associate with distinct signal transduction molecules in the plasma membrane. The BCR associates with Ig $\alpha$  and Ig $\beta$  chains that contain tyrosine activation motifs (TAMs) that are phosphorylated upon mIg cross-linking (16–19). Src family tyrosine kinases and Syk associate with the mIg receptor complex, are activated upon mIg cross-linking, and mediate the association and activation of other effector molecules in the mIg receptor complex (16, 20–26). The role of tyrosine kinases in CD40 signal transduction is controversial. Marked increases in tyrosine phosphorylation of cellular substrates following CD40 ligation has been reported in some systems, but not in others (27–31). CD40 is a member of the TNF receptor family and associates with novel signal transduction molecules termed TNF receptor-associated factors (TRAFs) (32–34). TRAF molecules contain zinc ring and zinc finger-like domains and in some cases an additional isoleucine zipper domain. It is important to note that TRAFs do not contain TAMs that would enable effector molecules containing SH2 domains to associate with the CD40 receptor complex. Truncated forms of TRAF molecules lacking the ring/finger/zipper domains inhibit CD40 signaling when overexpressed in cells, indicating that the zinc-containing structures and the isoleucine zipper are important for transduction of CD40 signals. TRAF-2, -3, -5, and -6 have been implicated in coupling of CD40 to NF- $\kappa$ B (35, 36), and TRAF2 may also couple CD40 to the Jun kinase (JNK) pathway (37).

Despite structural differences between mIg- and CD40-associated molecules, these receptors signal through some of the same signaling intermediates. Membrane Ig and CD40 activate a similar array of *trans*-acting factors, including NF- $\kappa$ B, NF-AT, and activating protein-1 (38–40). Early reports suggested that mIg and CD40 were specifically coupled to the ERK and JNK (MAPK)

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<sup>3</sup> Abbreviations used in this paper: BCR, B cell Ag receptor; mIg, membrane-bound Ig; TAM, tyrosine activation motif; TRAF, tumor necrosis factor receptor-associated factor; NF- $\kappa$ B, nuclear factor- $\kappa$ B; JNK, Jun N-terminal kinase; ERK, extracellularly regulated kinase; MAPK, mitogen activated protein kinase; CD40L, CD40 ligand; MBP, myelin basic protein; GST, glutathione S-transferase; PK-A, protein kinase A.

pathways, respectively (41–43). However, subsequent studies revealed that signals through either CD40 or mIg can activate both ERK and JNK in murine B cells, suggesting that differential coupling to these pathways is quantitative rather than qualitative (31, 44). Although both CD40 and mIg activate NF- $\kappa$ B and MAP kinases, selective inhibition of mIg responses by depletion of protein kinase C suggests that these receptors use distinct mechanisms for coupling to the same signal transduction pathway (31, 38, 45). Selective inhibition of mIg-induced DNA synthesis by elevation of cAMP also suggested utilization of distinct signaling pathways by CD40 and mIg in the regulation of B cell function (8, 46). In other receptor systems, elevation of cAMP inhibits receptor-mediated activation of the ERK pathway (47–52). In this study we examined coupling of CD40 to the ERK pathway and found that in contrast to mIg, activation of ERK by CD40 involves a novel signaling pathway that is insensitive to PK-A.

## Materials and Methods

### Reagents

Affinity-purified Abs were obtained from the following sources: anti-IgM plus IgD heavy and light chain (Fab')<sub>2</sub>, anti-IgM, Fc fragment, Jackson ImmunoResearch Laboratories (West Grove, PA); anti-murine CD40 (hamster IgM), PharMingen (San Diego, CA); polyclonal rabbit anti-Raf-1 (C-12; sc-133), MEK-1 (C-18; sc-219), JNK1 (sc-571), and polyclonal rabbit or goat ERK (C-14; sc-154-G), Santa Cruz Biotech (Santa Cruz, CA); anti-human SHC and anti-phosphotyrosine (4G10), Upstate Biotechnology, Inc. (Lake Placid, NY). Murine CD40L was derived from culture supernatants of J558L cells transfected with a plasmid encoding an mCD40L-CD8 $\alpha$  fusion protein, provided by Dr. Peter Lane (Basel, Switzerland) (53). J558L transfectants were cultured at  $8 \times 10^5$ /ml in Iscove's modified Dulbecco's medium, 1% FCS, 2 mg/ml G418 (Life Technologies, Gaithersburg, MD), and antibiotics. Supernatants were harvested after 24 h and extensively dialyzed against PBS followed by RPMI 1640 (Sigma Chemical Co., St. Louis, MO). Control supernatants were derived from untransfected J558L cells cultured under similar conditions in the absence of G418. PMSF was obtained from Sigma Chemical Co. (St. Louis, MO). Pefabloc SC (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride), aprotinin, leupeptin, pepstatin, and bestatin were purchased from Boehringer Mannheim (Indianapolis, IN). PMA and myelin basic protein (MBP) for in-gel kinase assays were purchased from Sigma Chemical Co. Ultrapure MBP for immune complex kinase assays was purchased from Upstate Biotechnology. Activators of adenylate cyclase, including cholera toxin, forskolin (6-*O*-[3'-piperidino]propionyl] hydrochloride), the inactive analogue, forskolin (1,9-dideoxy-), dibutyryl cAMP, and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine were purchased from Calbiochem (La Jolla, CA).

### Cells

The murine B lymphoma line, WEHI-231, was maintained in RPMI 1640 and 5% FCS/5% CS, supplemented with 50  $\mu$ M 2-ME, 2 mM glutamine, and antibiotics. The WEHI-231 cell line used in these studies exhibited anti-Ig-induced growth arrest followed by apoptosis and rescue from apoptosis by concomitant signaling through CD40 (54). Normal B cells were isolated from spleens obtained from C57BL/6, C57BL/10, or BALB/c mice (8–20 wk of age) and depleted of T cells by incubation at 4°C with a mixture of anti-T cell Abs followed by complement-mediated lysis at 37°C (55). Small, resting B cells were obtained from T-depleted spleen cells by either centrifugal elutriation or centrifugation through discontinuous Percoll gradients as described previously (56, 57). Cells were washed three times with serum-free medium (RPMI 1640 supplemented with 1% BSA (fraction V), 50  $\mu$ M 2-ME, 2 mM glutamine, and antibiotics) to remove serum-containing protease activity. After washing, cells were resuspended in serum-free medium and equilibrated to 37°C for 15 to 30 min before stimulation.

### Cell lysates

For immune precipitation of SHC, cells were lysed by the addition of 4 $\times$  modified RIPA buffer (final concentration of 1 $\times$  RIPA buffer: 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF or Peflabloc SC, and 1  $\mu$ g/ml of aprotinin, leupeptin, and pepstatin). For immune precipitation of ERK, MEK-1, and Raf-1, cells were lysed by addition of 1% Triton X-100, 150 mM NaCl, 10 mM Tris

(pH 7.4), 400  $\mu$ M EDTA, 1 mM PMSF, or Peflabloc SC, 2  $\mu$ g/ml of aprotinin, leupeptin, and pepstatin, 1 mM benzamide, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 400  $\mu$ M sodium vanadate, and 20 mM  $\beta$ -glycerophosphate. For in-gel kinase assays, cells were lysed in whole cell extract buffer (WCE buffer: 25 mM HEPES (pH 7.7), 0.3 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM  $\beta$ -glycerophosphate, 0.1 mM sodium vanadate, 1 mM PMSF, and 2  $\mu$ g/ml of aprotinin, leupeptin, and pepstatin). Cell suspensions were incubated on ice for 30 min and then clarified by centrifugation at 15,000  $\times$  g for 10 min.

### In-gel kinase assay

Activity of MAP kinases in SDS-PAGE gels was determined as described by Kameshita and Fujisawa with minor modifications (58). Polyacrylamide gels were cast with 0.5 mg/ml MBP (Sigma) added before gel polymerization. Following electrophoresis of cell lysates (10–50  $\mu$ g total protein), SDS was removed by rocking the gel in 20% isopropanol and 50 mM Tris-HCl, pH 8.0 (three times, 100 ml each time, 1-h total wash time), followed by 50 mM Tris-HCl, pH 8.0, and 1 mM DTT. Proteins in the gel were denatured by incubation in 100 ml of 6 M guanidine-HCl (Ultrapure, Life Technologies), 20 mM DTT, 2 mM EDTA, and 50 mM Tris-HCl, pH 8.0, for 1 h. Proteins were renatured by incubation at 4°C, without agitation, in 250 ml of 50 mM Tris-HCl, 1 mM DTT, 2 mM EDTA, and 0.04% Tween 20 for 12 to 18 h. Kinase assays were performed by equilibrating the gel in 100 ml of kinase buffer (1 mM DTT, 0.1 mM EGTA, 20 mM MgCl<sub>2</sub>, 40 mM HEPES (pH 8.0), and 100  $\mu$ M sodium vanadate) for 30 min, followed by incubation at room temperature in 10 to 25 ml kinase buffer supplemented with 50  $\mu$ M ATP and [ $\gamma$ -<sup>32</sup>P]ATP (5–10  $\mu$ Ci/ml, 3000 Ci/mmol; DuPont-New England Nuclear, Boston, MA) for an additional 30 min. The gels were washed extensively in 5% (w/v) TCA plus 1% sodium pyrophosphate (Sigma). Autoradiography of dried gels was performed using Kodak X-OMAT film (Eastman Kodak, Rochester, NY).

### Immune precipitation and Western blotting

Cell lysates (1 ml) were precleared by rotation with either protein A- or protein G-Sepharose (50  $\mu$ l of 50% slurry) for ~2 h at 4°C. In some experiments lysates were precleared with the combination of 1  $\mu$ g/ml non-specific rabbit IgG and protein A-Sepharose. Polyclonal rabbit Abs specific for SHC (5  $\mu$ g/ml), Raf-1 (1  $\mu$ g/ml), and MEK-1 (1  $\mu$ g/ml) or a polyclonal goat Ab specific for ERK (2  $\mu$ g/ml) were added to cell lysates along with protein A-Sepharose (50  $\mu$ l of 50%) for immune precipitations using rabbit Abs or protein G-Sepharose for immune precipitations with goat Abs and rotated for 4 to 16 h at 4°C. Beads were washed three times with lysis buffer followed by either a wash with kinase buffer (see below) in preparation for immune complex kinase assays or boiling following the addition of an equal volume of 2 $\times$  sample buffer (final concentrations: 50 mM Tris (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) in preparation for Western blotting. Boiled samples were resolved by SDS-PAGE (8–10% polyacrylamide gels). Gels were equilibrated in transfer buffer (10 mM CAPS (pH 11.0) and 10% methanol) and transferred (400 mA, for 2 h) to Immobilon-P (polyvinylidene difluoride) membranes (Millipore, Bedford, MA) using the Panther (HEP-1) semidry electroblotter (Owl Scientific, Cambridge, MA). Membranes were blocked by incubation without agitation in 1% BSA/PBS/0.05% sodium azide overnight at 4°C. Membranes were incubated with (0.5–1  $\mu$ g/ml) biotin-conjugated anti-phosphotyrosine (4G10), anti-SHC (0.5  $\mu$ g/ml), anti-Raf-1 (0.25–0.5  $\mu$ g/ml), or anti-ERK (1  $\mu$ g/ml) in PBS/0.05% Tween 20 for 2 to 4 h at room temperature. Polyclonal rabbit primary Abs were detected by 2- to 4-h incubation at room temperature with a 1/10,000 dilution of biotin-conjugated donkey anti-rabbit secondary Ab (Jackson ImmunoResearch, West Grove, PA). Incubation with biotin-conjugated Ab was followed by 0.5 to 1 h at room temperature incubation with (1/1000) streptavidin-horseradish peroxidase conjugate (Amersham, Arlington Heights, IL). All incubations were followed by five 100-ml washes with PBS/0.05% Tween 20 over a period of 30 min. Western blots were visualized by incubation with ECL (Amersham) or Renaissance chemiluminescence reagents (DuPont-New England Nuclear) according to the protocols recommended by the manufacturer followed by exposure (30–120 s) to Reflection film (DuPont-New England Nuclear).

### Immune complex kinase assays

Enzyme-Ab complexes were washed once with 1 ml of kinase buffer (Raf-1 buffer: 10 mM PIPES (pH 7.0), 100 mM NaCl, 5 mM MnCl<sub>2</sub>, and 2  $\mu$ g/ml aprotinin; ERK/MEK-1 buffer: 25 mM HEPES (pH 7.4), 1 mM DTT, and 10 mM MgCl<sub>2</sub>). Kinase reactions were performed by addition of 30  $\mu$ l of kinase buffer supplemented with substrate (ERK substrate: 5–10

$\mu\text{g}$  MBP (Upstate Biotechnology); Raf-1 substrate: 100–200 ng/ml GST-MEK-1 K97R (gift from K.-L. Guan, University of Michigan); MEK-1 substrate: 100–200 ng His-ERK (p42; sc-4024; Santa Cruz Biotechnology) plus 5–10  $\mu\text{g}$  MBP (Upstate Biotechnology), 25–50  $\mu\text{M}$  ATP, and 5  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP (3000 Ci/mmol), and the reaction mixtures were incubated at 30°C for 30 min. Kinase reactions were terminated by the addition of 2 $\times$  sample buffer and incubation at 100°C for 5 min. Substrates were resolved in SDS-PAGE gels (10% MBP and 8% GST-MEK-1), and incorporation of  $^{32}\text{P}$  into substrates was determined by autoradiography of dried gels using Reflection (DuPont-New England Nuclear) or Kodak X-OMAT film. Exposure time ranged from 0.5 to 4 h for ERK and MEK-1 assays and from 24 to 48 h with two intensifying screens for Raf-1 assays. Densitometric analysis of files generated by scanning autoradiographs (HP Scanjet II, Hewlett-Packard, Palo Alto, CA) was performed using National Institutes of Health Image 1.54 software (Bethesda, MD).

### Solid phase Jun kinase assays

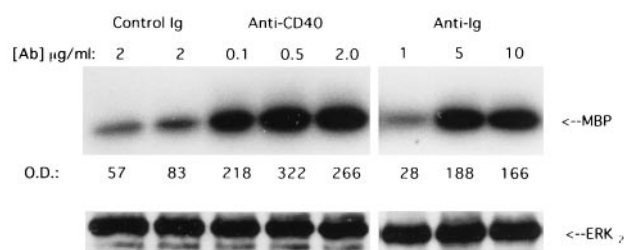
Activity of JNK in cell lysates was determined as described by Hibi et al. (59). Briefly, cell lysates prepared using WCE buffer were adjusted to a binding buffer composition of 20 mM HEPES (pH 7.7), 75 mM NaCl, 2.5 mM  $\text{MgCl}_2$ , 0.1 mM EDTA, 0.05% Triton X-100, 0.5 mM DTT, 20 mM  $\beta$ -glycerophosphate, 0.1 mM sodium vanadate, 1 mM PMSF, and 2  $\mu\text{g}/\text{ml}$  of aprotinin, leupeptin, and pepstatin. GST-Jun<sub>1–223</sub> bound to glutathione-agarose beads (Sigma) was incubated with cell lysates for 3 to 16 h. Beads were washed three times with binding buffer (20 mM HEPES (pH 7.7), 75 mM NaCl, 2.5 mM  $\text{MgCl}_2$ , 0.1 mM EDTA, and 0.05% Triton X-100). The relative amount of JNK enzyme bound to GST-Jun agarose beads for each sample was determined by Western blotting using polyclonal anti-JNK1 (Santa Cruz Biotechnology). Jun kinase activity was determined by incubating beads in 30  $\mu\text{l}$  of kinase buffer (20 mM HEPES (pH 7.6), 20 mM  $\text{MgCl}_2$ , 2 mM DTT, 20 mM  $\beta$ -glycerophosphate, and 0.1 mM sodium vanadate) supplemented with 25  $\mu\text{M}$  ATP and 5  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP for 30 min at 30°C. Reactions were terminated by the addition of 2 $\times$  Laemmli sample buffer and resolved on 8 to 10% SDS-PAGE gels. Incorporation of  $^{32}\text{P}$ -labeled phosphate into GST-Jun was quantitated by autoradiography using Reflection film (DuPont-New England Nuclear). Exposure time ranged from 0.5 to 4 h.

## Results

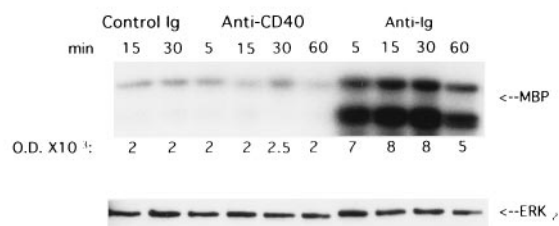
### CD40 is effectively coupled to the ERK pathway in normal splenic B cells, but not in WEHI-231

Whether CD40 effectively couples to the ERK pathway in B lymphocytes remains controversial (31, 41–44). To determine whether regulation of the ERK pathway is associated with a mitogenic response to CD40, the activation of ERK was examined in both the WEHI-231 B lymphoma and normal mouse B cells. Although signals through CD40 are mitogenic for normal mouse B cells (53), spontaneous cell cycle progression in WEHI-231 cells is unaffected by CD40 (54). The activity of ERK<sub>2</sub> was induced up to fivefold in splenic B cells 15 min after treatment with anti-CD40 (Fig. 1A). Levels of ERK<sub>2</sub> activity stimulated by cross-linking of CD40 were consistently higher than those induced by 1 to 15  $\mu\text{g}/\text{ml}$  of anti-Ig (Figs. 1 and 2). In WEHI-231 cells, anti-CD40 failed to stimulate ERK<sub>2</sub> activity over the time course examined (5–60 min), while cross-linking of mIg was an effective stimulus for ERK<sub>2</sub> activation, with maximal ERK<sub>2</sub> activity occurring 15 to 30 min after addition of anti-Ig (Fig. 1B). In an earlier study Kashiwada et al. reported that CD40 cross-linking induced rapid and transient activation of ERK (within 1–2 min following addition of anti-CD40) in WEHI-231 cells (44). An examination of ERK activity at points between 30 s and 5 min after addition of anti-CD40 failed to reveal CD40 coupling to the ERK pathway in the WEHI-231 cells used in this study (data not shown). In contrast, activation of ERK<sub>2</sub> in WEHI-231 cells was observed as early as 30 s to 1 min following addition of anti-Ig (data not shown). These WEHI-231 cells do respond to signals through CD40, since anti-CD40 treatment induces marked activation of *jun* kinases (Fig. 3C) and prevented mIg-induced growth arrest in these cells (data not shown). These data suggest that coupling of CD40 to the ERK pathway is associated with a mitogenic response in B lymphocytes. In addition,

### A) Normal B cells



### B) WEHI-231



**FIGURE 1.** Cross-linking CD40 activates ERK in normal B lymphocytes, but not in WEHI-231 cells. *A*, Splenic B cells ( $3 \times 10^7/\text{ml}$ ) were treated with control Ab (hamster Ig, 2  $\mu\text{g}/\text{ml}$ ), anti-CD40 (0.1–2  $\mu\text{g}/\text{ml}$ ), or anti-Ig (Fab')<sub>2</sub> (1–10  $\mu\text{g}/\text{ml}$ ) for 15 min. *B*, WEHI-231 cells ( $10^7/\text{ml}$ ) were treated for 5 to 60 min with control Ab (hamster Ig, 0.5  $\mu\text{g}/\text{ml}$ ), anti-CD40 (0.5  $\mu\text{g}/\text{ml}$ ), or anti-Ig (Fab')<sub>2</sub> (10  $\mu\text{g}/\text{ml}$ ). Activity of ERK in cell lysates was determined by an immune complex assay using MBP as a substrate (upper panels). Levels of ERK in immune precipitates were determined by Western blotting (lower panels).

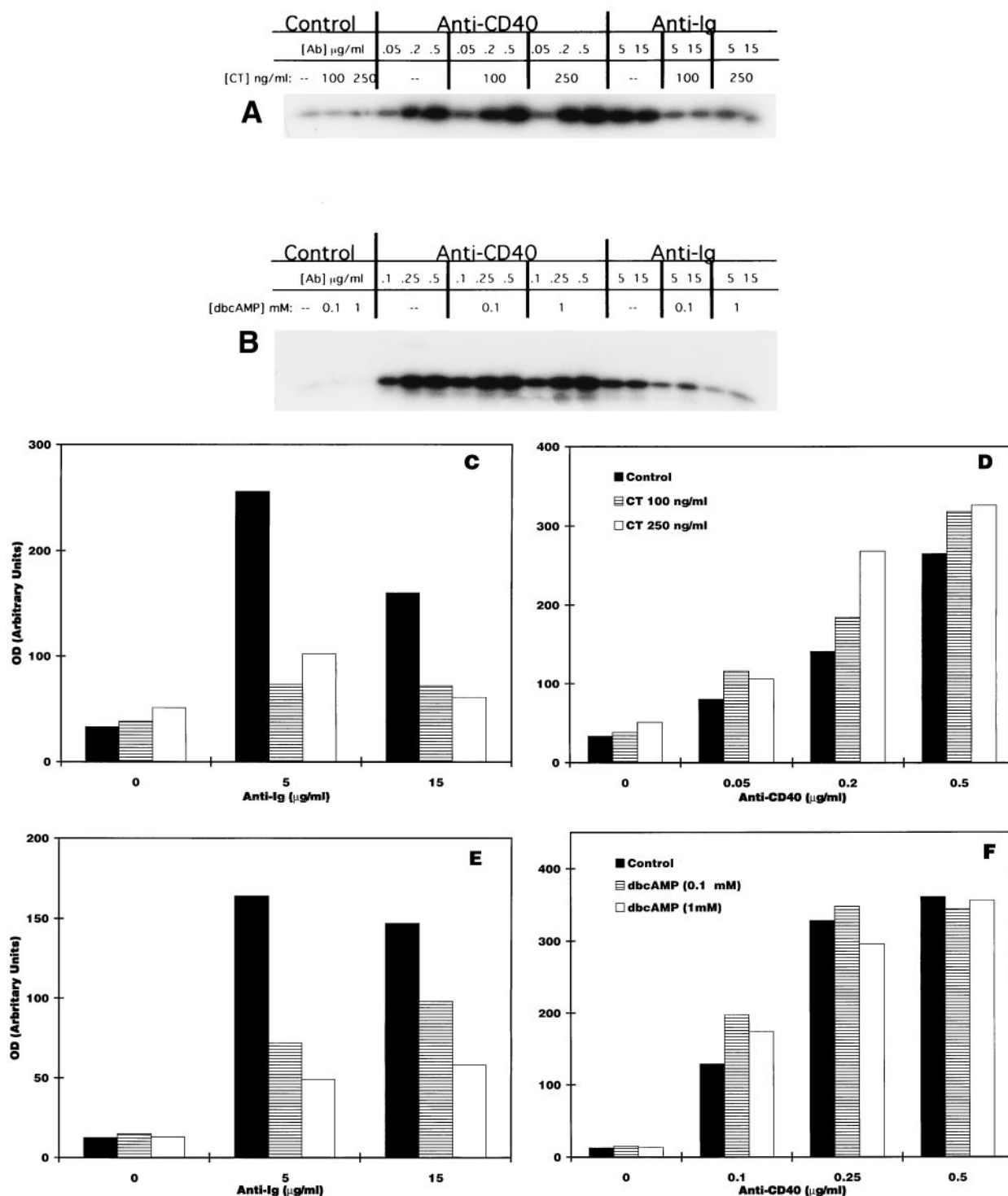
these results indicate that transformed B cell lines are not always suitable models for studying signal transduction in normal B cells.

### Elevation of cAMP inhibits ERK activation by signals through mIg, but not through CD40

Treatment of B lymphocytes with agents that elevate cAMP selectively inhibits DNA synthesis stimulated by signals through mIg, but not through CD40 (8, 46), suggesting that PK-A activation differentially inhibits signal transduction by mIg and CD40. The effects of agents that elevate cAMP on ERK<sub>2</sub> activation by CD40 was examined to determine whether CD40 regulates ERK<sub>2</sub> through a PK-A-sensitive pathway. As shown in Figure 2, *A*, *B*, *C*, and *E*, treatment of normal B cells with either cholera toxin or dibutyryl cAMP (0.1–1 mM) inhibited ERK<sub>2</sub> activation by two- to threefold in response to stimulation with optimal concentrations of anti-Ig (5–15  $\mu\text{g}/\text{ml}$ ). In contrast, ERK<sub>2</sub> activation induced by anti-CD40 was unaffected by treatment with agents that elevate cAMP in cells, as neither cholera toxin nor dibutyryl cAMP (Fig. 2, *A*, *B*, *D*, and *F*) inhibited the response to suboptimal concentrations of anti-CD40 (0.1  $\mu\text{g}/\text{ml}$ ). Similar results were obtained with forskolin-treated cells (not shown). Thus, CD40 regulates ERK<sub>2</sub> activity through a PK-A-insensitive pathway.

### Elevation of cAMP inhibits Raf-1 and subsequent ERK, but not JNK, activation by mIg

To define the mechanism(s) by which elevation of cAMP modulates signals through mIg, the effect of cholera toxin on mIg coupling to the ERK pathway was examined. Pretreatment of WEHI-231 cells with cholera toxin inhibited activation of Raf-1 and ERK

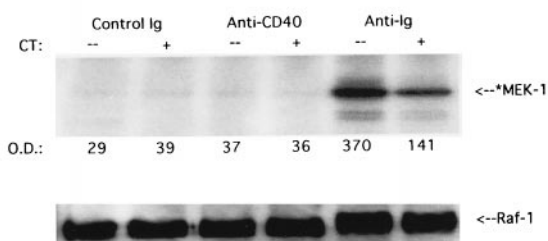


**FIGURE 2.** Elevation of cAMP does not inhibit ERK activation by signals through CD40. Splenic B cells ( $1-2 \times 10^7/\text{ml}$ ) were treated with 3-isobutyl-1-methylxanthine ( $200 \mu\text{M}$ ) and either cholera toxin ( $100-250 \text{ ng/ml}$ ) in *A* or dibutyryl cAMP ( $0.1$  or  $1 \text{ mM}$ ) in *B* for  $15 \text{ min}$  followed by the indicated concentrations of anti-Ig ( $\text{Fab}'_2$ ) or anti-CD40 for an additional  $15 \text{ min}$ . The activity of ERK in cell lysates was determined by an immune complex assay using MBP as a substrate. Results presented in *C* and *D* and in *E* and *F* are OD values generated by densitometric scans of autoradiograms shown in *A* and *B*, respectively. The results shown in *A* and *B* represent two independent experiments.

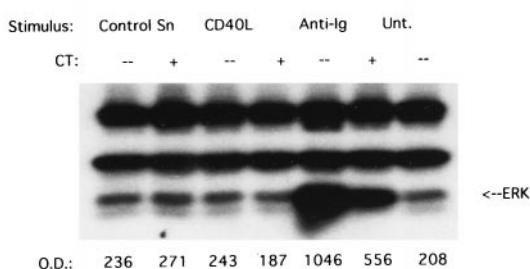
by mIg cross-linking (Fig. 3, *A* and *B*, respectively). Similar results were obtained with forskolin (data not shown). Consistent with results presented in Figure 1, treatment of B lymphoma cells with either soluble CD40L (Fig. 3*B*) or anti-CD40 (Fig. 3*A*) failed to induce Raf-1 and ERK activation in the presence or the absence of cholera toxin. In two independent experiments, treatment of WEHI-231 cells with either cholera toxin or forskolin did not af-

fect activation of JNK by either mIg or CD40 (Fig. 3*C*), demonstrating that elevation of cAMP does not affect the MEK kinase(s) that functions in the Jun kinase pathway. Forskolin had no effect on induction of SHC phosphorylation by anti-Ig, indicating that the effects of cAMP lie distal to recruitment and tyrosine phosphorylation of SHC (data not shown). Together these results suggest that inhibition of mIg-induced DNA synthesis by agents that elevate

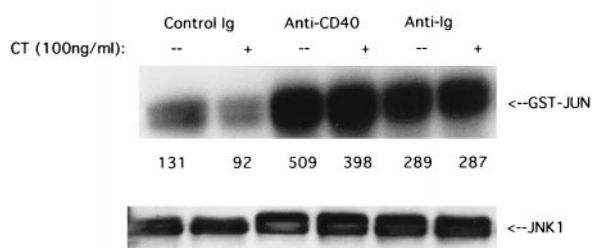
## A) Raf-1 Activity



## B) ERK Activity



## C) JNK Activity

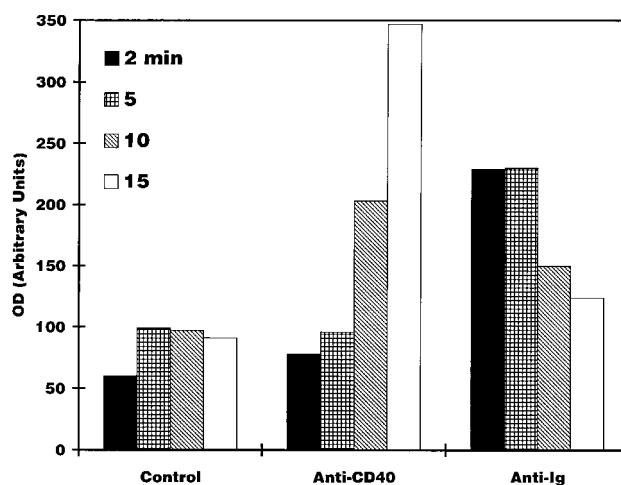


**FIGURE 3.** Cholera toxin inhibits Raf-1 and ERK activation by mIg in WEHI-231 cells. *A*, WEHI-231 cells ( $10^7$ /ml) were pretreated for 15 min with cholera toxin (100 ng/ml) or left untreated before the addition of control Ab (hamster Ig, 1  $\mu$ g/ml), anti-CD40 (0.5  $\mu$ g/ml), or anti-Ig (Fab')<sub>2</sub> (10  $\mu$ g/ml). After 5 min the activity of Raf-1 in cell lysates was determined by an immune complex kinase assay using kinase-inactive MEK-1 as a substrate (*upper panel*). Levels of Raf-1 in immune precipitates were determined by Western blotting (*lower panel*). *B*, WEHI-231 cells ( $10^7$ /ml) were treated with soluble CD40L-CD8 (20% (v/v) of 50-fold concentrated supernatant), control supernatant, or anti-Ig (30  $\mu$ g/ml) for 5 min. ERK activity in cell lysates was determined by an in-gel kinase assay using MBP as a substrate. Cholera toxin (10 ng/ml) was added 15 min before stimulation. *C*, WEHI-231 cells ( $10^7$ /ml) were treated as described in *A*. The activity of JNK in cell lysates was determined by a solid phase kinase assay using GST-Jun as a substrate (*upper panel*). The amount of JNK precipitated by GST-Jun agarose was determined by Western blotting using an anti-JNK1 Ab (*lower panel*).

cAMP may be explained at least in part by inhibition of the ERK pathway at the level of Raf-1. To date, efforts to measure Raf-1 activation in normal B cells have failed to reveal receptor regulation of this enzyme.

## Signals through either mIg or CD40 activate MEK-1

The results presented in Figures 2 and 3 indicate that CD40 regulates ERK activity through a Raf-1-independent pathway. In response to signals from a variety of receptors, activation of ERK is mediated by dual phosphorylation on tyrosine and threonine resi-

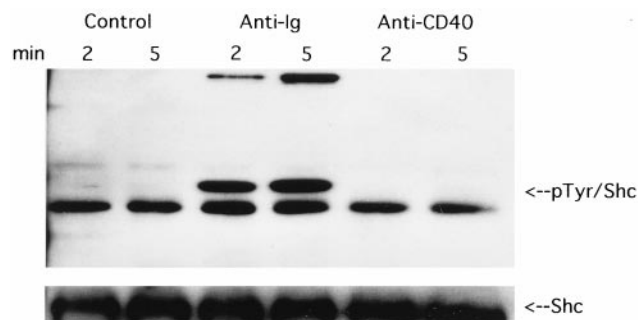


**FIGURE 4.** Signals through mIg and CD40 activate MEK-1 with different kinetics. Small B cells ( $1-2 \times 10^7$ /ml) were treated with either anti-CD40 (0.5  $\mu$ g/ml) or anti-Ig (Fab')<sub>2</sub> (10  $\mu$ g/ml) or were left untreated for 2, 5, 10, and 15 min. The activity of MEK-1 in cell lysates was determined by a linked immune complex assay using His-ERK<sub>2</sub> and MBP as substrates. The results presented are OD values generated by a densitometric scan of an autoradiogram.

dues by MEK-1 (Fig. 6). To determine whether MEK-1 functions in the pathway regulated by CD40, the effect of receptor cross-linking on MEK-1 activity was examined. Treatment of normal B cells with either anti-CD40 or anti-Ig stimulated MEK-1 activity two- to threefold (Fig. 4). Interestingly, MEK-1 activation by cross-linking of CD40 was delayed relative to the mIg response. MEK-1 activation was induced 10 min following addition of anti-CD40, and activity further increased by 15 min. Activation of ERK<sub>2</sub> by signals through CD40 exhibited similar kinetics (data not shown). In contrast, activation of MEK-1 was maximal 2 min following addition of anti-Ig and declined after 10 min. Interestingly, ERK<sub>2</sub> activity in cells treated with anti-Ig declined only slightly after 15 min, indicating that ERK<sub>2</sub> activation is maintained for several minutes following down-regulation of MEK-1 activity. (not shown). These data suggest that regulation of ERK by signals through either receptor is mediated by MEK-1, and thus, MEK-1 is the point of convergence between CD40 and mIg signaling pathways leading to ERK activation.

## Activation of ERK by CD40 is not associated with tyrosine phosphorylation of SHC

Differences in the kinetics of MEK-1 activation suggests that mIg and CD40 couple to the ERK pathway through distinct mechanisms. Cross-linking of mIg results in the recruitment of SHC to phosphorylated TAM sequences within the Ig $\alpha$  and Ig $\beta$  chains (60). Subsequent phosphorylation of SHC on tyrosine residues enables formation of the SHC-GRB2-SOS complex and activation of Ras through SOS-mediated guanine nucleotide exchange (61-64). Whether CD40 uses SHC as an adapter molecule to couple to the ERK pathway was examined by monitoring tyrosine phosphorylation of SHC in response to treatment of cells with anti-CD40. As shown in Figure 5, SHC was phosphorylated on tyrosine following treatment of splenic B cells with anti-Ig, but not with anti-CD40. A 130- to 150-kDa phosphoprotein coprecipitated with SHC from cells treated with anti-Ig, but not with those treated with anti-CD40. In anti-Ig-treated cells phosphorylation of SHC was maximal by 5 min, and SHC remained phosphorylated 15 min after addition of anti-Ig (data not shown). In contrast, SHC was not



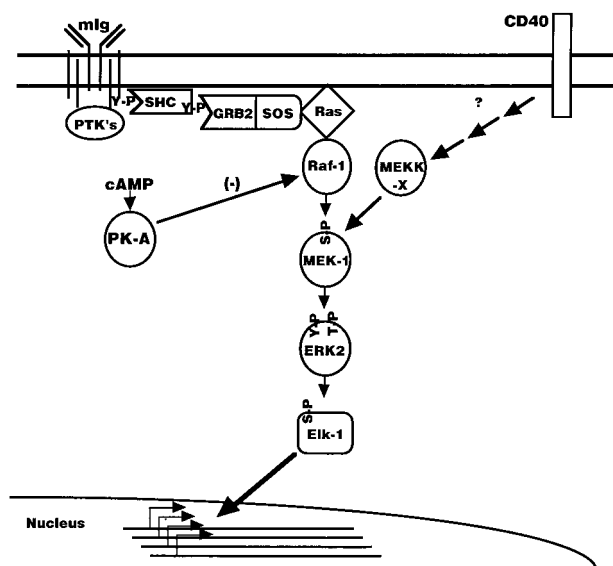
**FIGURE 5.** Cross-linking of mIg, but not CD40, induces tyrosine phosphorylation of SHC in normal B cells. Splenic B cells ( $3 \times 10^7$ /ml) were treated with anti-Ig (Fab')<sub>2</sub> (20  $\mu$ g/ml) or anti-CD40 (2  $\mu$ g/ml) or were left untreated for 2 or 5 min. SHC was immune precipitated from cell lysates and analyzed for phosphotyrosine content by Western blotting using the 4G10 Ab (upper panel). The amount of SHC protein in the immune precipitates was determined by Western blotting (lower panel).

phosphorylated on tyrosine 15 min following treatment with anti-CD40, at which time ERK<sub>2</sub> activation by signals through CD40 were maximal. The results presented in Figure 5 are representative of at least three independent experiments. These results indicate that CD40 uses adapter proteins other than SHC to couple to the ERK pathway.

## Discussion

Differential effects of pharmacologic agents on DNA synthesis stimulated by mIg and CD40 suggested that these receptors regulate B cell function through distinct signal transduction pathways (46). Subsequent studies have revealed that mIg and CD40 control B cell responses through activation of a similar array of *trans*-acting factors, including NF- $\kappa$ B, NF-AT, and activating protein-1 (39, 40, 45, 65). Receptor-specific regulation of *trans*-acting factors has been reported, and as studies of CD40 and mIg signal transduction continue, more differences are likely to be revealed (66, 67). Both receptors activate MAP kinases, including ERK, JNK, and p38 (31, 41–44). Differential activation of MAP kinases by signals through mIg and CD40 in B cells appears to be more a function of the cellular differentiation state than an intrinsic property of the receptor. Thus, while CD40 is not effectively coupled to the ERK pathway in transformed B cell lines, CD40 cross-linking stimulates ERK<sub>2</sub> activation in normal, murine B cells (Fig. 1). Although both mIg and CD40 activate ERK, Jun, and p38 kinases, differences in the kinetics and level of activation suggest distinct mechanisms of coupling to these pathways.

Characterization of the effects of cAMP elevation on MAP kinase activation revealed that CD40 and the BCR activate ERK<sub>2</sub> through different pathways that converge at MEK-1 (Fig. 6). Both CD40 and mIg induce MEK-1 activation in normal B cells (Fig. 4). A specific inhibitor of MEK-1, PD98059, blocks ERK<sub>2</sub> activation by signals through either receptor, indicating that MEK-1 activity is required for ERK<sub>2</sub> activation by signals through CD40 or mIg (31). Cross-linking of mIg induced Raf-1 activity in WEHI-231 cells, and elevation of cAMP inhibited Raf-1 activation by this receptor (Fig. 3A). The latter result is consistent with reports demonstrating that phosphorylation of Raf-1 by PK-A inhibits the MEK kinase activity of Raf-1 (47–52). Although induction of Raf-1 activity by cross-linking mIg was not observed in normal B lymphocytes, elevation of cAMP inhibited ERK<sub>2</sub> activation by signals through mIg (Fig. 2). The inability to measure Raf-1 activity in normal B cells might be explained by a PK-A-sensitive MEK



**FIGURE 6.** Membrane Ig and CD40 induce ERK activation through distinct pathways that converge at MEK-1. Signals through either CD40 or mIg activate MEK-1 and ERK. In contrast to mIg, regulation of MEK-1 by signals through CD40 involves an undefined MEK kinase that is insensitive to inhibition by PK-A.

kinase other than Raf-1 that functions in these cells; however, since Raf-1 activation involves dimerization and undefined interactions following Ras-dependent recruitment to the plasma membrane, immune complex kinase assays may not detect Raf-1 activity in normal B cells (68–71). Taken together, these results indicate that in normal B cells activation of MEK-1 by mIg is mediated by a PK-A-sensitive MEK kinase that is most likely Raf-1.

The evidence presented here indicates that CD40 uses a MEK-1 kinase activity other than Raf-1 in the regulation of ERK (Fig. 6). Elevation of cAMP did not affect ERK<sub>2</sub> activation by signals through CD40, demonstrating that the CD40 pathway involves a PK-A-insensitive MEK kinase (Fig. 2). The identity of the enzyme that regulates MEK-1 activity in the CD40 pathway remains to be established. One candidate enzyme is MEKK-1, a murine homologue of Byr2 and Ste11 kinases in yeast. Overexpression of MEKK-1 in cells induces activation of both ERK and JNK; however, at lower levels of expression, MEKK-1 selectively activates JNK (72, 73). In macrophages, activation of ERK by TNF- $\alpha$  correlates with increases in MEKK-1, but not in Raf-1, activity (74). Although cross-linking of CD40 is a very effective stimulus for JNK activation in B cells, receptor signals only slightly increase MEKK-1 activity in Ramos cells, an EBV-transformed B cell line (41). Furthermore, cross-linking of CD40 does not affect MEKK-1 activity in WEHI-231 cells under conditions that activate Jun kinases (J. M. Purkerson et al., unpublished observations). Although elevation of cAMP in PC12 cells inhibits MEKK-1 activation by epidermal growth factor, nerve growth factor, and tetradecanoyl phorbol acetate (75), elevation of cAMP does not inhibit JNK activation by CD40 (Fig. 3C). Thus, preliminary efforts to correlate MAPK activation with increases in MEKK-1 activity have failed to reveal a role for MEKK-1 in ERK or JNK activation by CD40. B-Raf is another candidate for a MEK kinase that is regulated by CD40. In PC12 cells B-Raf activity is positively regulated by elevation of cAMP, and B-Raf mediates activation of the ERK pathway by cAMP agonists (76). However, elevation of cAMP alone was not sufficient to activate MAP kinase pathways in mouse B

cells (Figs. 2 and 3), suggesting differences between PC12 cells and B cells with respect to coupling of PK-A to the ERK pathway. Interestingly, the CD40-associated molecule, TRAF-2, binds a kinase, termed NIK, that is related to mammalian and yeast MEK kinases (77). It is intriguing to speculate that CD40 is directly coupled to NIK through TRAF-2 and that NIK mediates activation of ERK<sub>2</sub> through phosphorylation of MEK-1. The role of NIK and other MEK kinases in the regulation of the MAP kinase pathway by CD40 is currently under investigation.

The function of guanine nucleotide binding proteins, such as Ras, in MAP kinase pathways is to recruit MEK kinases to the plasma membrane, where the kinase is activated via an undefined mechanism (68, 78). CD40 triggers guanine nucleotide exchange by Ras and Rac1 in an EBV-transformed B cell line, and expression of a dominant inhibitory mutant of Ras blocks MAP kinase activation by signals through CD40 (79). Since Ras mediates activation of MEK kinases other than Raf-1 (75), the results presented in this study are not inconsistent with Ras-dependent coupling of CD40 to a PK-A insensitive MEK kinase. A direct examination of Ras function in the ERK pathway of normal mouse B cells is under way to confirm the results obtained in human B cell lines.

CD40 and mIg use distinct adapter molecules to couple to GTP binding proteins in the MAP kinase pathway. As depicted in Figure 6, cross-linking of mIg promotes association of SHC with the Ig $\alpha$  and Ig $\beta$  chains and the formation of the SHC-GRB2-SOS complex that triggers guanine nucleotide exchange by Ras (61, 62). Signals through CD40 did not induce tyrosine phosphorylation of SHC, suggesting that CD40 does not use SHC as an adapter molecule to couple to the ERK pathway (Fig. 5). Although signals through CD40 induce phosphorylation of SOS, SOS phosphorylation is subsequent to ERK activation and results in feedback inhibition of the MAP kinase pathway (31, 49). Thus, whether SOS functions as a guanine nucleotide exchange factor in the ERK pathway regulated by CD40 remains to be established. Furthermore, coupling of CD40 to MAP kinase pathways may involve adapter molecules whose association with the CD40 receptor complex is not regulated by tyrosine phosphorylation. In contrast to the Ig $\alpha$  and Ig $\beta$  chains that interact with the BCR, TRAF molecules that associate with CD40 do not contain TAMs. TRAF-2 has been implicated in coupling of TNF receptor 1 to the JNK pathway (80, 81). Whether TRAF-2 directly or indirectly mediates association with GRB2/SOS or other adapter/guanine nucleotide exchange factors, thereby enabling coupling of CD40 to the ERK pathway, remains to be established.

Perhaps the most significant finding of this study is that CD40 regulates ERK through a PK-A-insensitive pathway. The absence of PK-A-mediated inhibition of mitogenic signal transduction through CD40 is significant because signals through receptors that couple to adenylate cyclase frequently enhance B cell responses to CD40 ligation. Elevation of cAMP synergizes with CD40 for induction of B7-1 and B7-2 expression (82, 83). In addition, agonists for adenylate cyclase promote Ig isotype production regulated by CD40 in human B lymphocytes.  $\beta_2$  adrenergic receptor agonists facilitate isotype switch to IgE stimulated by CD40 and IL-4 in cultures of human PBMC (84). PGE<sub>2</sub> or agents that elevate cAMP enhance proliferation of purified tonsillar B cells signaled through CD40 and promote the secretion of IgM, IgG, and IgA induced by IL-10 in conjunction with CD40, but inhibit IL-4-induced switching to IgG and IgE (85). Thus, agonists for adenylate cyclase can either promote or inhibit responses regulated by CD40 depending on the cellular and cytokine context in which cAMP synthesis is induced. Whether activation of adenylate cyclase is a direct result of CD40 ligation is unclear. Synthesis of cAMP in B cells treated

with membrane preparations from activated T cells has been observed in some laboratories (86, 87), but not in others (88). Interestingly, inhibitors of PK-A block DNA synthesis stimulated by membranes from activated T cells (87) and abrogate NF- $\kappa$ B dependent activation of the HIV-1 long terminal repeat induced by signals through CD40 (89). However, a direct effect of CD40 ligation on the regulation of adenylate cyclase activity has not been reported. Since ERK activation plays a central role in the transmission of mitogenic signals to the nucleus (90, 91), regulation of ERK activity through a cAMP-insensitive pathway enables agonists for adenylate cyclase to act in concert with signals through CD40 without inhibiting mitogenic signal transduction.

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