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Positive Signaling Through CD72 Induces Mitogen-Activated Protein Kinase Activation and Synergizes with B Cell Receptor Signals to Induce X-Linked Immunodeficiency B Cell Proliferation

Hsin-Jung Wu,† Chandrasekar Venkataraman,‡ Steven Estus,†‡ Chen Dong,§ Roger J. Davis,¶ Richard A. Flavell,§ and Subbarao Bondada *‡

CD72 is a 45-kDa B cell transmembrane glycoprotein that has been shown to be important for B cell activation. However, whether CD72 ligation induces B cell activation by delivering positive signals or sequestering negative signals away from B cell receptor (BCR) signals remains unclear. Here, by comparing the late signaling events associated with the mitogen-activated protein kinase pathway, we identified many similarities and some differences between CD72 and BCR signaling. Thus, CD72 and BCR activated the extracellular signal-regulated kinase (ERK) and the c-Jun N-terminal kinase (JNK) but not p38 mitogen-activated protein kinase. Both CD72- and BCR-mediated ERK and JNK activation required protein kinase C activity, which was equally important for CD72- and BCR-induced B cell proliferation. However, CD72 induced stronger JNK activation compared with BCR. Surprisingly, the JNK activation induced by both BCR and CD72 is Btk independent. Although both CD72 and BCR induced Btk-dependent ERK activation, CD72-mediated proliferation is more resistant to blocking of ERK activity than that of BCR, as shown by the proliferation response of B cells treated with PD98059 and dibutyryl cAMP, agents that inhibit ERK activity. Most importantly, CD72 signaling compensated for defective BCR signaling in X-linked immunodeficiency B cells and partially restored the proliferation response of X-linked immunodeficiency B cells to anti-IgM ligation. These results suggest that CD72 signals B cells by inducing BCR-independent positive signaling pathways.


Induction of proliferation and differentiation of small resting B lymphocytes into plasma cells depends on the integration of signals transmitted by the B cell receptor (BCR) and a variety of coreceptors. Coreceptors such as CD19, CD38, CD40, and CD72 have in the past been shown to induce positive signals that could enhance B cell responses (1–4). Coreceptors such as CD5, CD22, FcR, and paired Ig-like receptor B have classically been shown to provide negative signals that can attenuate signals provided by the BCR (5–9). However, it is not always clear whether coreceptors are acting as positive or negative modulators. For example, CD22 has been shown to be both a negative and a positive regulator based on the properties of B cells obtained from CD22-knockout mice (10–13). Similarly, the CD72 receptor is now suggested to have a dual role in B cell activation.

CD72 is a B cell transmembrane glycoprotein that is expressed in all stages of B cell development except plasma cells. Ligation of CD72 induces B cell proliferation, an increase in expression of surface MHC class II molecules, and an augmentation of IL-4-dependent CD23 expression (14–16). Results from CD72-knockout mice demonstrated that CD72 has a crucial and nonredundant role in B cell development and activation (17). These knockout mice exhibited a reduction in mature B cells in the spleen, presumably the result of a defect in bone marrow B cell development, but the remaining splenic B cells were slightly hyperresponsive to BCR cross-linking. CD72-knockout mice also have a slight increase in activated B cells, but no uncontrolled expansion as in Src homology 2 domain-containing protein tyrosine phosphatase (SHP)-1-deficient moth-eaten mice (17). Recently, two groups have shown that an immunoreceptor tyrosine-based inhibition motif of the CD72 cytoplasmic domain is associated with the protein tyrosine phosphatase SHP-1, also supporting a negative regulatory role for CD72 in BCR signaling (18, 19). Furthermore, it was shown that preligation of CD72 prevented BCR-induced apoptosis in a subline of WEHI-231 B cell lymphoma and in transitional B cells (19). However, the CD72 cytoplasmic domain contains another tyrosine region, which is capable of binding to Grb2 in vitro, giving CD72 the potential to regulate positive signaling via the Grb2-Sos-Ras pathway (19). Several biochemical changes induced by CD72 are supportive of a positive role for this molecule. Anti-CD72 Ab induces inositol turnover, Ca2+ elevation, and tyrosine phosphorylation of protein tyrosine kinases Lyn, Blk, and Btk but not Syk (20–22). Anti-CD72 Ab also induces transient association...
of CD72 with CD19, which is a positive regulator of BCR signaling (23). Finally, CD72-knockout mice have reduced numbers of IgM<sup>−</sup>IgD<sup>high</sup> mature B cells just like CD45<sup>−</sup>−/− and Btk-deficient mice, suggesting that CD72 has a positive role in B cell development (17, 24, 25).

In the context of a negative signaling role for CD72, it has been proposed that the positive effects of anti-CD72 Abs are the result of their ability to relieve CD72 inhibition of BCR signaling. Here, we studied the alternative possibility that CD72 behaves like CD22 in negatively regulating BCR signaling while initiating a positive signal by itself. In a number of systems, mitogen-activated protein kinase (MAPK) cascades are key intermediates coupling surface receptor ligation to transcriptional activation and proliferation (26). Comparing the late signaling events, such as the MAPK pathway, that follow CD72 and BCR ligation may help to define the relative importance of CD72 and BCR in CD72-mediated B cell activation. Because BCR ligation stimulates both Syk and Btk protein tyrosine kinases, whereas CD72 enhances Btk but not Syk activity (22), we were also able to delineate the relative roles of these protein tyrosine kinases in MAPK activation in normal splenic B cells.

In this study, we report that CD72 and BCR share many similarities in induction of the MAPK pathway, but they also exhibit some unique properties. Ligation of CD72 or BCR induces extracellular signal-regulated kinase (ERK) activation, which is found to be Btk dependent. In contrast, ligation of CD72 causes c-Jun N-terminal kinase (JNK) activation, which, surprisingly, is Btk independent. Interestingly, BCR-induced JNK activation is also Btk independent, but this JNK activation is not as strong as that induced by CD72. By using PD98059, an inhibitor of MAPK kinase (MEK)1/2 that blocks the ERK pathway, we show a differential requirement of ERK for CD72- and BCR-induced B cell proliferation. CD72 and BCR acted in synergy to induce proliferation of X-linked immunodeficiency (Xid) B cells, suggesting that they have independent roles in B cell activation.

Materials and Methods

Mice

Female CBA/Ca (non-Xid) and CBA/N (Xid) mice were obtained from the National Cancer Institute (Frederick, MD). DBA/2, CBA/J, CBA/CaJ, and BALB/c mice were purchased from the National Cancer Institute, National Institute on Aging, and The Jackson Laboratory (Bar Harbor, ME). All mice were kept in microisolator cages in our American Association for Laboratory Animal Accreditation and Certification-approved rodent facility. CBA/N × DBA/2F<sub>1</sub> male and female mice were bred by crossing female CBA/N and male DBA/2 in our own facility. The generation of JNK1<sup>−/−</sup> and JNK2<sup>−/−</sup> mice has been described previously, and JNK1<sup>−/−</sup> and JNK2<sup>−/−</sup> mice were bred in our own facility (27, 28). The JNK1<sup>−/−</sup> and JNK2-heterozygous mice in the (B6 × 129) background were intercrossed, and the offspring with JNK1- or JNK2-knockout phenotypes were selected by PCR.

Reagents

The monoclonal anti-CD72 Abs K10.6 and 10.1.D2 and the isotype-matched control Ab (Ab) MOPC195 (M195) used in this study have been previously described (14, 20, 22). Throughout these studies, anti-CD72 Ab refers to the clone K10.6 unless otherwise stated. Goat anti-mouse IgM F(ab′)<sub>2</sub> (μ-chain specific) was purchased from ICN Pharmaceuticals (Costa Mesa, CA). The 1C10 (anti-CD40) hybridoma was a gift from Dr. M. Howard (Corixa, Redwood City, CA), and 1C10 ascites were generated by injection of hybridoma cells into SCID mice. Abs to total p42/44/ERK1/2 and p38 MAPK were obtained from New England Biolabs (Beverly, MA). Ab to total p46/54 MAPK/JNK was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Abs to phosphorylated ERK, p38, and JNK were obtained from New England Biolabs. PD98059, U0126, dibutyryl cAMP (dbcAMP), and bisindoylmaleimide 1 hydrochloride (Bis) were purchased from Calbiochem (La Jolla, CA). Monoclonal anti-β-actin Ab and PMA were obtained from Sigma (St. Louis, MO).

B cell stimulation and Western blotting

Spleenic B cells were prepared by T depletion as described previously (20). Spleenic B cells in serum-free Iscove/F-12 medium were prewarmed at 37°C for 30 min and stimulated with 30 μg/ml of anti-IgM, anti-CD72, M195, or with a 1:50 dilution of anti-CD40 ascites for the time indicated. For experiments evaluating the role of protein kinase (PKC, K, cells were treated with PMA at 100 ng/ml for 20 h to down-regulate PKC activity. Alternatively, the PKC inhibitor Bis (1 μM) was added to the cells for 1 h at 37°C. For evaluating the impact of activated PKA, cells were preincubated with the PKA activator dbcAMP (1 mM) for 1 h at 37°C and later stimulated with the various Abs. Reactions of stimulation were stopped by adding ice-cold PBS containing 1 mM Na<sub>3</sub>VO<sub>4</sub> and 10 mM NaF (stop buffer). The cell pellets were collected by centrifugation and washed again with cold PBS stop buffer. Then the cells were lysed with lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 μg/ml leupeptin, and 1 mM PMSF for 30 min on ice. Lysates were cleared of nuclear debris by centrifugation at 12,000 × g for 10 min at 4°C. Total cell lysates were separated on SDS-PAGE using 10% polyacrylamide gels and analyzed by Western blotting using various primary Abs, followed by HRP-conjugated secondary Abs (Santa Cruz Biotechnology). The blots were developed with an enhanced chemiluminescence kit (DuPont/NEN, Boston, MA) and scanned with a flat-bed scanner (UMAX Technologies, Hsinchu, Taiwan). For reprobing, the membranes were stripped using a solution containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-ME at 65°C for 40 min. The relative integrated OD of the phosphorylated bands was estimated with the Scion Image program (Scion, Frederick, MD). The band intensities of phosphorylated MAPK in different treatment groups were normalized by dividing with the band intensity of total MAPK detected with an Ab that detects both phosphorylated and unphosphorylated forms of the enzyme. This corrected for any differences in protein loading. Fold induction is calculated as normalized band intensity in the experimental group to the band intensity in B cells treated with CAb, and these numbers are indicated beneath the phospho-MAPK bands in all the figures.

Apoptosis and cell cycle analysis

B cells (1 × 10<sup>6</sup>) were treated with various stimuli and cultures were harvested after 48 h. Cells were incubated with 10 μg/ml of the DNA-binding dye Hoechst 33342 (Molecular Probes, Eugene, OR) for 30 min at 37°C, washed with PBS, and stained for B cell marker B220 with anti-B220 Ab (clone RA3 6B2; Sigma). The level of Hoechst 33342 fluorescence measured using the MoFlo flow cytometer (Cytomation, Fort Collins, CO) is an indicator of the amount of DNA per cell, thus determining the position of each cell in the cell cycle at the time of harvest. The cells that were in the sub-G<sub>0</sub> region were considered to be apoptotic, as described earlier (29).

Proliferation assay

Cultures contained 2.5 × 10<sup>5</sup> purified B cells per 0.2 ml 5% FBS serum-free Iscove/F-12 medium in 96-well tissue culture plates. To determine cell proliferation, cultures were pulsed with 1 μCi [3H]thymidine (DuPont/NEN) 44 h after initiation of cultures unless indicated otherwise, and 4 h later, the cells were harvested onto filter mats (Skatron, Sterling, VA) using a cell harvester (Packard, Meriden, CT). The levels of radioactivity trapped in the mats were measured with a Matrix 96 β-counter particle counter (Packard).

Results

Stimulation of B cells with anti-CD72 Ab activates ERK1/2

Ligation of CD72 with most murine monoclonal anti-CD72 Abs induces B cell proliferation (Refs. 14, 22 and data not shown). Among the three major MAPKs (ERK1/2, JNK, and p38), activation of ERK1/2 is the most known for coupling surface receptor ligation to cell proliferation in numerous systems (26, 30, 31). Therefore, we examined the activation of ERK1/2 by detecting their dually phosphorylated (Thr<sup>202</sup>Tyr<sup>204</sup>) forms by Western blotting using Abs specific to the phosphorylated forms of ERK. Fig. 1A illustrates the kinetics of ERK1/2 activation after CD72 ligation. Treatment of B cells with anti-CD72 resulted in phosphorylation of ERK2 in 1 min. The ERK phosphorylation reached maximum levels by 15 min and decreased gradually over 60 min. To correct for protein loading, these blots were stripped and probed

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with an Ab to total ERK. The ratio of the density of the phospho-ERK2 band detected with the phospho-ERK Abs to that of the total ERK2 was calculated as described in Materials and Methods. The fold induction of phospho-ERK after being normalized to total ERK (as described in Materials and Methods) is shown directly below each band. Similar results were obtained in two other experiments. B, Dose-dependent response of ERK and JNK activation by CD72 ligation. B cells were stimulated with various concentrations of anti-CD72 Ab (0.01–30 μg/ml) for 1 min and tested for their ERK and JNK activity. The blots were then stripped and probed with anti-β-actin Ab to correct for changes in protein loading in different lanes. C, B cells were stimulated with 5, 10, or 50 μg/ml of anti-IgM, 50 μg/ml of anti-CD72, or 10 μg/ml of anti-CD72 plus 20 μM IL-4 in the presence of increasing concentrations of PD98059. The stimulated cells were harvested after 2 or 3 days of culture. At day 2, the proliferation responses of B cells treated with medium, 5 μg/ml anti-IgM, 10 μg/ml anti-IgM, 50 μg/ml anti-IgM, anti-CD72, and anti-CD72 plus IL-4 in the absence of PD98059 were 800 ± 116; 5,364 ± 1,048; 17,459 ± 2,884; 20,587 ± 2,849; 3,153 ± 270; and 14,812 ± 1,354 cpm, respectively. At day 3, the proliferation responses of B cells treated with medium, 5 μg/ml anti-IgM, 10 μg/ml anti-IgM, anti-CD72, and anti-CD72 plus IL-4 in the absence of PD98059 were 774 ± 34; 2,490 ± 775; 14,088 ± 3,481; 2,253 ± 296; and 18,021 ± 2,447 cpm, respectively. Proliferation was measured as described in Materials and Methods. Results were expressed as percentage of inhibition (mean ± SD of triplicate cultures) when compared with cells that were not treated with PD98059. Percentage of inhibition is defined as [1 − (cpm in the treatment group/cpm in the presence of vehicle control)] × 100. D, B cells were stimulated with 10 μg/ml of anti-IgM or 50 μg/ml of anti-CD72 in the presence of increasing concentrations of U0126. The stimulated cells were harvested after 2 days of culture. The proliferation responses of B cells treated with medium, anti-IgM, and anti-CD72 in the absence of U0126 were 2,394 ± 426; 4,462 ± 784; and 5,092 ± 123 cpm, respectively.

FIGURE 1. Cross-linking CD72 activates ERK in splenic B cells, and this ERK activation is required for optimal CD72-mediated B cell proliferation. A, Splenic B cells from DBA/2 mice were treated with CAb or anti-IgM for 10 min or anti-CD72 for indicated time points. Cell lysates were analyzed by immunoblotting with Abs to phosphorylated ERK1/2. The membrane was stripped and reprobed with anti-ERK1/2 Ab. The fold induction of phospho-ERK after being normalized to total ERK (as described in Materials and Methods) is shown directly below each band. Similar results were obtained in two other experiments. B, Dose-dependent response of ERK and JNK activation by CD72 ligation. B cells were stimulated with various concentrations of anti-CD72 Ab (0.01–30 μg/ml) for 1 min and tested for their ERK and JNK activity. The blots were then stripped and probed with anti-β-actin Ab to correct for changes in protein loading in different lanes. C, B cells were stimulated with 5, 10, or 50 μg/ml of anti-IgM, 50 μg/ml of anti-CD72, or 10 μg/ml of anti-CD72 plus 20 μM IL-4 in the presence of increasing concentrations of PD98059. The stimulated cells were harvested after 2 or 3 days of culture. At day 2, the proliferation responses of B cells treated with medium, 5 μg/ml anti-IgM, 10 μg/ml anti-IgM, 50 μg/ml anti-IgM, anti-CD72, and anti-CD72 plus IL-4 in the absence of PD98059 were 800 ± 116; 5,364 ± 1,048; 17,459 ± 2,884; 20,587 ± 2,849; 3,153 ± 270; and 14,812 ± 1,354 cpm, respectively. At day 3, the proliferation responses of B cells treated with medium, 5 μg/ml anti-IgM, 10 μg/ml anti-IgM, anti-CD72, and anti-CD72 plus IL-4 in the absence of PD98059 were 774 ± 34; 2,490 ± 775; 14,088 ± 3,481; 2,253 ± 296; and 18,021 ± 2,447 cpm, respectively. Proliferation was measured as described in Materials and Methods. Results were expressed as percentage of inhibition (mean ± SD of triplicate cultures) when compared with cells that were not treated with PD98059. Percentage of inhibition is defined as [1 − (cpm in the treatment group/cpm in the presence of vehicle control)] × 100. D, B cells were stimulated with 10 μg/ml of anti-IgM or 50 μg/ml of anti-CD72 in the presence of increasing concentrations of U0126. The stimulated cells were harvested after 2 days of culture. The proliferation responses of B cells treated with medium, anti-IgM, and anti-CD72 in the absence of U0126 were 2,394 ± 426; 4,462 ± 784; and 5,092 ± 123 cpm, respectively.
response induced by anti-IgM (at doses of 5, 10, and 50 μg/ml) was inhibited by PD98059 in a dose-dependent manner with ~90% inhibition at 25 μM PD98059 (Fig. 1C). Interestingly, the CD72-induced response was also inhibited in a dose-dependent manner by PD98059, but the maximal inhibition was only ~50% even at a 25-μM concentration of PD98059 (Fig. 1C). Higher doses of PD98059 could not be tested because they were cytotoxic. Notably lower doses of PD98059 had minimal effects on anti-CD72-induced response while inhibiting anti-IgM response by 60–70%. The differential susceptibility of anti-IgM- and anti-CD72-induced responses to the MEK inhibitor appeared to be independent of the magnitude of the proliferative response as well as the time of the assay (day 2 vs 3) (Fig. 1C). Thus, 5 μg/ml anti-IgM and 50 μg/ml anti-CD72 induced a low level of proliferation, whereas higher doses of anti-IgM or anti-CD72 plus IL-4 induced a high level of proliferation. Data from one experiment are shown in Fig. 1C, and the average of several experiments show that the inhibition was 40 ± 16% for anti-CD72 (n = 7) and 86 ± 9.4% for anti-IgM (n = 5), and the difference between the two levels of inhibition was statistically highly significant (p < 0.001). U0126, another MEK1/2-specific inhibitor (35), also inhibited BCR-induced B cell proliferation responses to a higher degree than those induced by anti-CD72 (Fig. 1D). Thus, there is a significantly distinct requirement of MEK1/2-activated ERK for BCR- and CD72-induced B cell mitogenesis response. There are at least two possible explanations for the partial resistance of the CD72 response to inhibition by PD98059. First, ERK activation may be the critical signaling event required for BCR proliferation, whereas CD72-mediated proliferation is mediated by a combination of ERK together with other unidentified signaling mediators. Alternately, CD72 may activate MEK1/2 as well as an unknown MEK that can phosphorylate ERK1/2, accounting for partial resistance of CD72 response to PD98059.

CD72-induced ERK activity is dependent on MEK1/2

To determine whether there was a PD98059-resistant MEK activated by CD72, MEK activity was evaluated by testing the ability of MEK to phosphorylate its substrate ERK after PD98059 treatment. B cells were pretreated for 1 h with 25 μM PD98059 or DMSO (vehicle control) and stimulated for 10 min with anti-IgM or anti-CD72 Ab. Cells were lysed, and the phosphorylation of ERK was determined. As demonstrated in Fig. 2, CD72-induced ERK1 phosphorylation is completely abolished with PD98059 treatment, and CD72-induced ERK2 activation was reduced by 70% when compared with the activity seen in DMSO-treated B cells and was below the level of unstimulated cells. Treatment of B cells with PD98059 (25 μM) inhibited BCR-mediated ERK2 phosphorylation by 70% and suppressed the phospho-ERK1 to undetectable levels, which is in agreement with previous observations (36). Hence, ERK1/2 activation by CD72 appears to be regulated only by MEK1/2, and the resistance of CD72-induced B cell activation to PD98059 could result from other positive signaling events that are activated after CD72 ligation.

Elevation of cAMP inhibits ERK activation by both CD72 and BCR

Previously, we showed that CD72-mediated proliferation was more resistant to dbcAMP at any given dose when compared with BCR (20, 37), suggesting that PKA activation might differentially inhibit signal transduction through CD72 and BCR. Purkerson and Parker demonstrated that inhibition of BCR signaling by PKA resulted in down-regulation of ERK activity (32). Therefore, it was interesting to see whether PKA activation by cAMP treatment differentially down-regulated CD72- or BCR-induced ERK activation. B cells were treated with or without 1.0 mM dbcAMP for 1 h at 37°C before stimulation with different stimuli for the indicated time points (Fig. 3). The activation of ERK was measured using phospho-ERK-specific Abs; the blots were stripped and reprobed with ERK2-specific Abs to correct for differences in protein loading. CD72- as well as BCR-induced ERK activation was reduced by dbcAMP close to the levels in cells treated with CAb (Fig. 3). Thus, dbcAMP inhibited both CD72- and BCR-induced ERK activity. However, unlike BCR stimulation, CD72 stimulation still induced partial B cell proliferation in the presence of dbcAMP (H.-J. Wu, L. Long, and S. Bondada, data not shown, and Ref. 20).

Stimulation of B cells by anti-CD72 activates JNK but not p38

In addition to ERK, there are two other MAPK family members, JNK and p38 MAPK. In B cells, CD40 is a much stronger activator of JNK and p38 MAPK, whereas BCR is a much stronger activator...
Ligation of various cellular surface receptors results in different activation patterns of the MAPK family, leading to differential gene expression and functional responses. Hence, we tested whether partial resistance of CD72 responses to ERK inhibition was due to its ability to activate JNK or p38 MAPK. We examined the activation of the JNK by testing for the appearance of its dually phosphorylated (Thr183 Tyr185) form by Western blotting using specific anti-phospho JNK Abs and correcting for the differences in protein loading by probing for total JNK enzyme. As shown in Fig. 4A, anti-CD72 treatment for 1 min resulted in a maximum phosphorylation of p56 JNK, and the levels of phosphorylation decreased gradually over 30 min. At 10 min, CD72 ligation resulted in a 6.2-fold increase in p56 JNK phosphorylation, whereas BCR ligation induced a 3.1-fold increase at 10 min. Stimulation through CD72 and CD40 also activated the p46 JNK isoform, but only to a minor extent (H.-J. Wu and S. Bondada, data not shown). Anti-CD72-mediated JNK activation was dose dependent, suggesting that induction of phospho-JNK is a CD72-specific event (Fig. 1B). Because CD72 activated JNK as well as ERK, we determined whether the partial inhibition of CD72 proliferation response by the MEK inhibitor (Fig. 1) PD98059 was due to its effect on ERK or JNK or both. As shown in Fig. 4E, PD98059 did not interfere with anti-CD72-induced JNK activation, whereas ERK activation was completely blocked. In contrast to JNK, ligation of either CD72 or BCR failed to induce p38 MAPK activation as examined by anti-phospho-p38 Ab (Fig. 4B). Only anti-CD40 induced a 1.5-fold induction of p38 MAPK at 10 min. The lack of BCR-induced p38 MAPK activation was surprising because several studies using B cell lines demonstrated such induction (40, 41). Interestingly, very few studies used normal B cells for this measurement. A positive p38 MAPK induction was seen in tonsillar but not peripheral blood B cells (36, 42). In summary, CD72 ligation induced both ERK and p56 JNK but not p38 MAPK activation in murine splenic B cells.

Lack of JNK2 or JNK1 does not affect CD72- and BCR-induced B cell proliferation

JNK activation could be the positive signal that enables CD72-ligated B cells to respond even when ERK is inhibited by PKA or PD90859. JNK has been implicated in playing several positive roles in B cell activation. For example, JNK is required for IL-3-induced proliferation in a pre-B cell line, and activation of JNK is detected in Ag-responsive naive B cells but not tolerant B cells after BCR ligation (43, 44). Three genes encode JNK in mammals, of which JNK1 and JNK2 but not JNK3 are expressed in the immune system (45). We used JNK1- and JNK2-knockout mice to...
ask whether activation of one of these two enzymes is essential for CD72-induced proliferation. JNK isozymes are produced in 56- and 46-kDa isoforms. Splenocytes from wild-type mice express predominantly 56-kDa JNK2 isofrm and the 46-kDa JNK1 isoform (27, 28, 46). Thus, in B cells, the p56 phospho-JNK induced by anti-CD72 probably was the JNK2 isofrm. In JNK2−/− mice, the CD72-induced proliferation response is indistinguishable from that of wild-type mice at any given dose of the anti-CD72 Ab (Fig. 4C). Similar results were obtained when B cells were stimulated with anti-IgM (H.-J. Wu, S. Estus, and S. Bondada, data not shown). Consistent with the data from Sabapathy et al., JNK2−/− B cells proliferated normally to LPS, IL-4, and anti-CD40 (Ref. 46 and data not shown). Similar results were obtained by using JNK1−/− mice to measure CD72- or BCR-induced B cell proliferation (Fig. 4D). Deficiency of JNK2 or JNK1 alone does not adversely affect CD72-induced B cell proliferation responses, which may be due to the ability of JNK1 and JNK2 to compensate each other (28, 46). Mice deficient for both JNK1 and JNK2 could not be tested because they are not viable.

PKC is involved in CD72- and BCR-transduced proliferation signal

Activation of PKC plays an important role in the signaling pathways activated by BCR ligation. Because we are interested in the similarities and differences of the signaling events activated by CD72 and BCR, we determined the importance of PKC in CD72- and BCR-mediated proliferation using the PKC specific inhibitor Bis (47). There was a dose-dependent inhibition of CD72-stimulated proliferation in the presence of Bis similar to that of BCR-stimulated cells (Fig. 5A). Thus, unlike PKA and ERK, PKC activation appears to play an equal role in CD72- and BCR-induced B cell proliferation.

Induction of ERK and JNK activity by CD72 ligation is PKC dependent

Several studies have suggested that, in B cells, there are both PKC-dependent (such as BCR-induced MAPK) and PKC-independent (such as CD40-induced MAPK activation) MAPK activation (33, 36, 48). We addressed the role of PKC in CD72-mediated ERK activation by using the PKC inhibitor Bis. B cells were pretreated with Bis for 1 h before stimulation. The PKC inhibitor abolished ERK phosphorylation in response to anti-CD72 as well as anti-IgM (Fig. 5B). We further addressed the role of PKC in CD72-mediated ERK activation by using overnight treatment with PMA to deplete PKC. B cells were pretreated with 100 ng/ml PMA for 20 h to induce the membrane translocation and subsequent degradation of PKC and were then stimulated with anti-CD72 Ab. In agreement with the Bis pretreatment experiments, the PMA treatment abolished ERK phosphorylation in response to anti-CD72 as well as anti-IgM (data not shown). Because ERK is required for maximal CD72-induced B cell proliferation, the reduction of CD72-induced proliferation by Bis shown in Fig. 5A may at least be partially due to a lack of PKC-dependent ERK activation.

We next addressed whether PKC is also required for CD72-mediated JNK activation by treating cells with Bis. There was a clear down-regulation of CD72-induced JNK activity in B cells treated with anti-CD72 for 1 min as well as 10 min (Fig. 5C). BCR-induced JNK activity was also PKC dependent, which is consistent with other published results (33, 36).

Induction of ERK1/2 but not JNK activity by both BCR and CD72 is Btk dependent

Xid in mice is an immunodeficiency disease caused by a mutant Btk gene resulting in intrinsic B cell defects (49). Ligation of CD72

FIGURE 5. A, The PKC inhibitor Bis inhibits CD72- and BCR-induced proliferation to similar levels. B lymphocytes from DBA/2 mice were incubated with various doses of Bis and anti-IgM (50 µg/ml) or anti-CD72 Ab (1/200 dilution). Proliferation of B cells to anti-IgM and anti-CD72 without Bis were 52,211 ± 12,076 cpm and 6,514 ± 448 cpm, respectively. Medium alone response was 2,129 ± 170. Percentage of inhibition was calculated as described in Fig. 1. B and C, ERK and JNK activation by CD72 and BCR signaling is PKC dependent. B cells from DBA/2 mice were treated with Bis or vehicle control (medium) for 1 h and then stimulated with Cab for 10 min or with anti-CD72 Ab or anti-IgM for the indicated time periods. Cell lysates were analyzed by immunoblotting with Abs to phosphorylated ERK1 and ERK2 (B) or phosphorylated JNK (C). Fold induction is calculated as described in Materials and Methods. Normalized phospho-ERK and phospho-JNK ratio for B cells treated with Cab in the presence of medium or Bis is set to 1.
or BCR failed to induce B cell proliferation in Btk-deficient Xid (CBA/N) mice (14, 22). Because Btk is thought to be critical for B cell activation, we tested the role of Btk in CD72- and BCR-induced MAPK activation in murine B cells. Ligation of CD72 in Xid B cells displayed no sign of phospho-ERK induction at any time point studied in the experiment shown in Fig. 6, whereas minimal activation was seen in other experiments. Results from several experiments comparing MAPK activation in normal and Xid B cells are summarized in Table I. CD72-induced ERK activation in Xid mice was 84% less than in wild-type mice (Table I). BCR or anti-CD40 cross-linking resulted in a slightly higher ERK phosphorylation in Xid B cells compared with CD72 ligation. However, the BCR-mediated ERK activity in Xid mice was 78% less compared with that in the wild-type mice (Fig. 6A vs Fig. 6 and Table I), suggesting that Btk is important for both BCR- and CD72-mediated ERK activation in B cells.

In various systems, JNKs have been shown to be activated through a different pathway from ERKs. Thus, we were interested in knowing whether CD72-mediated p56 JNK activation had the same Btk requirement as CD72-mediated ERK activation. Xid B cells were purified and stimulated with anti-CD72, and JNK phosphorylation was determined. CD72-mediated p56 JNK activation was maintained in Btk-deficient B cells with maximum 5.9-fold activation at 1 min, which decreased gradually over 30 min (Fig. 6). Also, the kinetics of CD72-induced p56 JNK activation in B cells from Xid mice (Fig. 6) were similar to B cells from CBAJ and CBA/Ca mice (Fig. 4A and Table I), which are the normal counterparts. Although BCR-induced JNK activity was again less than that induced by anti-CD72, this low activity was preserved in CBA/N mice, suggesting its Btk independence. Thus, unlike ERK, p56 JNK activation induced by anti-CD72 or BCR is independent of Btk.

**CD72 and BCR signals cooperate in the induction of proliferation of B cells from Btk-defective mice**

Previously, CD72 Ab has been shown to block BCR hypercross-linking-induced apoptosis in mature B cells as well as anti-IgM-induced apoptosis in immature B cells in normal mice (19). Because CD72 ligation induced Btk-independent signals such as JNK activation, we tested the ability of CD72 to overcome BCR-induced growth arrest in Btk-defective B cells. To rule out the possibility that the percentage of Xid B cells after T depletion is less than in wild-type mice, resulting in an unfair comparison, B cells were enriched to 98% by sorting for IgM+ B cells on a flow cytometer. Anti-CD72 plus IL-4 or anti-IgM plus IL-4 induced very little B cell proliferation in Btk-defective (CBA/N×DBA/2)F1, male mice (Xid), which is consistent with previous results (Fig. 7) (14, 22, 50). Interestingly, there was a synergy between CD72 and BCR in that the proliferation of Xid B cells in the presence of IL-4 to these two stimuli was 2.3-fold more than the sum of the response obtained with each individual Ab (arrow in Fig. 7A shows the sum of individual response to anti-IgM plus IL-4 and K10.6 plus IL-4) (Fig. 7A). Although IL-4 was added to the cultures to increase the magnitude of B cell proliferation, without IL-4 the fold difference between different groups remained the same. Such a synergy was almost nonexistent in the phenotypically normal (CBA/N×DBA/2)F1 female mice because both anti-CD72 and anti-IgM on their own induced a robust B cell growth response (Fig. 7B). Thus, although anti-CD72 dramatically increased responses of Xid B cells to anti-IgM, the rescue of Xid B cells was only partial because the resulting response was still less than that in the wild type.

Because Xid B cells have been shown to have a defect in cell cycle progression (51, 52), we examined whether anti-CD72 overcame the cell cycle defect and reduced the BCR-induced apoptosis of Xid mice. B cells were treated with medium, anti-IgM, or anti-CD72 Ab or a combination of anti-IgM and anti-CD72 in the presence or absence of IL-4 and were analyzed for DNA content using flow cytometry. As presented in Fig. 7C, IL-4 alone reduced apoptotic cell number and slightly increased cycling cell number. Treatment with IL-4 plus anti-IgM or anti-CD72 treatment did not further trigger cell cycle entry of Xid B cells. Addition of anti-CD72 Ab into Xid B cell cultures containing anti-IgM and IL-4 dramatically increased the number of cycling cells (5.4 or 3.7% when treated with IL-4 plus anti-IgM or anti-CD72, respectively, to 16.7% in the presence of anti-CD72 plus anti-IgM plus IL-4). In the phenotypically normal (CBA/N×DBA/2)F1 female mice, the effects of the two Abs on B cells appeared to be nearly additive (22.5 or 6.6% when treated with IL-4 plus anti-IgM or anti-CD72, respectively, changes to 32.3% in the presence of IL-4 plus both Abs) (Fig. 7D). Therefore, the synergy in the proliferation response of Xid B cells seen with anti-CD72 plus anti-IgM appeared to be a result of a relief from cell cycle block but not a reduction of apoptosis.

**Discussion**

CD72 is an important coreceptor on B cells that has been associated with positive signaling events, such as protein tyrosine phosphorylation, leading to B cell proliferation (14, 20, 22, 23). Recent findings on its association with SHP-1 and gene deletion studies have suggested a negative signaling role for this coreceptor (17–19). However, little is known about the downstream signaling events mediated by CD72 that can help distinguish between positive and negative roles of this molecule in B cell activation. Here we have shown for the first time that Abs to CD72 can induce activation of ERK1/2 and JNK1/2 but not p38 MAPK. In B cells, BCR cross-linking induces ERK activation via the Ras/Raf/1-MEK pathway, which has been associated with positive signaling through the BCR. CD72 may also use this pathway to activate ERK because its cytoplasmic region associates with Grb2, a major adaptor protein required to activate the Ras pathway (19). More recently, CD72-associated Grb2 has been shown to recruit B cell linker protein (53), an adaptor protein that is known to deliver positive signals from the BCR (54). Consistent with the idea that CD72 is a positive regulator, we found that optimal B cell proliferation induced by CD72 required ERK activity. The MAPKs are often involved in transcriptional activation of the cell cycle components such as cyclin D, E1, and p27kip1 (55).

We propose that CD72 activates JNK through its association with CD19 based on our previous result that CD72 ligation induced phosphorylation of CD19 and recruitment of the lipid kinase
phosphatidylinositol 3-kinase (PI-3K) to CD19 (23). CD19-associated PI-3K has been shown to activate Rho and Rac proteins that are upstream of JNK (56, 57). In support of this hypothesis, we found that inhibition of PI-3K disrupted CD72-mediated B cell proliferation (23). Furthermore, there was a significant reduction in the proliferation response of CD19−/− B cells to CD72 stimulation. The JNK activation by CD72 is in accord with a positive role for this coreceptor because JNK activity has been shown to be critical for proliferation of pre-B cells, lung cancer cells, germinal center B cells, and EBV latent membrane protein-1-induced long-term growth of human lymphoblastoid cells (43, 58–60). For the first time, we have shown that ERK activation by both CD72 and BCR is Btk dependent in murine B cells, which is consistent with the failure of Xid B cells to proliferate in response to CD72 or BCR ligation. Surprisingly, JNK activation by CD72 or BCR was not affected by Btk mutation. Moreover, we found that simultaneous ligation of BCR and CD72 on Xid B cells restored a strong proliferation response in Xid B cells. Thus, both BCR and CD72 can provide partial activation signals to Xid B cells that can cooperate to restore a mitogenic response. This is consistent with previous reports that Xid B cells exhibit changes in early signaling events but not mitogenesis when triggered with soluble anti-Ig Ab (61, 62). Our finding that Xid B cells exhibit some ERK activity, albeit reduced, upon BCR ligation and near normal levels of JNK activity upon CD72 ligation suggests that these two MAPKs may act in synergy to promote B cell mitogenesis. Requirement for both ERK and JNK for B cell growth response is also supported by the finding that anergic B cells exhibit ERK but not JNK activation (66). The effect of CD72 ligation in Xid B cell proliferation is not simply due to the proliferation response of CD19−/− B cells to CD72 stimulation. CD72 ligation rescues Xid B cell response to anti-IgM by relieving the block in cell cycle. B lymphocytes from (CBA/N × DBA/2)F1 male mice (Xid) (19, 65). Based on CD72-SHP-1 association, Adachi et al. (Fig. 8A) (19, 65). In this model, the positive growth responses induced by anti-CD72 Ab in mature B cells may be due to interruption of BCR-CD72 association by the anti-CD72 Ab and prevention of BCR-SHP-1 association (65). Because no other ligand is intentionally added in B cell cultures treated with anti-CD72 Ab, one has to postulate that constitutive signaling via BCR should be

<table>
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<tr>
<th>Fold Induction Of</th>
<th>Wild Type</th>
<th>Xid</th>
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<tbody>
<tr>
<td>Phospho-ERK2</td>
<td>4.4 ± 2.3 (n = 5)</td>
<td>6.5 ± 1.6 (n = 5)</td>
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<tr>
<td>Phospho-JNK</td>
<td>6.1 ± 2.9 (n = 3)</td>
<td>1.9 ± 1.2 (n = 3)</td>
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* *Ligation of BCR or CD72 induces Btk-dependent ERK activation and Btk-independent JNK activation. Wild-type mice include CBA/Ca, CBA/J, and DBA/2. Fold induction is calculated as described in Materials and Methods. Results represent the mean ± SD from the number of experiments indicated as n in parentheses.

In contrast to the present data, which are highly supportive of a positive signaling role for CD72, two views of negative regulation of BCR signaling by CD72 were proposed by Adachi et al. and Wu et al. (Fig. 8A) (19, 65). Based on CD72-SHP-1 association, Adachi et al. suggest that CD72 inhibits BCR signaling by recruiting SHP-1 to BCR. An increase in CD72 expression in anergic B cells further supports a potential negative regulatory role for CD72 in B cell activation (66). In this model, the positive growth responses induced by anti-CD72 Ab in mature B cells may be due to interruption of BCR-CD72 association by the anti-CD72 Ab and prevention of BCR-SHP-1 association (65). Because no other ligand is intentionally added in B cell cultures treated with anti-CD72 Ab, one has to postulate that constitutive signaling via BCR should be

FIGURE 7. CD72 ligation rescues Xid B cell response to anti-IgM by relieving the block in cell cycle. B lymphocytes from (CBA/N × DBA/2)F1 male mice (Xid) (44) and phenotypically normal (CBA/N × DBA/2)F1 female mice (B) were stimulated with IL-4 plus anti-CD72 (K10.6) or anti-IgM or both Ab. Arrows in both panels indicate the sum of individual response to anti-IgM plus IL-4 and anti-CD72 plus IL-4. The effect of CD72 ligation on cell cycle stage in B cells from both Xid (C) and normal (D) mice is shown. B cells were treated with either medium, IL-4, IL-4 plus anti-IgM, anti-CD72 Ab, or a combination of anti-IgM and anti-CD72 for 2 days. The amount of DNA in the B220+ cells was quantified by flow cytometry as described in Materials and Methods.
FIGURE 8. Two models postulating the mechanisms for CD72-induced signal transduction in B cells. A, Negative signaling model of CD72. Positive signaling through CD72 is induced by BCR ligation, which triggers the association of CD72 with SHP-1. The positive responses induced by CD72 ligation are likely to be due to enhanced constitutive BCR signals that result from sequestration of the negative SHP-1-CD72 complex away from the BCR. The mechanism for negative signaling by CD72 is the same as in A. However, in B, the positive effect of anti-CD72 is due to the independent and unique signaling of CD72, which involves phosphorylation of CD19 and other signaling intermediates by CD72 ligation. 

A. Negative signaling model of CD72

B. Dual signaling model of CD72

responsible for the CD72-induced growth responses. However, this appears to be unlikely for four reasons. First, in vivo studies suggest that constitutive signaling by BCR is important for B cell survival but not for proliferation (67). Constitutive signaling by definition is weak and unlikely to constitute a full mitogenic signal unless supplemented by positive signals provided by CD72 ligation. The second and most compelling argument is that Xid B cells are normally unresponsive to BCR cross-linking. Therefore, addition of anti-CD72 to prevent BCR-CD72 association cannot restore any mitogenic constitutive signal in these B cells because of their Btk defect. The major functional outcome of BCR signaling in Xid B cells is apoptosis (68), which should be enhanced by CD72-SHP-1 sequestration in a model that views CD72 as a guard molecule that attenuates any signal from BCR (Fig. 8A). As shown here, adding anti-CD72 to anti-IgM plus IL-4-treated B cell cultures results in a vigorous growth response, suggesting that CD72 must be providing a positive signal that is independent of BCR. This signal is likely due to, but not limited to, JNK activation. In agreement with this notion, Wu et al. found that, unlike CD22, CD72 ligation did not enhance BCR-induced apoptosis of the WEHI-231 B cell lymphoma (19). In contrast, ligation of CD72 enhanced growth response of WEHI-231 B cells to BCR ligation (19). Third, CD72 and BCR ligation display a differential signaling pattern. If ligation of CD72 is just to increase weak BCR constitutive signals, we will predict that CD72 ligation would display a weaker but similar pattern of kinase activation in comparison to BCR (i.e., SYK and ERK > JNK) activation. However, CD72 induces stronger JNK than ERK and no SYK activation, which would not be predicted by a model that proposes that sequestration of negative signals by CD72 enables constitutive signaling via BCR (Fig. 8A). Also, CD72 growth response is more resistant to blocking of ERK activity by PD98059 and dbcAMP. Finally, it is worth noting that, unlike CD22, CD72 was not found to associate with BCR upon BCR ligation (69–71). Thus, how the CD72-SHP-1 complex inhibits BCR signals needs to be further clarified. 

While this paper was in review, one group reported that CD100 is the natural ligand for CD72 (72). They found that ligation of CD72 by CD100 delivered positive signals into B cells by turning off phosphorylation of CD72 that leads to its dissociation from SHP-1 but is not simply an effect of sequestration of CD72-SHP-1 away from the BCR. B cells from CD100−/− mice are defective in CD40-induced B cell proliferation (73). Because BCR is not involved, these results suggest that CD72 ligation could deliver unique positive signals that are independent of BCR.

CD22, another receptor shown to associate with SHP-1, has been found to have both a positive and a negative regulatory role in B cell responses, as evidenced by a decrease in BCR-induced B cell proliferation in some CD22-knockout mice but up-regulation of B cell responses in vitro by CD22 sequestration from BCR (9, 11, 12). Thus, CD22 and CD72 may be similar in mediating positive signals despite their association with SHP-1.

We would like to propose the following based on the results reported here and other data in the literature (Fig. 8B). Treatment of mature B cells with unconjugated anti-IgM evokes proliferation, a condition under which CD72 was not found to be tyrosine phosphorylated (Ref. 20 and Venkatraman et al., unpublished observations). On the contrary, in immature B cells or B cell lines, BCR ligation alone was sufficient to induce CD72 tyrosine phosphorylation and its association with SHP-1 accompanied by apoptosis. Thus, BCR-induced binding of SHP-1 to CD72 may depend on the B cell developmental stage and/or the strength of stimulation. This implies that the function of CD72 might be context dependent but not fixed as a totally negative or positive regulator. Therefore, we hypothesize that CD72 serves as an independent positive signaling molecule as well as a negative regulator of BCR signaling. CD72
ligation may transduce the positive signal through a specific phosphorylation of the Grb2 binding site and recruiting Grb2 and/or through interaction with CD19, which in turn activates downstream signals such as ERK and JNK. In contrast, BCR ligation induces phosphorylation of CD72 at its SHP-1 binding site and thus recruits SHP-1 to the CD72 cytoplasmic domain. SHP-1, in turn, can dephosphorylate its targets and thus negatively regulate BCR signaling. In agreement with the hypothesis that CD72 can deliver positive signals independent of BCR (Fig. 8B), we recently showed that CD72 induced ERK activation in A20.2-2.4, a BCR-deficient B cell line (our unpublished data). In CD72-/- mice, the normal humoral immune response to T-dependent and T-independent Ag or for isotype switching might be the result of a compromise between the positive and negative roles of CD72 on B cell activation. Which signal is evoked by CD72 might depend on the microenvironment surrounding B cells, such as the accessibility of B cell to CD72 ligand and Ag. Recent identification of CD100 as a ligand for CD72 may help in resolving these issues (72, 73).

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References


