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Convergence of CD19 and B Cell Antigen Receptor Signals at MEK1 in the ERK2 Activation Cascade

Xiaoli Li* and Robert H. Carter2*†‡

CD19 plays a critical role in regulating B cell responses to Ag. We have studied the mechanism by which coligation of CD19 and the B cell Ag receptor, membrane Ig (mIg), augments signal transduction, including synergistic enhancement of release of intracellular Ca2+ and extracellular signal-regulated protein kinase 2 (ERK2) activation, in Daudi human B lymphoblastoid cells. The pathway leading to ERK2 activation was further dissected to determine how signals derived from CD19 and mIgM interact.

The best-defined pathway, known to be activated by mIgM, consists of the sequential activation of the mitogen-activated protein kinase (MAPK) cascade that includes Ras, Raf, MAPK kinase 1 (MEK1), and ERK2. Ligation of CD19 alone had little effect on these. CD19-mIgM coligation did not increase activation of Ras or Raf beyond that induced by ligation of mIgM alone. In contrast, coligation resulted in synergistic activation of MEK1. Furthermore, synergistic activation of ERK2 occurred in the absence of changes in intracellular Ca2+, and was not blocked by inhibition of protein kinase C activity and represents a separate pathway by which CD19 regulates B cell function. Thus, the CD19-dependent signal after CD19-mIgM coligation converges with that generated by mIgM at MEK1. The intermediate kinases in the MAPK cascade leading to ERK2 integrate signals from lymphocyte coreceptors. * The Journal of Immunology, 1998, 161: 5901–5908.

The functional complexity of lymphocytes requires the integration of multiple regulatory signals. CD19 is a component of a multimeric complex on mature B cells that includes CD21, CD81, and Leu-13 (1, 2). Coligation of this complex and membrane Ig (mIg) powerfully augments B cell activation. In vitro, such coligation results in enhanced release of intracellular calcium, activation of ERK2, induction of DNA synthesis, and Ab production (3–8). In vivo, CD19 is required for normal germinal center formation and Ab responses to T-dependent Ags and for maintenance of the B1 subpopulation, and appears to act to amplify B cell Ag receptor (BCR)-driven responses (9–11). Purposeful coligation of CD21 and mIg in mice reduces by orders of magnitude the amount of Ag necessary to induce an Ab response (12).

Stimulation of B cells through the Ag receptor results in tyrosine phosphorylation of CD19 and binding of cytoplasmic signaling molecules, including phosphatidylinositol 3-kinase (PI3K) and Vav, to those residues (13–17). Ligation of CD19 or coligation of CD19 and mIgM results in enhanced tyrosine phosphorylation of Vav, a possible mechanism of enhancement of signaling (6, 16, 18–20). We have used Daudi human B lymphoblastoid cells expressing mutant variants of CD19 to determine which pathways linked to CD19 enhance mIg-induced signaling. Activation of ERK2 in these cells is similar to that in normal mature mouse and human B cells in that coligation of CD19 and mIgM results in synergistically enhanced activity (6, 17). In Daudi cells, ERK2 activity was maximal at 1 min and persisted for 30 min following such stimulation. The synergistic activation of ERK2 by coligation of CD19 and mlg was blocked by mutation of CD19 Y391 (6).

The current studies were designed to further our understanding of how synergy is produced between CD19 and mIg by determining the point at which their respective pathways leading to ERK2 intersect. ERK2 is the terminal kinase in the well-defined mitogen-activated protein kinase (MAPK) cascade that consists of Ras, Raf, MEK1, and ERK2. Activation of Ras and Raf by mIg has been reported previously, most likely through the Shc/SOS/Grb2 complex (21–23).

Where CD19 impacts on this series is unknown. CD19 might enhance Ras activation by enhanced formation of a Grb2/SOS/Grb2 complex (24, 25). Alternatively, CD19 may activate members of the Rac/cdc42 family (17, 19, 26), which in turn activate the Jun N-terminal kinase (JNK) MAPK cascade (27), members of which may phosphorylate MEK1 in the ERK2 cascade (28–31). Finally, the increase in intracellular Ca2+ that results from CD19-mIg coligation could enhance the activity of enzymes such as protein kinase C that phosphorylate Raf (21, 32, 33).

We find that ligation of mIgM alone, but not CD19 alone, results in activation of Ras, Raf, and MEK1. Neither Ras nor Raf activation is enhanced after coligation of CD19 and mIgM, relative to mIgM alone. In contrast, MEK1 is activated synergistically by CD19-mIgM coligation, and thus is an intersection point of the signal transduction pathways activated by these two receptor complexes. We also show that the CD19 component of the enhanced activation of ERK2 is predominantly independent of changes in intracellular calcium and PKC activity.

Beyond the relevance for CD19 itself, this provides a model for how signals for lymphocyte coreceptors are integrated in the multienzyme cascades. To our knowledge, this represents the first
demonstration of synergistic activation of MEK1 by surface receptors. In addition to providing an amplification of a signal by a multienzyme cascade, individual members of the cascade may serve to integrate signals from different receptors.

Materials and Methods

Abs, reagents, plasmids, and cells
Polyclonal rabbit Abs to MEK1 (C18), Raf-1 (C12), and ERK2 (C14) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Murine [K77A]MEK1 (catalytically inactive) glutathione S-transferase (GST)tag-rose conjugate, mouse MEK1, mouse p42 MAP kinase, ERK1-GST, and ERK1 [K71A]GST were purchased from Upstate Biotechnology (Lake Placid, NY). Peroxidase-coupled polyclonal mouse anti-rabbit IgG was purchased from Jackson ImmunoResearch (West Grove, PA). Streptavidin (Sigma, St. Louis, MO), myelin basic protein (MBP, Sigma), PMA (Sigma), indo-1-AM (Molecular Probes, Eugene, OR), 1,2-bis(2-aminophenoxy)ethane-N,N'N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM, Calbiochem, La Jolla, CA), bisindoylmaleimide I (Calbiochem), and bisindoylmaleimide V (Calbiochem) were purchased. Biotinylated Fab(α)′, DA4.4 mouse anti-human IgM and biotinylated Fab(α)′, ADF4.2 anti-CD19 were prepared as described previously (34). The pGEX-RBD plasmid was a gift from Dr. Stephen J. Taylor (Columbia University, Ithaca, NY). Daudi B lymphoblastoid cells were obtained from American Type Culture Collection (Manassas, VA).

Cell activation and immunoblotting
Daudi cells, 1–2 × 10⁷/ lane, were preincubated in HBSS containing 1 mg/ml BSA, 1 mM MgCl₂, and 1 mM CaCl₂ (except as described with biotinylated Fab(α)′, DA4.4, biotinylated Fab(α)′, ADF4.2, or combinations of both Abs for 10 min and washed (preincubation with these Abs fragments had no significant effect on intracellular calcium concentration or ERK2 activity without the addition of a cross-linker (not shown)). The cells were suspended at 5 μg/ml of streptavidin for 1 min. The cells were spun down, and the pellet was lysed with Triton X-100 lysis buffer, as previously described (6). Lysates were cleared by centrifugation at 14,000 rpm for 10 min at 4°C. Immunoprecipitations were prepared by addition of appropriate Abs (2 μg/ml), followed by protein A-Trisacryl (Pierce, Rockford, IL). The precipitates were washed five times with wash buffer and then boiled in 2× Laemmli sample buffer for 5 min. Eluted proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with polyclonal Abs (1:1,000). The Abs were detected by peroxidase-coupled anti-rabbit (1:10,000), followed by chemoluminescence (Amersham, Arlington Heights, IL).

Assay for detection of activated Ras
Activated Ras interaction assays were performed as described previously (35–37). A GST fusion protein containing the Ras-binding domain (RBD) of Raf (amino acids 1–149 of c-Raf-1), which binds only GTP-bound (active) Ras, was prepared as described using the plasmid pGEX-RBD. For affinity precipitation of active Ras, 1–2 × 10⁷ Daudi cells were stimulated for 1 min and lysed. Clarified lysates were incubated with GST-RBD immobilized on glutathione-agarose beads (25 μl packed beads containing 50 μg protein) for 30 min at 4°C with rocking. The GST-RBD beads were washed three times with lysis buffer. Bound proteins were eluted by boiling in SDS-PAGE sample buffer, resolved on 10% acrylamide gels, and transferred to nitrocellulose. Affinity-precipitated Ras was detected by immunoblotting using anti-Ras antisera to confirm similar kinase activity (Transduction Labs, Lexington, KY).

Raf1 immunoprecipitation cascade kinase assay
Lysates of Daudi cells (1 × 10⁷/ lane) were clarified by centrifugation at 10,000 × g for 10 min at 4°C. Raf1 kinase in the supernatants was immunoprecipitated with 2 μg/ml of polyclonal anti-c-Raf-1 or normal rabbit IgG for 1 h at 4°C and 20 μl of protein A-Trisacryl beads (Pierce, Rockford, IL) for 1 h at 4°C. The beads were washed three times with lysis buffer and two times with Raf-1 kinase assay buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na₂VO₃, 1 mM DTT, 0.167 mM ATP, and 1 mM MgCl₂). Raf-dependent activation of inactive GST-ERK2 was measured using a c-Raf1 immunoprecipitation kinase cascade assay (33) (Upstate Biotechnology). Except where indicated, the kinase cascade reactions were conducted in a mixture containing 30 μl of kinase assay buffer, immunoprecipitated Raf1, 0.4 μg inactive MEK1, and 1 μg inactive GST-ERK2 at 30°C for 30 min in a shaking incubator. Phosphorylation of MBP by activated GST-ERK2 was conducted in a mixture containing 4 μl of the above mixture, 15 μl ERK2 kinase assay buffer (20 mM HEPES, pH 7.6, 20 mM MgCl₂, 20 mM β-glycerophosphate, 20 mM p-nitrophenylphosphate, 0.1 mM Na₂VO₃, and 2 mM DTT), 20 μg MBP, and 10 μCi [γ-³²P]ATP at 30°C for 10 min. The reaction was terminated by adding 20 μl of 2× Laemmli sample buffer and boiling for 5 min. Samples were resolved on a 10% SDS-polyacrylamide gel and analyzed by autoradiography.

Raf1 kinase activity assay
Raf1 kinase activity was determined as described by Niculescu et al. (38). Raf1 immunoprecipitates were prepared from Daudi cells (1 × 10⁷/ lane), as described above. The immunoprecipitates were washed three times with lysis buffer and twice with cold 50 mM Tris-HCl, pH 7, and suspended in 50 μl of 2× PIPES reaction buffer, pH 7, containing 10 mM MnCl₂, 10 μCi [γ-³²P]ATP, and 0.5 μg [K97A]MEK1-GST as a Raf-1-specific substrate. The samples were incubated for 30 min at 30°C in a shaking incubator. The reaction was terminated by adding 50 μl of 2× Laemmli sample buffer and boiling for 5 min. Samples were resolved on a 10% SDS-polyacrylamide gel and analyzed by autoradiography.

MEK1 immunoprecipitation cascade kinase assay
MEK1 was immunoprecipitated from lysates of Daudi cells (1 × 10⁷/ lane). The immunoprecipitates were suspended in 30 μl assay buffer (20 mM HEPES, pH 7.6, 20 mM β-glycerophosphate, 20 mM p-nitrophenylphosphates, 0.1 mM Na₂VO₃, 20 mM MgCl₂, and 2 mM DTT) containing 10 μCi of [γ-³²P]ATP, 0.4 μg of inactive GST-ERK2, and 20 μg of MBP, except as indicated. The reaction was conducted at 30°C for 30 min in a shaking incubator. The reaction was terminated by adding 30 μl of 2× Laemmli sample buffer and boiling for 5 min. Samples were resolved on a 10% SDS-polyacrylamide gel. The phosphorylation of MBP was analyzed by autoradiography and by phosphor imager analysis.

ERK2 immune complex kinase assay
In vitro protein kinase assays of ERK2 immunoprecipitates were performed as described previously (6). Incorporation of [³²P] into MBP was measured by autoradiography and by phosphor imager analysis. The amount of ERK2 in the immunoprecipitates was shown to be similar by immunoblotting using anti-ERK2 antisera. The data shown in Fig. 4 are representative; blots are not shown in other figures.

Flow cytometric analysis of intracellular Ca²⁺
Daudi cells (5 × 10⁶/ml) were loaded with 1 μM indo-1 AM at 37°C for 40 min. BAPTA-AM (5–25 μM final concentration, from stock in DMSO) or DMSO was added during the indo-1 loading. DMSO was added to all samples to yield equal final DMSO concentrations. Cells pretreated with DMSO vehicle only were washed and resuspended in HBSS containing 0.1% BSA, 1 mM MgCl₂, and 1 mM CaCl₂. Cells pretreated with BAPTA-AM were washed and resuspended in HBSS containing 1 mM EGTA, 0.1% BSA, 1 mM MgCl₂, and no Ca²⁺. Changes in indo-1 fluorescence were monitored by flow cytometry (FACS Vantage, Becton Dickinson, San Jose, CA). After a 20-s baseline, polyclonal goat anti-human IgM (20 μg/ml) was added, and analysis continued for a total of 4 min.

Results

CD19-mIgM coligation does not result in enhanced Ras activation
Activation of Ras by stimulation with anti-mIgM leads to the rapid activation of the Raf/MEK/ERK cascade in human B lymphocytes (22). The synergistic activation of ERK2 by coligation of CD19 with mIgM might result from enhanced activation of Ras. To test this hypothesis, Daudi B lymphoblastoid cells were incubated with biotinylated Fab(α)′, anti-CD19 or biotinylated Fab anti-IgM, or the combination of the two, washed, stimulated with avidin for 1 min, and lysed. Active (GTP-bound) Ras was detected using an assay based on the known specificity of the interaction between Ras-GTP and the RBD of Raf1 (35–37). GTP-Ras is affinity precipitated with GST-RBD and detected by immunoblotting using anti-Ras antisera. Active Ras increased by 2.9-fold following ligation of mIgM for 1 min (Fig. 1A, top). CD19 ligation alone induced no increase in active Ras. The level of active Ras was no greater
following coligation of CD19 and mIgM than that induced by ligation of mIgM alone.

To verify that ERK2 was synergistically activated under the stimulation conditions described, ERK2 immunoprecipitates from aliquots of the same lysates as were used in the active Ras assay were assayed for ERK2 kinase activity (Fig. 1A, bottom). The results show synergistic enhancement of ERK2 following CD19-mIgM coligation occurred in the same lysates used to test Ras activation.

Similar results were obtained in six experiments. For each of these, the fold increase in GTP-Ras in stimulated cells relative to cells treated with PBS only was calculated. The mean ± SD of the fold increase for each condition in the six experiments is shown (Fig. 1B). Coligation of CD19 and mIgM does not result in greater activation of Ras than ligation of mIgM alone. These results demonstrate that while ERK2 activation following ligation of mIgM alone correlates with an increase in GTP-Ras, the further, enhanced activation of ERK2 by CD19-mIgM coligation does not, suggesting that the CD19-dependent component of the synergistic interaction is not mediated by Ras.

**CD19-mIgM coligation does not result in synergistic Raf1 activation**

Raf1 is the most upstream protein kinase in the ERK MAPK cascade, and its activation is sufficient for ERK activation. The synergistic activation of ERK2 might result from synergy between...
CD19 and mlgM in activation of Raf1. We measured the activity of Raf1 after stimulation with PBS only or by ligation of CD19, mlgM, or both together under conditions shown to induce synergistic ERK2 activation. Immunoprecipitates formed with anti-Raf1 or control Abs were incubated with purified MEK1 and GST-ERK2. The incorporation of $^{32}$P into MBP, a substrate of ERK2, was analyzed by autoradiography and phosphor image analysis. As shown in Fig. 2A, while anti-CD19 alone induced a small increase in Raf1 activation, stimulation with anti-mlgM resulted in a 3.5-fold increase in Raf1 activation. In three independent experiments, levels of Raf1 activation after CD19-mlgM coligation were 11–14% higher than that induced by anti-mlgM alone, less than the sum of the increases induced by CD19 alone and mlgM alone. The measured kinase activity was specifically related to Raf1, as when normal rabbit IgG was used as a negative control for the immunoprecipitates, little kinase activity was detected (Fig. 2A).

In a separate experiment, we compared the activation of Raf1 and ERK2 in the same lysates following stimulation of Daudi cells with PBS only or by ligation of either CD19 alone or mlgM alone or by coligation of CD19 and mlgM (Fig. 2B). The activity of ERK2 was synergistically enhanced following CD19-mlgM coligation (3.38-fold, compared with 1.28-fold with mlgM alone, relative to the activity in cells stimulated with PBS only). In contrast, the activity of Raf1 precipitated from the same lysates was increased by 1.83-fold following ligation of mlgM alone and 1.99-fold after CD19-mlgM coligation. The small increase in Raf1 activity is unlikely to account for the large increase in ERK2 activity following CD19-mlgM coligation. Experiments in which the cells were lysed 30 s after stimulation gave similar results (not shown).

Additionally, Raf1 activation under similar stimulation conditions was determined in a Raf1 immunocomplex kinase assay using [K97A]MEK1-GST (lacking kinase and autophosphorylation activities due to mutation of lysine 97 to alanine in the ATP binding site) as a substrate (38). Fig. 3 shows the mean (±SD) of the fold increase in Raf1 kinase activity for each stimulation condition, relative to activity in PBS-treated cells, in four separate experiments. The results parallel those observed in the immunoprecipitation cascade assay, mlgM alone induced a 2.4-fold increase in Raf1 kinase activity. CD19-mlgM coligation resulted in only a minimal further increase (to 2.5-fold).

**MEK1 was synergistically activated in response to CD19-mlgM coligation**

MEK1 is phosphorylated and activated by Raf (39) and in turn phosphorylates and activates ERK2 (40, 41). To determine whether MEK1 functions as a convergent point for distinct signaling pathways mediated by CD19-mlgM coligation, MEK1 activation was measured in Daudi cells under the same stimulation conditions shown to induce synergistic ERK2 activation. To monitor MEK1 activation, we performed an immunoprecipitation kinase cascade assay measuring the ability of MEK1 to activate a downstream substrate, ERK2. Immunoprecipitates formed with either anti-MEK1 or control Ab were incubated with GST-ERK2, MBP, and [γ-$^{32}$P]ATP. The activity of the GST-ERK2 in incorporating $^{32}$P into MBP was measured. In the absence of precipitated MEK1, the GST-ERK2 was minimally active (Fig. 4A, rabbit IgG lanes). Anti-CD19 stimulation by itself had little effect on MEK1 activity (Fig. 4A, anti-MEK1 lanes). Anti-mlgM induced an increase in specific MEK1 activity toward ERK2. CD19 and mlgM coligation resulted in synergistic activation of MEK1 (in replicate experiments, 3–3.5-fold by anti-IgM alone versus 5.3–7.3-fold by anti-CD19 plus anti-IgM). The level of synergistic activation of MEK1 is comparable with that of ERK2 by CD19-mlgM coligation (Figs. 1A, 2B, and 4A).

MEK1 is a dual kinase that acts on ERK with narrow specificity. The substrate specificity of the MEK1 assay was examined by assessing the requirement for the GST-ERK2. MBP was phosphorylated in the presence of immunoprecipitated MEK1 and added GST-ERK, or with directly precipitated ERK2, from cells stimulated by CD19-mlgM coligation. In the absence of GST-ERK2, however, little kinase activity was detected in MEK1 immunoprecipitate from cells stimulated by CD19-mlgM coligation, demonstrating that the phosphorylation of MBP required ERK2 and thus reflected MEK1 activity (Fig. 4B). Experiments were also performed comparing the activity measured after addition of either unmutated or kinase-dead [K71A]ERK. Both the Raf and MEK (Fig. 5) cascade assays required activatable ERK, further demonstrating the specificity of the assays as well as the synergistic activation of MEK1, but not Raf.

**Synergistic activation of ERK2 by CD19-mlgM coligation does not require increased intracellular Ca$^{2+}$ or PKC activity**

CD19-mlgM coligation induces a synergistic increase in intracellular free Ca$^{2+}$ (34), which may in turn stimulate activity of certain kinases, including some isoforms of PKC, including PKC$\alpha$, which may regulate ERK2 activation by the BCR (32, 42). To begin to understand the mechanism by which CD19 up-regulates the ERK MAPK pathway, we asked whether the intracellular Ca$^{2+}$ flux is required for synergistic ERK2 activation. Daudi cells were pretreated with 20 $\mu$M BAPTA-AM, an intracellular Ca$^{2+}$ chelator, or DMSO vehicle only, washed, and then resuspended in HBSS with 1 mM Mg$^{2+}$ and either 1 mM EGTA or 1 mM Ca$^{2+}$, respectively. In preliminary experiments, we found that the calcium flux induced by stimulation with polyclonal anti-IgM was partially suppressed by 5 $\mu$M BAPTA-AM and completely suppressed by 10 $\mu$M BAPTA-AM under these conditions (not shown).

Daudi cells were stimulated with anti-CD19 or anti-mlgM or the combination of both, and ERK2 activity in these cells was measured. ERK2 activity in cells stimulated by ligation of mlgM alone

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**FIGURE 3.** Analysis of Raf activation by in vitro kinase assay. Daudi cells (1 x 10$^6$/lane) were incubated with buffer only (PBS), biotinylated anti-CD19 mAb (5 $\mu$g/ml) alone, biotinylated anti-lgM (1 $\mu$g/ml) alone, or both biotinylated anti-lgM (1 $\mu$g/ml) and anti-CD19 mAb (5 $\mu$g/ml) at 25°C for 10 min. Cells were stimulated by the addition of avidin (5 $\mu$g/ml) at 25°C for 1 min and lysed. Immunoprecipitates formed from the lysates with anti-Raf1 antisera (2 $\mu$g/ml) were analyzed for kinase activity, using [K97A]MEK1-GST as a substrate. For each stimulation condition, the fold increase, relative to PBS only, was calculated. The mean ± SD fold increase from four independent experiments is shown.
was diminished, but not blocked by BAPTA-AM + EGTA. Although the absolute level of ERK2 activity after CD19-mIgM coligation was diminished to a similar degree by BAPTA-AM + EGTA, the ability of CD19 coligation to enhance ERK2 activation was preserved in BAPTA-AM + EGTA-treated cells (Fig. 6A, top). The relative enhancement or suppression by BAPTA-AM + EGTA, compared with DMSO only, of the increase in ERK2 activity was calculated for mlgM alone, relative to PBS, and for CD19-mlgM coligation, relative to the increase over PBS observed with CD19 alone and mlgM alone. For three experiments, the average change in the increase over PBS induced by mlgM alone in the BAPTA-AM + EGTA samples was 0.59 ± 0.13 relative to DMSO samples. The average synergistic change induced by CD19-mlgM coligation was 1.29 ± 0.33 in the BAPTA-AM + EGTA samples, relative to DMSO samples. Thus, the stimulation of ERK2 by mlgM was reduced by 41% by chelation of calcium, but the relative enhancement of ERK2 activation by CD19 was unchanged. Pre-treatment of the cells with BAPTA-AM did not alter the amount of ERK2 immunoprecipitated from the cells (Fig. 6A, bottom). The concentration of BAPTA-AM used in these experiments (20 μM) was shown to be sufficient to prevent an increase in intracellular free Ca\(^{2+}\) with the Ab concentrations used for the ERK2 assays (Fig. 6B). A synergistically enhanced Ca\(^{2+}\) flux was observed in cells treated with DMSO and stimulated by coligation of CD19 and IgM. No increase in calcium was observed after any stimulation in cells treated with 20 μM BAPTA-AM + EGTA. The synergistic activation of ERK2 after CD19-mlgM coligation, therefore, occurred under conditions that blocked the increase in intracellular Ca\(^{2+}\).

The inhibition of increased calcium would block activation of calcium-dependent isoforms of PKC, but other isoforms are calcium independent. Therefore, we performed a similar analysis in cells treated with either bisindoylmaleimide I, which inhibits all isoforms of PKC tested, or bisindoylmaleimide V, a related compound that serves as a control (43). The results were similar to those observed following inhibition of the increase in calcium. The activation of ERK2 following ligation of mlgM alone was partially suppressed (1.3- versus 1.9-fold) by pretreatment with bisindoylmaleimide I (Fig. 7). However, the ability of CD19 to enhance activation when coligated with mlgM was predominantly intact (1.93- versus 2.05-fold in the presence of control versus active inhibitor). Activation of ERK2 by PMA was blocked by bisindoylmaleimide I, demonstrating effective inhibition of PKC. The activity of bisindoylmaleimide I against PKC\(_{\mu}\), which is activated by CD19 (44), has not been reported. Therefore, we also tested the ability of another PKC inhibitor, Gö 6976, which is active against this isoform, and again no suppression of the CD19 component was observed (not shown).
Requirement for intracellular Ca\(^{2+}\) in the synergistic activation of ERK2 by CD19-mlgM coligation. A, Requirement for intracellular Ca\(^{2+}\) in ERK2 activation. Daudi cells (1 × 10\(^7\)/lane) were pretreated with either 20 μM BAPTA-AM or an equal volume of DMSO vehicle only at 37°C for 40 min, washed, and resuspended in HBSS containing either 1 mM EGTA, 0.1% BSA, 1 mM MgCl\(_2\), and no Ca\(^{2+}\) (BAPTA-AM-treated cells), or 0.1% BSA, 1 mM MgCl\(_2\), and 1 mM CaCl\(_2\) (DMSO only cells). The cells were then incubated with buffer only (PBS), anti-CD19 mAb (5 μg/ml) alone, anti-IgM (1 μg/ml) alone, or both anti-IgM (1 μg/ml) and anti-CD19 mAb (5 μg/ml) at 25°C for 10 min. Cells were stimulated by the addition of avidin (5 μg/ml) for 1 min and lysed. Immunoprecipitates formed with anti-ERK2 were analyzed for kinase activity. Gels were analyzed by autoradiography and with the phosphor imager (top). The fold increase in incorporation of \(^{32}\)P into MBP for each stimulation condition, relative to that in cells stimulated with PBS only, is shown. Equivalent kinase loading in all samples was confirmed by immunoblotting with anti-ERK2 antisera (bottom).

**FIGURE 6.** Analysis of the requirement for an increase in intracellular Ca\(^{2+}\) in the synergistic activation of ERK2 by CD19-mlgM coligation. A, Requirement for intracellular Ca\(^{2+}\) in ERK2 activation. Daudi cells (1 × 10\(^7\)/lane) were pretreated with either 20 μM BAPTA-AM or an equal volume of DMSO vehicle only at 37°C for 40 min, washed, and resuspended in HBSS containing either 1 mM EGTA, 0.1% BSA, 1 mM MgCl\(_2\), and no Ca\(^{2+}\) (BAPTA-AM-treated cells), or 0.1% BSA, 1 mM MgCl\(_2\), and 1 mM CaCl\(_2\) (DMSO only cells). The cells were then incubated with buffer only (PBS), anti-CD19 mAb (5 μg/ml) alone, anti-IgM (1 μg/ml) alone, or both anti-IgM (1 μg/ml) and anti-CD19 mAb (5 μg/ml) at 25°C for 10 min. Cells were stimulated by the addition of avidin (5 μg/ml) for 1 min and lysed. Immunoprecipitates formed with anti-ERK2 were analyzed for kinase activity. Gels were analyzed by autoradiography and with the phosphor imager (top). The fold increase in incorporation of \(^{32}\)P into MBP for each stimulation condition, relative to that in cells stimulated with PBS only, is shown. Equivalent kinase loading in all samples was confirmed by immunoblotting with anti-ERK2 antisera (bottom).

**FIGURE 7.** Analysis of the requirement for PKC activity in the synergistic activation of ERK2 by CD19-mlgM coligation. Daudi cells (1 × 10\(^7\)/lane) were pretreated with 10 μg/ml of either bisindoylmaleimide I (BIM) or bisindoylmaleimide V (BIM-V) for 1 h. The cells were then incubated with buffer only (PBS), anti-CD19 mAb (5 μg/ml) alone, anti-IgM (1 μg/ml) alone, or both anti-IgM (1 μg/ml) and anti-CD19 mAb (5 μg/ml) at 25°C for 10 min. Cells were stimulated by the addition of avidin (5 μg/ml) for 1 min and lysed. Immunoprecipitates formed with anti-ERK2 were analyzed for kinase activity. Gels were analyzed by autoradiography and with the phosphor imager (top). The fold increase in incorporation of \(^{32}\)P into MBP for each stimulation condition, relative to that in cells stimulated with PBS only, is shown. Equivalent kinase loading in all samples was confirmed by immunoblotting with anti-ERK2 antisera (bottom).

Discussion

Stimulation through the BCR elicits signal transduction events that affect fundamental biologic responses of the B cell, including proliferation, differentiation, and apoptosis. The complex responses of B cells to various environmental stimuli occur by the integration of signals from the BCR and its coreceptors. Currently, three paradigms for coreceptor modulation of B cell activation have been described. The B cell-specific membrane protein CD22 forms a complex with the tyrosine phosphatase Src homology 2-containing protein tyrosine phosphatase (SHP-1) upon ligation of mlgM, which negatively regulates signaling and activation of ERK2 by the BCR (8, 45). Similarly, coligation of BCR with the FcR for IgG (FcγRII) inhibits B cell activation and proliferation by activating SHP-1 and/or Src homology 2-containing inositol phosphatase (SHIP) (46). Coligation of FcγRII with mlgM has also been shown to disrupt Shc-Grb2 interactions, which blocks Ras activation (47). Thus, CD22 and FcγRII function in negative signaling, including inhibition of MAPK activation, by modifying signals from the BCR. In contrast, CD40, a transmembrane glycoprotein expressed on several types of APCs, mediates signaling in B cells through BCR-independent pathways. CD40 engagement alone induces rapid and sustained increase in the tyrosine phosphorylation of distinct signaling proteins; activates the transcription factor, nuclear factor-κB; and promotes B cell survival and proliferation (48, 49). In addition, CD40 ligation alone induces ERK activation through a PKC-independent pathway, distinct from the mechanism of induced by mlgM (42, 50). Although the CD19/CD21/CD81 complex also may serve other functions (51, 52), CD19 positively amplifies BCR signaling, but in a BCR-dependent manner. Ligation of mlgM results in tyrosine phosphorylation in the intracellular domain of CD19, which recruits cytoplasmic signaling molecules that enhance the activation of downstream pathways such as the MAPK pathways (6, 16, 17, 24). The association of PI3K with CD19 is required for optimal signaling following ligation of mlgM alone (53). In contrast, CD19 Y391 is critical for the enhanced calcium release and ERK2 activation that follow coligation of CD19 and mlgM (6, 17).

The MAP kinase pathway links many extracellular stimuli to transcriptional regulation and is critically involved in mediating cellular proliferation and differentiation. The convergence of the
BCR and BCR/CD19-derived signaling pathways in activation of ERK is likely to play an important role in B cell function. ERK kinases are capable of phosphorylating and activating a number of transcription factors, including c-Myc and p62TCF (54–56), and may augment the transcription of early genes, including c-fos (55). Transcription of the early gene egr-1 in immature B cells after mlgM ligation is mediated by activation of the Ras-MAP kinase pathway (57). Therefore, the purpose of this study is to use ERK2 activation by CD19-mlgM coligation as a model of how the additional pathways that are activated by CD19 integrate with signals generated by mlgM alone.

We have provided evidence that, in Daudi cells, ligation of mlgM alone, but not CD19 alone, results in activation of the Ras/Raf1/MEK1 cascade. Minimal further activation of Ras and Raf is detected after CD19-mlgM coligation. This conclusion is supported by three different assays: analysis of association of Ras-GTP with the Ras-binding domain of Raf, the kinase activity of Raf for its substrate MEK, and the ability of Raf to activate the terminal components of this cascade. Synergistic activation of ERK was demonstrated in the same lysates in which little CD19 had little effect on Ras or Raf, indicating that the coligation enhances ERK2 activation through a pathway separate from Ras.

In contrast, MEK1 is synergistically activated in response to CD19-mlgM coligation. Thus, the distinct signals from the two receptor complexes converge at MEK1 to provide synergy in stimulating ERK2 activation. These findings are consistent with our previous observations, using the selective MEK inhibitor PD98059, that synergistic activation of ERK2 acts through MEK1 (6).

Chelation of intracellular calcium with BAPTA-AM, under conditions that blocked the increase in calcium induced by coligation, reduced the absolute level of activation of ERK2 following coligation of CD19 and mlgM, but did not reduce the fold increase induced by coligation relative to individual ligation of CD19 and mlgM alone. We conclude that the CD19 component of synergy is not dependent on calcium-dependent kinases. This would include PKCα, which phosphorylates and activates Raf (32), a possible intermediary in this pathway. This is consistent with the lack of effect of coligation on Raf. A similar result was observed with bisindolylmaleimide I, a more general inhibitor of PKC isoforms, and with Gö 6976, which inhibits PKCμ. Again, the overall ERK2 activity induced by coligation was reduced in cells treated with bisindolylmaleimide I, compared with the bisindolylmaleimide V control. However, while the increase in ERK2 activity induced by ligation of mlgM alone, relative to unstimulated cells, was reduced by 66% in Fig. 7, the further increase induced by coligation, relative to cells stimulated with anti-IgM alone, was reduced by only 5%. Thus, while the activation of Raf by ligation of mlgM alone is highly dependent on intracellular calcium and PKC activity, the CD19 component of the synergistic activation appears to be largely independent of these.

The linkage between CD19 and MEK remains to be determined. Our current data suggest that the mechanism involves factors that act distal to Ras and Raf. We have shown previously that, in transfected Daudi cells, the synergy is blocked by mutation of CD19 tyrosine 391, the site of Vav association (6). Vav is a GTP exchange factor for the Rac1/Cdc42 family of GTP-binding proteins, which have been implicated in the activation of p21-activated kinase (MEKK1) (19). MEKK1 may only phosphorylate one of two critical residues on MEK1, but in the presence of a second signal, derived through Raf, this may up-regulate MEK1 activation (28–30, 58). p21-activated kinase 1 also acts synergistically with Raf to activate MEK1 by direct phosphorylation (30, 31, 59). However, in mice, disruption of the Vav gene did not block synergistic activation of ERK2 (17). In preliminary experiments, Raf activation was analyzed in five preparations of normal human B cells, prepared from tonsils, in which synergy was observed in activation of ERK2 after coligation of CD19 and mlgM. In four such preparations, no such synergy was detected in Raf activity, but synergy was observed in one. Thus, we conclude that the Raf-independent activation of ERK2 by CD19 occurs in normal cells. However, the one exception, and the small but consistent decrease in synergy following treatment with PKC inhibitors, leads us to suspect that there may be some redundancy. At present, we do not exclude the possibility that an alternative signaling molecule(s) regulated by CD19 is involved in the enhanced activation of MEK1 by CD19. In addition, the interaction between Vav, PI3K, PKC, Ras, and Rac/cdc42 is likely to be complex (20, 60).

The most important conclusion from these findings is that optimal ERK activation requires at least two signals. One signal, activated by mlgM alone, targets Ras and leads to an intermediate level of ERK activity. The second signal, generated upon CD19-mlgM coligation, acts through a separate pathway that converges at MEK1 and induces enhanced ERK2 activation. Our findings provide a model for how signal input from a coreceptor is integrated in a multienzyme cascade. Intermediate members of the MAPK pathways can serve as integration sites for modification of signals, derived from ligation of Ag receptors, by cell surface coreceptors that modulate lymphocyte activation in response to the context in which Ag is recognized. Convergence of distinct, coreceptor-dependent pathways feeding into the ERK cascade during BCR signaling would provide multiple levels of regulation and allow more effective and precise control of B cell activation.

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References


