Impairment of B cell receptor-mediated Ca\(^{2+}\) influx, activation of mitogen-activated protein kinases and growth inhibition in CD72-deficient BAL-17 cells

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**Keywords:** B cell antigen receptor, calcium entry, CD19, PI3 kinase

**Abstract**

CD72 is a 45 kDa B cell-specific type II transmembrane protein of the C-type lectin superfamily. It was originally defined as a receptor-like molecule that regulates B cell activation and differentiation; however, its precise function remains unclear since more recent functional analyses, including a gene targeting study, suggest that CD72 may serve as a negative or a positive regulator of B cell signaling. In the present study, we analyzed the cell-autonomous function of CD72 in B cell receptor (BCR) signaling using CD72-deficient cells generated from mature BAL-17 cells. We found that BCR-mediated phosphorylation of CD19, Btk, Vav and phospholipase C\(_\gamma\)\(^2\) and association of CD19 with phosphatidylinositol-3 kinase were impaired in CD72-deficient cells. Inositol trisphosphate synthesis was normally induced initially but ablated at 1 min of stimulation in CD72-deficient cells. In the event, Ca\(^{2+}\) release from intracellular stores remained intact, though influx of extracellular Ca\(^{2+}\) was severely impaired in CD72-deficient cells. Furthermore, BCR-evoked activation of mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase and c-Jun NH\(_2\)-terminal kinase, and growth inhibition in BAL-17 cells were blocked in the absence of CD72. Significantly, these effects were largely reversed by re-expression of CD72. Thus, CD72 appears to exert a positive effect on BCR signaling pathways leading to Ca\(^{2+}\) influx and MAPK activation, which in turn may determine the fate of BAL-17 cells.

**Introduction**

B cell antigen receptor (BCR) ligation induces activation of three families of protein tyrosine kinases (PTKs): Src-family PTKs (Lyn, Fyn, Lck, Fgr and Blk), Syk and Btk. Activation of these enzymes initiates a number of downstream biochemical events that ultimately determine the final outcomes of B cells, which include activation, proliferation, survival, differentiation into plasma or memory cells, anergy and apoptosis (1–4). Also participating in this process are various coreceptors, including CD19, CD21, CD22, CD40 and CD72 (4–8).

Major signaling pathways triggered by BCR ligation involve activation of phospholipase C\(_\gamma\)\(^2\) (PLC\(_\gamma\)\(^2\)), of Ras and Ras-related small G proteins, and of phosphatidylinositols-3 kinase (PI3K) (9). Activated PLC\(_\gamma\)\(^2\) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) to inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol, which respectively induce Ca\(^{2+}\) mobilization and activation of protein kinase C. For activation of Ras (10,11), tyrosine phosphorylated adaptor protein Shc recruits Grb2, which binds to Sos, a guanine nucleotide exchange.
factor (GEF) that interacts with Ras, forming a trimolecular complex (12,13). Subsequent translocation of the complexed Sos to the plasma membrane leads to Ras activation, which in turn activates Raf/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (ERK) pathways. In addition, phosphorylation of Vav, which serves as a GEF for Rho family GTPases (14), leads to activation of c-Jun NH2-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK). Rho family GTPases also promote sustained PI3K synthesis by activating PI 4-phosphate 5-kinase, while activation of PI3K leads to accumulation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) in the plasma membrane (15), which in turn induces recruitment of signaling molecules containing pleckstrin homology domains—e.g. Btk, PLCγ2 and Vav—leading to their full activation (2).

CD72 is a 45 kDa type II transmembrane protein belonging to the C-type lectin superfamily (16,17) that is expressed on all B cells except plasma cells (18,19) and has a receptor recently identified as CD100, a class IV semaphorin (20). We originally reported that CD72 is involved in the regulation of antibody responses to T-dependent antigens (21), and that it most likely exerts its effect by modulating an early phase of B cell activation (22,23). Ligation of CD72 with anti-CD72 antibody, alone or together with anti-IgM antibody or IL-1, induces a variety of effects including cell proliferation (23,24), expression of major histocompatibility complex class II (25), IL-4-dependent expression of CD23 (26), rescue of splenic B cells from BCR-mediated apoptosis (27), activation of Lyn, Btk, Btk, PLCγ2 and CD19 (28), association of CD72 with CD19 (29), phosphatidylinositol (PI) turnover (30), Ca2+ mobilization (31) and MAPK activation (32). In other words, mAb ligation of CD72 delivers positive regulatory signals in B cells.

On the other hand, recent biochemical studies have provided evidence suggesting CD72 serves as a negative regulator of BCR signaling. CD72 contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic domain and, in WEHI-231 B cells, BCR ligation induces tyrosine phosphorylation of CD72 and recruitment of the protein tyrosine phosphatase SHP-1, for which CD72 serves as a substrate (33,34). Because SHP-1 is a negative regulator of BCR signaling (35), this suggests that CD72 may negatively regulate BCR signaling through the action of SHP-1. In fact, expression of CD72 in K46 mouse B lymphoma cells, which expresses hapten-specific μ heavy and λ light chains, reduces BCR-induced ERK activation and Ca2+ mobilization, as does preligation of CD72 in splenic B cells (36). CD72 expression in BCR-expressing myeloma J558L cells also reduces BCR-induced phosphorylation of Ig-α/β. Syk and B cell linker protein (BLNK, also named SLP-65) (37). This profile of responses suggests CD72 serves as a negative regulator of B cell activation. A recent gene-targeting study showed that there is a reduction of mature recirculating B cells and an accumulation of pre-B cells in the bone marrow of CD72-deficient (CD72−/−) mice; that there is a reduction in mature B-2 cells and an increase in B-1 cells in the periphery of these mice; and that responses to anti-IgM antibody and a mitogen are augmented in CD72−/− B cells, as is BCR-mediated Ca2+ mobilization (38).

To resolve this discrepancy, we re-evaluated the cellular autonomous function of CD72 in BCR signaling using CD72−/− deficient cells generated from BAL-17 mature B cells. Our results show that CD72 positively regulates signaling pathways leading to Ca2+ influx and MAPK activation, which may ultimately dictate the fate of BAL-17 cells.

Methods

Antibodies and reagents

Goat anti-mouse IgM antibody F(ab′)2 fragments were purchased from ICN Pharmaceuticals, Inc. (Aurora, OH). Antibody CD72ab mAb (9–6.1) (21), CD72a/Ly-19.2 mAb (K10.6) and CD72b/Ly-32.2 mAb (B9.320) (39) were used. Anti-BLNK antibody was described previously (40). Anti-phosphotyrosine mAb (PY20) and rabbit anti-mouse antibodies against Lyn, Syk, ERK-2, JNK, p38, PLCγ2 and Btk were all from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti- phospho-Lyn (Tyr507), anti-phospho-Src (Tyr416) and anti-CD19 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rat anti-mouse CD19 mAb (BD Pharmingen, San Diego, CA) was used for immunoprecipitation. Rabbit anti-mouse phospho-specific p38 antibody and phospho-specific JNK antibody were from New England BioLabs (Beverly, MA), and rabbit anti-mouse phospho-specific ERK antibody was from Promega (Madison, WI). Rabbit anti-PI3K regulatory subunit p85 (p85-PI3K) and anti-Vav antibodies were from Upstate Biotechnology (Lake Placid, NY).

Generation of CD72-deficient clones

BAL-17 cells, a murine mature B cell line (IgM-positive, IgD-positive, CD72b), were cultured as described elsewhere (41,42). Initially, the cells were treated with N-methyl-N′-nitro-N-nitrosoguanidine (2 μg/ml) (Sigma, St Louis, MO) for 45 min at 37°C under an atmosphere of 5% CO2/95% air. After washing, the cells were cultured in RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, 100 μg/ml streptomycin and 100 U/ml penicillin (complete medium) for 1–2 weeks. To eliminate CD72-positive cells, the cells were incubated with anti-mouse CD72ab/Ly-19.2 mAb for 30 min on ice and then with complement for 45 min at 37°C. Viable cells were then cloned by limiting dilution. The process of elimination and limiting dilution was repeated three times.

Flow cytometric analysis

To examine the surface phenotype, cells were first incubated with anti-CD72a mAb, anti-CD72b/Ly-19.2 mAb, anti-CD72b/Ly-32.2 mAb, anti-H-2d mAb (34-1-2S), anti-Iad (MK-D6), anti-CD16/CD32 mAb (2.4G2) or buffer alone for 20 min at 4°C and then with FITC-conjugated secondary antibodies for 20 min at 4°C. FITC-conjugated anti-mouse IgGs (ICN Pharmaceuticals, Aurora, OH), FITC–protein A (PA) (Amersham Pharmacia Biotech, Uppsala, Sweden), FITC–mouse anti-rat Igκ chain mAb (Zymed, San Francisco, CA) and FITC–avidin (ICN Pharmaceuticals) were used as secondary antibodies. To detect surface expression of IgM, sIgD, CD19 and CD22, FITC–conjugated F(ab′)2 fragments of anti-mouse IgM antibody (ICN Pharmaceuticals), FITC-conjugated anti-IgD allotype (Igh-5a) mAb, FITC-conjugated anti-CD19 mAb, and FITC-conjugated anti-CD22 mAb (BD PharMingen) were...
used. After washing, the FITC-labeled cells were analyzed with a Beckman-Coulter ELITE flow cytometer (Coulter, Miami, FL).

RT–PCR analysis
Total RNA from BAL-17 and 9-59 cells was isolated using the single-step acid guanidium thiocyanate method. cDNA synthesis and RT–PCR were performed as previously reported with slight modifications (43). Five micrograms of total RNA from each sample was reverse transcribed at 42°C for 1 h in 20 µl of buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl and 3 mM MgCl₂) containing 5 µM random primer, 200 U Superscript II reverse transcriptase (Life Technologies, Grand Island, NY), 0.5 mM dNTP and 10 mM dithiothreitol. Primer sequences for amplification of full-length CD72 and internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows. CD72: sense, 5’-CGGAATTCCGTATGGGCTGACGCTA-3’; antisense, 5’-CTTATGGCCATGCCCGAGA-GAG-3’. GAPDH: sense, 5’-CATCCACCTCTCCAGGAG-3’; antisense, 5’-CTGCTTACACCTCTTGTG-3’. The PCR conditions were 25 cycles of denaturation (94°C, 0.5 min), annealing (58°C, 1 min) and extension (72°C, 1 min), followed by a final 5 min extension at 72°C. PCR products were subsequently electrophoresed on 1% agarose gels containing ethidium bromide.

Cell stimulation
Cells were initially harvested from log phase cultures, resuspended in fresh prewarmed complete medium, and incubated for 3 h at 37°C. They were then stimulated with anti-IgM antibody F(ab’)₂ fragments for 1–30 min, after which the reactions were terminated with ice-cold PBS containing 2 mM Na₃VO₄ and 2 mM EDTA (PBS-VE). After centrifugation, the cells were solubilized in TNE lysis buffer (1% Nonidet P-40, 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM Na₃VO₄, 2 mM EDTA, 1 mM NaF, 10 mM sodium pyrophosphate) supplemented with protease inhibitors (1 mg/ml E64, 1 mg/ml pepstatin A, 10 mM benzamidine, 2 mg/ml aprotinin and 100 mg/ml TPCK). The resultant lysates were centrifuged at 10 000 g at 4°C for 30 min, and the supernatants were subjected to further analysis.

Measurement of [Ca²⁺]
Assays were performed as previously described (41). Briefly, cells were loaded with membrane-permeant acetoxy-methyl ester of the fluorescent Ca²⁺ indicator Fluo-3 (Fluo-3AM) (Molecular Probes, Eugene, OR) in 1 ml of Krebs–Ringer–Hepes (KRH) buffer (6 mM Hepes–NaOH, 125 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 2 mM CaCl₂, 6 mM glucose, pH 7.4) for 45 min at 37°C. After loading, the cells were washed three times and resuspended in KRH buffer at a density of 10⁶ cells/ml. The cells were then stimulated with anti-mouse IgM antibody F(ab’)₂ fragments (10 µg/ml), and fluctuations in [Ca²⁺] were measured over a 5–10 min period using an ELITE flow cytometer; mean fluorescence intensity was determined using the Multimite program (Phenix Flow Systems, San Diego, CA). In some experiments, EGTA (2 mM) was added to chelate the extracellular Ca²⁺ prior to making the measurements. To deplete intracellular calcium stores, 0.2 µM thapsigargin (Tg) was added to cells suspended in Ca²⁺-free KRH buffer, after which capacitative Ca²⁺ influx was measured following addition of 0.2 mM CaCl₂ to the cell suspension.

Western blot analysis
The NP-40-soluble supernatants from unstimulated and anti-IgM-stimulated cells were separated on 10% SDS–PAGE gels and transferred to nitrocellulose membranes for 2 h at 80 V. The membranes were blocked with 5% non-fat dried milk; incubated overnight at 4°C with respective antibodies in buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Tween-20 and 5% BSA, and then incubated for an additional 1 h at room temperature with alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies. The blots were visualized using an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad Laboratories, Hercules, CA) or ECL Western Blotting Detection Reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturers’ protocols.

IP₃ measurement
IP₃ production was measured as described by Choi et al. (44), with a minor modification. Briefly, cells (10⁶/ml) were stimulated with anti-mouse IgM antibody F(ab’)₂ fragments (20 µg/ml), after which the reaction was terminated by adding ice-cold 100% trichloroacetic acid. IP₃ was then extracted from the samples and quantified using an IP₃ [3H] radio-receptor assay kit (PerkinElmer Life Sciences, Boston, MA).

Stable transfection
cDNA encoding mouse CD72a (a generous gift from Dr. J. R. Parnes, Stanford University Medical Center, Stanford, CA) was subcloned into pEGFP-C3 expression vector. This construct was then transfected into CD72b-deficient 9-59 cells by electroporation, and transfectants were selected in the presence of G418 (1 mg/ml). Expression of the transfected cDNA was confirmed by staining cells with anti-CD72a mAb.

Assay for DNA synthesis
Cells (5 × 10⁵) were cultured for 2 days in 0.2 ml of complete medium in triplicate with goat anti-mouse IgM antibody F(ab’)₂ fragments (5–20 µg/ml). To assess DNA synthesis, 0.5 µCi of [³H]thymidine was added to each well for the last 4 h. The cells were then harvested on glass fiber filters using a semiautomatic Skatron harvester, and thymidine incorporation was measured in a Beckman liquid scintillation counter. The results were expressed as percent inhibition of DNA synthesis = 100 [1 – (anti-IgM antibody added)/(no antibody added)].

Results
Generation and characterization of CD72-deficient clones
CD72-deficient clones were isolated from negatively selected BAL-17 cells following mutagenesis (see Methods). Using this approach, we obtained a CD72-deficient clone 9-59 (Fig. 1). This clone was not reactive with two CD72a (anti-Ly-19.2 and anti-Ly-32.2) mAbs directed against different epitopes of CD72 (Fig. 1A). Furthermore, reverse transcription (RT)–PCR analysis with primers that cover the whole CD72 sequence
Positive regulation of BCR signaling by CD72

A

BAL-17

9-59

CD72b/Ly-19.2

CD72b/Ly-32.2

Fluorescence intensity

B

BAL-17

9-59

CD72

GAPDH

C

BAL-17

9-59

IgM

IgD

H-2d

I-a\(^d\)

CD45

CD16/CD32

CD19 & CD22

Fluorescence intensity
demonstrated expression of CD72 at mRNA level was almost undetectable in 9-59 cells (Fig. 1B). Thus, we conclude 9-59 cells express a negligible level of CD72 protein. The cell surface expression of various proteins was also examined in 9-59 cells as compared to BAL-17 parental cells. As shown in Fig. 1(C), molecules such as IgM, IgD, H-2, Ia, CD45, CD16/CD32, CD19 and CD22 were expressed in 9-59 cells, comparable to the level in BAL-17 cells. These results suggest, but do not necessarily prove, that 9-59 cells are selectively defective in CD72 expression. Therefore, to unequivocally demonstrate the function of CD72, we investigated whether the phenotypes observed in 9-59 cells were reversed by re-expression of CD72.

BCR-induced tyrosine phosphorylation is minimally affected in CD72-deficient cells

We first examined early signaling events induced by BCR ligation. As shown in Fig. 2(A), the level of total protein tyrosine phosphorylation was not significantly different in the parental and CD72-deficient 9-59 cells within 5 min. This is also the case for 15–30 min (data not shown). The tyrosine phosphorylation of Syk was not affected by the absence of CD72 (Fig. 2B). The phosphorylation of Lyn seemed to be slightly reduced at 2–5 min in 9-59 cells. We then examined the phosphorylation of the autophosphorylation site, an indicator of activation of Src-PTKs, by immunoblotting with anti-phospho-Src autophosphorylation site antibody that cross-reacts with phosphorylated Y397 of Lyn. As shown in Fig. 2(B), there was little difference between the parental cells and 9-59 cells. The phosphorylation of the negative regulatory site of Lyn was also comparable to the parental cells. Thus, CD72 exerts minimal effects on the phosphorylation and activation of Lyn and Syk.

BCR-induced calcium influx is selectively impaired in CD72-deficient cells

BCR ligation stimulates synthesis of IP3, which leads to the release of Ca2+ from intracellular stores. It has been proposed that this event is coupled to influx of extracellular Ca2+ through store-operated Ca2+ channels (SOCs) or Ca2+ release-activated Ca2+ channels (CRACs) in the plasma membrane. Our analysis of the Ca2+ responses of parental BAL-17 cells and CD72-deficient 9-59 cells revealed that BCR-mediated release of Ca2+ from intracellular stores (initial transient rise in [Ca2+]i) was comparable in the two cell types, but that the later sustained influx of extracellular Ca2+ was markedly diminished in 9-59 cells (Fig. 3A). Chelation of extracellular Ca2+ using 2 mM EGTA blocked the late phase of the Ca2+ response in the parental cells, but had only a minimal effect on the responses in 9-59 cells. Indeed, the profile of untreated 9-59 cells was virtually superimposable on that of EGTA-treated BAL-17 cells. Apparently, Ca2+ influx is selectively impaired in 9-59 cells.
To examine the function of SOCs in 9-59 cells further, cells were initially treated with Tg, an inhibitor of sarco-endoplasmic reticulum Ca2+-ATPases that depletes intracellular Ca2+ stores and elicits capacitative Ca2+ influx. In the absence of extracellular Ca2+, Tg (0.2 μM) induced a transient rise in [Ca2+]i due to release from internal stores (Fig. 3B). Once [Ca2+]i had returned to baseline, addition of 0.2 mM CaCl2 elicited a second peak, due to Ca2+ entry through SOCs, that was only slightly smaller in 9-59 cells. This suggests that the function of the SOCs themselves remained intact in 9-59 cells, but that signals leading to Ca2+ influx were in some way altered. Treatment with vehicle (DMSO) had no effect on either

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**Fig. 3.** BCR-mediated Ca2+ influx is selectively impaired in CD72-deficient cells. (A) BAL-17 parental cells and clone 9-59 cells were loaded with Fluo-3AM and then stimulated with anti-IgM antibody F(ab')2 fragments (10 μg/ml) in the absence or presence of 2 mM EGTA. The results are representative of four separate experiments. (B) Tg-induced Ca2+ transients are minimally affected by CD72 deficiency. Fluo-3-loaded BAL-17 and 9-59 cells were stimulated with 0.2 μM Tg or its vehicle (DMSO) in Ca2+-free KRH buffer. After the Tg-induced Ca2+ mobilization had subsided, Ca2+ influx via SOCs was monitored following addition of 0.2 mM CaCl2. The results are representative of four separate experiments. (C) Ca2+ influx is partially recovered in 9-59/CD72a cells. Fluo-3-loaded BAL-17 parental cells, 9-59 cells and 9-59/CD72a cells were incubated for 5 min with anti-IgM antibody F(ab')2 fragments (10 μg/ml) in the absence of EGTA. The results are representative of three separate experiments. The right panels show the CD72 expression profile in the parental and CD72-deficient cells by staining with anti-CD72 antibody and the expression of exogenously-introduced CD72a in 9-59/CD72a cells as revealed by EGFP fluorescence.
Ca^{2+} release or Ca^{2+} influx upon CaCl_2 addition. Significantly, the impaired Ca^{2+} influx was partially restored by re-expression of CD72 (Fig. 3C).

BCR-induced phosphorylation of Btk, PI3K and Vav is reduced in CD72-deficient cells
Btk appears to be involved in regulating Tg-sensitive, IP_3-gated Ca^{2+} stores and therefore Ca^{2+} influx through SOCs (45). Activation of Btk is thought to be regulated by phosphorylation and membrane localization via binding of its pleckstrin homology domain to PIP_3, which is a phosphoinositol product of PI3K. When we assessed levels of Btk activation in parental and 9-59 cells by monitoring its phosphorylation state, we found that in the parental cells Btk phosphorylation was sustained for 5 min after BCR ligation whereas the phosphorylation declined at 2–5 min in 9-59 cells (Fig. 4). Similarly, BCR-induced tyrosine phosphorylation of p85-PI3K was significantly diminished after 5 min in CD72-deficient cells, and these phenotypes were reversed by re-expression of CD72 (Fig. 4). The Vav family of GEFs for Rho family GTases is also thought to be involved in the regulation of Ca^{2+} mobilization and to be regulated by tyrosine phosphorylation (14,46,47). We therefore assayed BCR-mediated tyrosine phosphorylation of Vav and found it to be significantly diminished within 1 min of stimulation in 9-59 cells (Fig. 4). Again, introduction of CD72 into 9-59 cells restored the parental phenotype.

Tyrosine phosphorylation of CD19 and association of CD19 with PI3K are impaired in CD72-deficient cells
CD19 is implicated in the recruitment and subsequent activation of PI3K, PI hydrolysis and calcium mobilization upon BCR ligation (48,49). We thus examined whether the positive regulatory loop of CD19–PI3K association may be disrupted in the absence of CD72. As shown in Fig. 5, phosphorylation of CD19 was strongly induced at 1 min and diminished within 5 min in the parental cells, but this event was severely impaired in 9-59 cells. Accordingly, association of CD19 with PI3K was significantly reduced in CD72-deficient cells, as evidenced by blotting CD19 immunoprecipitates with anti-PI3K antibody (Fig. 5A) and by probing PI3K immunoprecipitates with anti-CD19 antibody (Fig. 5B). This impairment was partially restored by re-expression of CD72.
BCR-induced phosphorylation of PLCγ2 and IP3 production is reduced in CD72-deficient cells

Receptor-mediated Ca\textsuperscript{2+} mobilization is also regulated by BLNK and PLCγ2. A current model holds that, upon phosphorylation by Syk and Btk, BLNK recruits PLCγ2 and Btk, which leads to phosphorylation and activation of PLCγ2 by Btk (50). In addition, CD72 is known to recruit SHIP-1 and Grb2 via its ITIMs, enabling Grb2 to associate with BLNK via its SH3 domain (51). We therefore wondered whether CD72 deficiency would affect the phosphorylation states of BLNK and PLCγ2. As shown in Fig. 6(A), BCR-mediated phosphorylation of BLNK was not significantly different, but that of PLCγ2 was reduced at 5 min in the absence of CD72. Since the phosphorylation state of PLCγ2 does not necessarily reflect its activity (45,52,53), we further assayed IP3 production as a downstream indicator of PLCγ2 activation and a determining factor for Ca\textsuperscript{2+} responses. Figure 6(B) shows that IP3 production was not affected at 30 s of stimulation but almost completely ablated at 1 min in 9-59 cells. These phenotypes were reversed by re-expression of CD72. Thus, BCR-mediated PLCγ2 activity is positively regulated by CD72, and diminished IP3 production at the peak may contribute to the reduction of Ca\textsuperscript{2+} influx in CD72-deficient cells.

BCR-induced activation of ERK and JNK, but not p38, is blocked in CD72-deficient cells

We further examined MAPK activation in CD72-deficient cells using phospho-specific MAPK antibodies. In the parental cells, BCR-ligation mediated transient phosphorylation of ERK that peaked within 5 min (Fig. 7). No induction ERK phosphorylation was observed in 9-59 cells; likewise, phosphorylation of JNK was completely blocked in 9-59 cells. Phosphorylation of ERK and JNK was restored by re-expression of CD72. By contrast, phosphorylation of p38 MAPK was minimally affected in 9-59 cells, suggesting that CD72 positively regulates activation of ERK and JNK, but not p38 MAPK, in BAL-17 cells.

BCR-induced inhibition of DNA synthesis is reduced in CD72-deficient clones

We finally examined the effect of CD72 deficiency on the final outcome. We have previously showed that BCR ligation induces inhibition of DNA synthesis in BAL-17 cells (41,54). Likewise, in the present study, stimulation with anti-IgM antibody induced a dose-dependent 60–80% reduction in DNA synthesis in the parental cells (Fig. 8). On the other hand, this inhibition was reduced by 50–60% in the CD72-deficient 9-59 clone at all concentrations of anti-IgM antibody tested, and this inhibition was restored by re-introduction of CD72. These results suggest CD72 is positively involved in BCR-induced signaling pathways leading to inhibition of cell proliferation.

Discussion

The fate of B cells following antigen binding is determined by many factors, including the strength of the binding, the maturational stage of the cells and the contribution of coreceptors such as CD72, CD22 and CD40. The results of some previous studies suggest that CD72 negatively regulates BCR signaling by recruiting and then serving as a substrate for the protein tyrosine phosphatase SHP-1 (33,34). Consistent with those studies, gene-targeting experiments demonstrated that B cells from CD72 ±/± mice are hyperproliferative and exhibit somewhat enhanced Ca\textsuperscript{2+} responses (38). On the other hand, other reports suggest that CD72 mediates positive regulatory signaling activating a variety of biochemical events, including Ca\textsuperscript{2+} mobilization and activation of tyrosine kinases, PI3K, PLCγ2 and MAPKs (28–32). To resolve this discrepancy, we re-addressed the cell-autonomous function of CD72 using CD72-deficient cells generated from mature BAL-17 cells. Our results show that BCR-mediated initial events, such as total protein tyrosine

![Image](https://academic.oup.com/intimm/article-abstract/16/7/971/839168/12513707189189166866866866866686)
phosphorylation and activation of Lyn and Syk kinases were not significantly affected by the absence of CD72. BCR ligation induces biphasic Ca^{2+} mobilization: initial transient increases in [Ca^{2+}]_i due to IP_3-evoked release from intracellular stores followed by sustained influx of extracellular Ca^{2+} through SOCs or CRACs in the plasma membrane. CD72 deficiency almost completely inhibits the Ca^{2+} influx without affecting the initial Ca^{2+} release from the intracellular stores. Given that Tg-induced capacitative Ca^{2+} entry was minimally affected in CD72-deficient cells and that re-expression of CD72 reversed the defective BCR-mediated Ca^{2+} entry, it appears the impairment of Ca^{2+} influx is not due to a defect in the SOCs per se, but to defective signaling leading to Ca^{2+} influx.

To explore which signaling pathways are regulated by CD72, we examined signaling events thought to be situated upstream of Ca^{2+} influx. CD72 contains two ITIMs in its cytoplasmic domain; upon tyrosine phosphorylation, the first (5ITYADL10) recruits SHP-1 (33,34) and the second (37LTYENV42) recruits Grb2, an adaptor protein required for activation of the Ras/MEK/ERK pathway (34). Previous studies, including ours, have shown that SHP-1 does not act on Lyn or Syk but directly dephosphorylates BLNK (40), and that BLNK associates with CD72 via the SH3 domain of Grb2 (51). It was thus possible to speculate that in the absence of CD72, reduced SHP-1 recruited to the membrane may lead to hyperphosphorylation of BLNK. However, phosphorylation of BLNK was not affected by the absence of CD72. Earliest events affected by the absence of CD72 were tyrosine phosphorylation of CD19 and Vav and recruitment of PI3K to CD19. Vav has been implicated in the regulation of PIP 5-kinase, which catalyzes the synthesis of PIP_2. This means that a reduction in Vav phosphorylation would be expected to diminish PIP_2 availability and therefore IP_3 production. Additionally, given that recruitment of PI3K to CD19 may activate its enzymatic activity (48), impaired recruitment of

**Fig. 7.** BCR-mediated activation of ERK and JNK, but not p38 MAPK, is positively regulated by CD72. (A) BAL-17 parental cells, 9-59 cells and 9-59/CD72^a^ cells were incubated with anti-IgM antibody F(\(\text{ab}\)^\(^{-}\))\(_2^\) fragments (10 \(\mu\)g/ml) for the indicated times, after which the total cell lysates were immunoblotted with antibodies against ERK, JNK and p38 and their phosphorylated forms (pERK, pJNK and pp38). The results are representative of four separate experiments. (B) Summarized data are shown as means of fold increase \(\pm\) SEM in MAPK phosphorylation in the parental cells (solid bars), 9-59 cells (open bars) and 9-59/CD72^a^ cells (gray bars) calculated by densitometric analysis of four separate experiments.
PI3K would be expected to lead to decreased production of PI_P and perhaps diminished membrane targeting of signaling molecules required for PI3 production, e.g. Btk and Vav. Indeed, IP3 production was ablated at 1 min without being compromised initially in CD72-deficient cells. This train of initial events explains very well the pathways leading to calcium mobilization, and may be targets of CD72 regulation.

Signaling events occurring further downstream from BCR ligation than Ca2+ mobilization—e.g. activation of ERK and JNK (but not p38 MAPK) and inhibition of DNA synthesis—are also affected by CD72 deficiency. Impaired activation of ERK and JNK in CD72-deficient cells may be due respectively to the loss of the Grb2 docking site and the reduced activation of PI3K, which activates Rho and Rac upstream of JNK. Our earlier studies demonstrated that BCR ligation inhibits proliferative responses as measured by DNA synthesis by the concerted actions of JNK and p38 MAPK (41,42,54). The present study showed that BCR-mediated inhibition of DNA synthesis is reduced by 50±60% in CD72-deficient clones. In any case, all of these results indicate CD72 to be a positive regulator of cellular responses induced by BCR ligation.

It is notable that BCR signaling pathways affected by CD72 deficiency are reminiscent of pathways affected by CD72 ligation. Treatment with anti-CD72 antibody stimulates Btk but not Syk PTK, resulting in Syk-independent PLCγ2 activation, Ca2+ mobilization and B cell proliferation (24,28). It also induces activation of PI3K recruited to CD19, a transient association of CD72 with CD19 (29), and activation of ERK and JNK but not p38 MAPK (32). Moreover, the observations that CD72-induced B cell proliferation is blocked by PI3K inhibitors and is reduced in B cells from CD19+/− mice (29) suggest that activation of CD19-associated PI3K is critical for CD72-triggered B cell responses.

In CD72+ mice, B cell development is blocked at the transitions from pre-B cells to immature B cells and from immature to mature B cells. In the periphery of CD72+ mice, the numbers of mature, long-lived B-2 cells and follicular B cells are reduced, whereas the numbers of B-1 cells and marginal zone B cells are increased. Thus CD72 seems to exert positive and negative effects at distinct maturational stages. In vitro studies of B cells from CD72+ mice show increased proliferation in response to anti-IgM antibody and lipopolysaccharide, and slightly enhanced Ca2+ responses. Given the biased B cell subpopulations in the CD72+ spleen—i.e. greater numbers of marginal zone B cells and fewer follicular B cells—the alterations in Ca2+ mobilization may be masked in part by differences in the responsiveness of the various subpopulations. However, enhanced proliferative responses of resting B cells in CD72+ mice are difficult to reconcile in this study.

There are other indications that CD72 serves as a negative regulator of B cell as revealed by experiments using cell lines. For example, expression of CD72 in K46 mouse B lymphoma cells, into which hapten-specific μ heavy and λ light chains were introduced, reduces BCR-induced ERK activation and Ca2+ mobilization (36). Moreover, introduction of CD72 in BCR-expressing myeloma cells (J558L p3) also inhibits BCR-induced phosphorylation of Ig-α/Ig-β, Syk and BLNK (37). One caveat is that the cellular milieu of myeloma cells is different from B cells and both of these cell lines lack expression of CD72. This may suggest that CD72 does not have a significant function in these cells. These factors may delicately contribute to the apparently discrepant capacity of CD72 to stimulate or inhibit BCR signaling in different cell types. Additionally, which regulatory molecules, including SHP-1, Grb2 or even as-yet-unidentified proteins, are dominantly recruited to CD72 may dictate the mode of CD72 action in a cell.

In summary, CD72 apparently positively regulates BCR-mediated signaling pathways including phosphorylation of CD19, Vav, Btk and PLCγ2, recruitment of PI3K to CD19, IP3 production and Ca2+ influx. Consequently, the signals are relayed to activate ERK and JNK, determining the fate of BAL-17 cells. Given that CD72 is able to recruit both positive and negative regulatory molecules, signals generated by CD72 may be intricately balanced by the stoichiometry of the molecules recruited to it.

**Acknowledgements**

We thank Dr Janes Parnes for plasmid containing CD72a gene, Dr David Saffen for preprints and informative discussion, Dr Tatsuo Katagiri for his initial contribution and Dr Haruo Takemura for technical advice. This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BCR</td>
<td>B cell antigen receptor</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton’s tyrosine kinase</td>
</tr>
<tr>
<td>[Ca2+]i</td>
<td>cytosolic free Ca2+ concentration</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol 1,4,5-triphosphate</td>
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<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
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**Fig. 8.** BCR-mediated inhibition of DNA synthesis is blocked in CD72-deficient clone. BAL-17, clone 9-59 and 9-59/CD72a cells were cultured in the presence of graded concentrations of anti-mouse IgM antibody F(ab)2 fragments, and [3H]thymidine incorporation was determined on day 2. The results are expressed as means of percent inhibition of DNA synthesis ± SEM of two separate experiments.

<table>
<thead>
<tr>
<th>% Inhibition of DNA synthesis</th>
<th>F(ab)2 anti-IgM antibody (μg/ml)</th>
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<tbody>
<tr>
<td>5</td>
<td>0</td>
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<tr>
<td>10</td>
<td>0</td>
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<tr>
<td>20</td>
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MAPK  
mitogen-activated protein kinase

PKC  
phosphatidylinositol 3-kinase

PIP\_3  
phosphatidylinositol 4,5-bisphosphate

PIP\_2  
phosphatidylinositol 3,4,5-trisphosphate

PLC\_y  
phospholipase C-y2

PTK  
protein tyrosine kinase

SOC  
store-operated channel

Tg  
thapsigargin

References


