Selective Activation of c-Jun Kinase Mitogen-activated Protein Kinase by CD40 on Human B Cells*

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The B cell surface antigen receptor, surface IgM (sIgM), is involved in B cell activation and proliferation. CD40 is involved in regulating IgE production and B cell survival. Cross-linking of B cell sIgM activates the Ras/Raf/ERK2 pathway. In contrast, ligation of CD40 by antibody or soluble gp39 (CD40 ligand) leads to activation of the c-Jun kinase (JNK)/stress-activated protein kinase pathway. JNK/stress-activated protein kinase activity correlated with the stimulation of MEK kinase activity. CD40 does not activate the ERK2 pathway, and sIgM fails to regulate the JNK/stress-activated protein kinase pathway in B cells. Thus, two important cell surface receptors involved in controlling specific B cell response differentially regulate sequential protein kinase pathways involving different members of the mitogen-activated protein kinase family. Anti-CD40 also rescued B cell apoptosis induced by anti-IgM. CD40 ligation did not affect the sIgM stimulation of ERK2 activity. Conversely, sIgM ligation did not influence CD40 stimulation of JNK/stress-activated protein kinase. These results suggest that independent, parallel protein kinase response pathways are involved in the integration of sIgM and CD40 control of B cell phenotype and function.

The B lymphocyte surface antigen receptor, membrane immunoglobulin, has important functions in the binding and internalization of antigen as well as in transducing signals through the plasma membrane that lead to cell activation, differentiation, and apoptosis (1, 2). Cross-linking of the receptor stimulates the Ras/Raf/ERK2/MAP kinase and p90SRK (3). A second important B cell surface antigen receptor is CD40. CD40 is a 45–50-kDa transmembrane glycoprotein expressed on all mature B cells (4). CD40 is a member of the TNF receptor family and has homology to the receptors for nerve growth factor (5), TNF-α (6–8), Fas (9), and CD30 (10). The ligand for CD40 (CD40L, gp39) is expressed on activated T cells (11), and activation through CD40 plays an important role in T cell-dependent immunoglobulin isotype switching (12, 13). In contrast to cross-linking of sIgM, which can cause apoptosis, CD40 can rescue cells from apoptosis (13–17). The signal transduction pathways through CD40 are not well delineated, but may induce protein tyrosine phosphorylation of a number of substrates (18–20).

In this paper, we demonstrate that c-Jun amino-terminal kinases (J NKS)/stress-activated protein kinases are activated following CD40 ligation of human B cells. J NKS are members of the MAP kinase family (21) and are activated by stresses such as UV irradiation (22–24), osmotic change (25, 26), and heat shock (21). In contrast to CD40, signaling through the antigen receptor activated p42 and failed to activate J NK.

MATERIALS AND METHODS

Cells—The human Burkitt’s lymphoma cell line Ramos was obtained from the American Type Culture Collection (Rockville, MD), and cells were maintained in RPMI 1640 medium supplemented with 50 units/ml penicillin/streptomycin, 2 mM glutamine, and 10% fetal calf serum. Exponentially growing cells were used in all experiments. Human primary B cells were prepared from tonsils as described previously (27).

Reagents—Anti-IgM (Fab′2) goat anti-human IgM antibody was purchased from Zymed Laboratories, Inc. (South San Francisco, CA). The mouse monoclonal anti-human CD40 antibody (G28-5) was generously provided by Dr. E. A. Clark (Washington University, Seattle). The monoclonal anti-gp39 antibody (39-1.106) and recombinant soluble gp39 protein were prepared as described previously (28, 29). Monoclonal mouse anti-ERK2 antibody was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal rabbit anti-Raf-1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). GSH-Sepharose was obtained from Pharmacia Biotech (Uppsala), and protein A-Sepharose and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma. EGFR (662–681)-peptide (IRRELVEPLTGPSEAPNQALLR) was synthesized as described (3).

Immunoblot Analysis—Cells (10^6/ml) were treated with anti-IgM or anti-CD40 antibody (G28-5) for various times. Cells were lysed in 100 μl of lysis buffer (25 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, and 50 μl of leupeptin). Lysates were centrifuged for 10 min at 14,000 rpm in an Eppendorf microcentrifuge. 90 μl of the supernatants were mixed with 30 μl of 4 × Laemmli sample buffer (59). Samples were boiled for 5 min. Twenty μl of the prepared samples were electrophoresed through a 12% SDS-polyacrylamide gel, and proteins were transferred to nitrocellulose membranes. Membranes were incubated in blocking buffer (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.05% Tween 20) and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Promega; 1:10,000 in TBST) for 1 h at room temperature. The blots were washed three times in TBST (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.025% Tween 20) and incubated with alkaline phosphatase-conjugated goat anti-mouse Ig (Promega; 1:1000 in TBST) for 1 h at room temperature. The blots were washed three times in TBST and developed with the colorigenic substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Promega Protoblot alkaline phosphatase system).

ERK Kinase Assay—Kinase activity was evaluated using EGFR-P

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1 The abbreviations used are: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; MAP, mitogen-activated protein; TNF, tumor necrosis factor; IgM, surface IgM; JNK, c-Jun kinase; ERK, extracellular signal-regulated kinase; PMA, phorbol 12-myristate 13-acetate; EGFR, epidermal growth factor receptor; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; KMEKK, catalytically inactive MEK; MEKK, MEK kinase; J NKK, J NK kinase; PBS, phosphate-buffered saline.
ERK 1 is Activated by Anti-IgM, but Not by Anti-CD40—The Ramos cell line expresses both IgM and CD40 on the cell surface. When these cells were stimulated by cross-linking slgM and immunoblotting was carried out using a monoclonal anti-ERK2 antibody, we detected a second lower mobility species of ERK2 (Fig. 1A). This lower mobility form represents the activated form of this kinase as described previously (30) and was also stimulated in response to PMA (Fig. 1A). In contrast to stimulation with anti-IgM antibody, immunoblotting analysis following treatment with 5 μg/ml (or 10 μg/ml [data not shown]) anti-CD40 antibody (G28-5) showed no lower mobility shift in p42\textsuperscript{erk2} (Fig. 1A). These data indicate that anti-CD40 antibody activates p42\textsuperscript{erk2}, but anti-CD40 antibody fails to activate p44\textsuperscript{erk2}. Similar results were shown when kinase activities were measured using EGF-R-(662–681)-peptide as a substrate. Activation of p42\textsuperscript{erk2} by anti-IgM was confirmed by increases in EGF-R-(662–681)-peptide phosphorylation (Fig. 1B). In contrast, the addition of anti-CD40 antibody at concentrations up to 10 μg/ml failed to activate p42\textsuperscript{erk2} in fresh isolated tonsilar B cells (Fig. 1C).

J NK Is Activated by Anti-CD40 and Soluble gp39, but Not by Anti-IgM Antibody—J NK activity was measured by solid-phase kinase assay using GST-C-jun UV1 as a substrate following treatment with anti-IgM or anti-CD40 antibody. A rapid and marked increase in J NK activation was detected within 1 min of treatment with 1 μg/ml anti-CD40 antibody (Fig. 2A), reached peak levels within 15 min, and then began to decline by 30–60 min. Fig. 2B illustrates CD40-activated J NK activity in Ramos cells treated with various concentrations of anti-CD40 antibody for 15 min. In the presence of 0.5 μg/ml anti-CD40 antibody, levels of \textsuperscript{32}P incorporation were 5-fold higher than control samples. J NK activity increased in a dose-dependent fashion, with peak levels (7-fold) observed at a concentration of 2–5 μg/ml antibody. A dose-dependent response to anti-CD40 antibody was also detected in tonsilar B cells (Fig. 2C). Throughout the dose-response curve, lower levels of activation were observed in tonsilar B cells relative to Ramos cells, but both cell types clearly respond to CD40 ligation with a significant J NK activation. Recombinant soluble gp39 also activated J NK in a dose-dependent fashion in Ramos cells (Fig. 2D) and tonsilar B cells (data not shown). J NK activation was significantly higher using soluble gp39, and the response was specific in that anti-gp39 antibody prevented activation of J NK by soluble gp39 (Fig. 2E). Anti-gp39 antibody failed to block UV irradiation-induced activation of J NK (Fig. 2E).
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JNK activity was not increased following surface IgM cross-linking even in the presence of 10 μg/ml anti-IgM antibody in Ramos cells (Fig. 2F) and tonsil B cells (Fig. 2G). These anti-IgM antibody concentrations were effective in ERK activation (Fig. 1A). The results demonstrate that JNK is activated by anti-CD40, but not anti-IgM, indicating that anti-CD40 activates JNK through a different signaling pathway than that which mediates ERK activation by anti-CD40. We therefore investigated the signaling pathways that lead to ERK and JNK activation following treatment with anti-IgM or anti-CD40 antibody.

Anti-CD40 Activates JNK through a Ras-independent Pathway—It is known that Ras is involved in the signaling pathway that activates ERKs following treatment with PMA or antigen-receptor ligation of human B cells (3). Metabolically 32P-labeled Ramos cells were treated with 10 μg/ml anti-IgM or 5 μg/ml anti-CD40 for 1, 5, and 10 min. Ras was immunoprecipitated, and radioactive GTP and GDP bound to Ras were measured (31). Anti-IgM treatment activated Ras as reported previously (3). However, anti-CD40 treatment failed to activate Ras at concentrations that were effective in JNK activation (Fig. 3). Our results demonstrate that the signals transduced following CD40 engagement lead to JNK activation through a pathway that does not involve Ras activation.

Raf-1 Does Not Participate in the CD40-activated JNK Pathway—The activation of ERKs by Ras is mediated via Raf-1 (36). We investigated Raf-1 activity in order to confirm that the signals leading to JNK activation were mediated via a different pathway than the one leading to ERK activation. The Raf-1 assay was carried out using catalytically inactive MEK (KMMEK) as a substrate (32). KMMEK is a recombinant mutant form that lacks both kinase and autophosphorylation activities due to a mutation of lysine 97 to methionine in the ATP-binding site (32). Fig. 4 illustrates that the levels of KMMEK phosphorylation following treatment with 5 μg/ml anti-CD40 were not different than control samples, whereas cells treated with anti-IgM antibody resulted in increased KMMEK phosphorylation. To verify similar loading of immunoprecipitated Raf-1, an immunoblot was concomitantly performed using the same antibody as was used for the immunoprecipitates (Fig. 4). The Raf-1 mobility shifts (Fig. 4, lower panel) were consistent with the increased levels of kinase activity (upper panel) measured using KMMEK. As previously reported (3), PMA gives a very robust Raf-1 activation. Nonetheless, the magnitude of anti-IgM activation of Raf-1 is sufficient to activate ERK2 similarly compared with PMA, Raf-1, which is an efficient activator of the ERK pathway, is not measurably activated during JNK activation in response to CD40 ligation.

p90Rsk Mobility Shift following ERK but Not JNK Activation—p90Rsk is a known downstream kinase of the ERK pathway (37). Following treatment with anti-IgM or anti-CD40, cell lysates were assayed for p90Rsk activation using the mobility shift of the kinase as a measure of increased activity. Anti-IgM treatment induced a shift in the mobility of p90Rsk, whereas in the samples treated with anti-CD40 antibody, no shift in the mobility of p90Rsk protein was detected (data not shown). This finding is consistent with the differential signaling of slgM and CD40 involving MAP kinase pathways in B cells.

Anti-CD40 Activates MEKK—JNK activation involves a sequential protein kinase pathway including JNKK (34, 38) and MEKK (40). We measured MEKK activity following the addition of anti-CD40 antibody (2 μg/ml) to Ramos cells. At different time points, cell lysates were immunoprecipitated with anti-MEKK antibody and subjected to an MEKK kinase assay was carried out using catalytically inactive MEK (KMMEK). As previously reported (3), PMA gives a similar level of kinase activity (data not shown). This data indicate that in B lymphoblastoid cells, a MEKK is present that regulates the JNK pathway and is activated in response to CD40 ligation.

Anti-CD40 Rescues Apoptosis Induced by Anti-IgM—Anti-IgM treatment induces apoptosis in B lymphoblastoid cells (13–17). For detection of DNA breaks derived from anti-IgM-induced apoptosis, an in situ terminal deoxynucleotidyltransferase assay (35) was employed. Fig. 6A shows that DNA breaks were detected in 64.2% of Ramos cells 18 h following treatment with 10 μg/ml anti-IgM antibody, whereas there was no shift in fluorescence intensity in control (untreated) cells or in cells treated with 2 μg/ml anti-CD40 antibody. However, in the presence of 2 μg/ml anti-CD40 antibody preincubated for 30
min prior to the addition of anti-IgM antibody, DNA breaks induced by anti-IgM antibody were reduced to 3.5% of the cells. Under identical conditions (Fig. 6), an immunoblot using monoclonal anti-ERK2 antibody indicated the mobility shift in p42 erk2 protein 5 min following treatment with 10 μg/ml anti-IgM antibody in the presence of 2 μg/ml anti-CD40 antibody preincubated for 30 min. In addition, JNK activity, measured by solid-phase kinase assay using GST-c-Jun fusion protein, was increased 15 min following treatment with anti-CD40 antibody in the presence of anti-IgM antibody. Thus, CD40 ligation does not affect p42 erk2 activation by sIgM, and sIgM ligation does not affect CD40 activation of JNK.

**DISCUSSION**

Many growth factors and cytokines activate MAP kinase family members, including ERKs and JNKs. Many growth factors have been shown to activate ERKs, and the signaling cascade has been well characterized. Similar to other members of the MAP kinase family, JNKs are activated through phosphorylation at conserved Thr and Tyr residues (41). The pathways leading to JNK activation are less well understood and may function as a protective response against environmental stresses and may influence the apoptotic response.

We previously showed (3) and confirmed here that following triggering of B cells through the surface antigen receptor, Ras, Raf-1, and MEK are all activated and participate in p42 erk2 activation. Following stimulation with anti-IgM, Ras activation was observed, and the ability of Raf-1 to phosphorylate recombinant and kinase-inactive MEK was increased. In parallel, MEK activity toward kinase-active or -inactive recombinant
MAP kinase also increased. Under conditions where anti-IgM increased phosphorylation of p42<sup>erk2</sup>, we were unable to detect any activation of JNK in either a B lymphoblastoid cell line or freshly isolated tonsillar B cells.

In contrast to anti-IgM, the addition of anti-CD40 antibody or recombinant soluble gp39 activated JNK in a dose- and time-dependent fashion. Qualitatively, the results were similar when studied in Ramos cells or tonsillar B cells. The higher level of JNK activation in Ramos cells may reflect higher levels of CD40 expression on the B cell line compared with freshly isolated tonsillar B cells (42). As reported previously (43) for activation of NF-κB or B cell proliferation, soluble gp39 resulted in greater increases in JNK activation than did the addition of anti-CD40 antibody. This may be due to the fact that soluble gp39 is highly aggregated, resulting in oligomerization of CD40 and in a stronger signal. Activation of B lymphocytes through CD40 did not result in any detectable increase in phosphorylation or activation of p42<sup>erk2</sup> or its downstream substrate, p90<sup>rsk</sup>. Furthermore, the addition of anti-CD40 failed to affect Ras or Raf-1 activation. These data indicate that signaling of B cells through CD40 leads to JNK activation by a Ras-independent pathway.

These results are similar to what has been described for TNF-α stimulation of PC12 cells (40). In these cells, activation of JNK by epidermal growth factor or nerve growth factor was dependent on Ha-Ras, while activation by TNF-α was Ras-independent. It appears that Ras activates two kinases, Raf-1 and MEK kinase (MEKK1) (44). Although Raf-1

**FIG. 4.** Activation of Raf-1 following treatment with anti-IgM, but not with anti-CD40. Ramos cells were untreated (control (C)) or treated with 100 ng/ml PMA, 10 μg/ml anti-IgM, or 5 μg/ml anti-CD40 for the indicated times (in minutes). Raf-1 was immunoprecipitated, and a kinase assay was performed as described under “Materials and Methods.” After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes. T<sup>2</sup>P incorporation into catalytically inactive MEK (KMMEK) was measured by autoradiography (upper panel). The membrane was also probed with the same anti-Raf-1 antibody used for immunoprecipitation, and immunoreactivity was visualized by the alkaline phosphatase system to verify similar loading of immunoprecipitated Raf-1 (lower panel). Results are representative of two separate experiments.

**FIG. 5.** Activation of MEKK following treatment with anti-CD40 antibody. Ramos cells were treated with 2 μg/ml anti-CD40 antibody for the indicated times (in minutes). MEKK was immunoprecipitated, and a kinase assay was performed as described under “Materials and Methods.” After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes. T<sup>2</sup>P incorporation into JNKK was measured by autoradiography (upper panel). COS cells transfected with full-length MEKK were used to localize JNKK. The level of T<sup>2</sup>P incorporation (± S.D.) into the substrate from three independent experiments was evaluated by the PhosphorImager and then illustrated as the ratio of MEKK activity to that of untreated samples (lower panel). Statistically significant differences from untreated (lane 0) samples are represented by an asterisk (p < 0.05).

**FIG. 6.** Rescue from anti-IgM-induced apoptosis by anti-CD40 antibody. Ramos cells were untreated (control) or treated with 10 μg/ml anti-IgM antibody or 2 μg/ml anti-CD40 antibody; costimulation of cells consisted of a 30-min preincubation with anti-CD40 antibody followed by anti-IgM antibody. A, after an 18-h culture, DNA breaks derived from anti-IgM-induced apoptosis were evaluated using an in situ terminal deoxynucleotidyltransferase assay as described under “Materials and Methods.” B, p42<sup>erk2</sup> activation (5 min following treatment) and JNK activation (15 min following treatment) were evaluated under identical conditions as described under “Materials and Methods.” The level of T<sup>2</sup>P incorporation (± S.D.) from two independent experiments was evaluated by the PhosphorImager and then illustrated as the ratio of JNKK activation to that of untreated samples. Statistically significant differences from untreated samples are represented by an asterisk (p < 0.05).
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Contributes to ERK but not JNK activation (40). MEKK is involved in JNK activation (40, 45). In the absence of measurable Ras activation, as observed with TNF-α in PC12 cells and signaling through CD40 in B cells, the pathway leading to MEKK activation is presently unclear (34, 38).

The B cell antigen receptor (surface immunoglobulin) is important for binding and internalization of antigen as well as transducing signals through the plasma membrane, resulting in cell activation and differentiation (1, 2). Anti-Ig antibodies trigger the rapid activation of phospholipase C and several tyrosine kinases, increases in cytosolic Ca2⁺ concentrations, and increased transcription of a number of early genes including Egr, c-fos, and c-myc (46–54). Ligation of CD40 appears to initiate a distinct series of events with no increase in cytosolic Ca2⁺ concentrations and inconsistent results from different groups on Src kinase activation (18–20). Together with the absence of Ras activation, it appears that CD40 does not signal transduction pathways resulting in MEKK and JNK activation distinct from responses controlled by sIgM. Whether these transduction pathways resulting in MEKK and JNK activation to some degree in the presence of anti-IgM antibody. The apoptotic response of the B cell. Genetic manipulation of JNK pathway with sIgM responses dramatically alters the function of the JNK MAP kinase pathway that is important for binding and internalization of antigen as well as transduction of the apoptotic response. This is not the case in B lymphocytes, where JNK activation is not observed in sIgM-stimulated apoptosis. Rather, JNK is activated in association with the protective response mediated by CD40 and even possibly augmented to some degree in the presence of anti-IgM antibody. The apparent independence of CD40 and sIgM signaling involving MAP kinase pathways suggests that the integration of the JNK pathway with sIgM responses dramatically alters the functional response of the B cell. Genetic manipulation of JNK activation in B cells, including altering the apoptotic response to sIgM ligation, will define whether this is a dominant pathway in CD40 function.

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