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Activation of Lyn, Blk, and Btk But Not Syk in CD72-Stimulated B Lymphocytes¹

Chandrasekar Venkataraman, Natarajan Muthusamy,² Subramanian Muthukkumar,³ and Subbarao Bondada⁴

CD72 is a B cell-specific glycoprotein that has been shown to be important for activation of mature B cells. Previously we showed that some of the early signaling events, such as calcium mobilization and phospholipase- γ activation, were similar in B cell Ag receptor (BCR)- and CD72-stimulated B cells and that BCR- but not CD72-mediated early signaling events were blocked by protein kinase A activation. The present report shows that CD72 ligation induces a variety of tyrosine-phosphorylated proteins, most of which were of the same molecular mass as those seen in anti-IgM-treated B cells, except for a 72-kDa protein. Further analysis showed that the tyrosine kinases lyn and blk were activated in CD72-ligated B cells. Interestingly, the non-src kinase syk was not activated in CD72-stimulated cells whereas the tec family kinase btk was activated in both CD72- and BCR-stimulated B cells. Furthermore, B cells from *xid* mice were unresponsive to CD72-induced proliferation, indicating an essential role for btk in CD72-induced signaling events. Surprisingly, tyrosine phosphorylation of phospholipase C- γ 2 was normal in CD72-stimulated cells in spite of a lack of activation of syk. Furthermore, B cell proliferation through CD72 was blocked by the immunosuppressive agents cyclosporin A and FK506, indicating the important role for Ca²⁺-regulated activation events similar to BCR-stimulated cells. We propose that btk can substitute for syk in inducing phospholipase C- γ 2 tyrosine phosphorylation and initiating calcium mobilization in CD72-stimulated B lymphocytes. *The Journal of Immunology*, 1998, 160: 3322–3329.

Ligation of the clonotypic B cell Ag receptor (BCR)⁵ and various coreceptors CD19, CD40, CD72, and CD38 results in the generation of signals leading to cell proliferation, differentiation, and growth inhibition (1–5). These events are primarily initiated by activation of protein tyrosine kinases (PTKs) of the src-family (lyn, blk, and fyn), non-src family (syk), and the tec-family kinase (btk) (6, 7). Subsequently, several substrates including phospholipase C- γ 2 (PLC- γ 2) (8), phosphatidylinositol 3-kinase (9), and ras-GAP (10) undergo tyrosine phosphorylation leading to generation and amplification of diverse signals into the cell. For BCR-induced signaling events, initial activation of the src-kinase lyn is essential for activation of syk and btk (11, 12), which in turn control tyrosine phosphorylation and activation of PLC- γ 2 (13, 14). However, recent evidence demonstrates a lyn-independent activation of syk (15) in BCR-stimulated B lymphocytes. Thus coordinate activation and interaction of different sig-

naling molecules tightly regulate cellular responses to various signals.

The B cell specific coreceptor CD72 is a 45-kDa type II transmembrane glycoprotein expressed in all stages of B cells except plasma cells. Stimulation through CD72 induces B cell proliferation (16–18), an increase in levels of surface MHC class II (19, 20), and augmentation of IL-4-dependent CD23 expression (21). CD72 has an important role in differentiation of B cells into Ab-secreting cells in responses to both thymic-independent and thymic-dependent Ags (20, 22, 23). Thus, activation of Ag-specific B lymphocytes by the thymic independent Ag TNP-Ficoll was enhanced by signaling through CD72 (22).

Although the exact mechanisms involved in CD72-mediated B cell activation are unknown, ligation of CD72 induces hydrolysis of inositol phospholipids (18, 24) and elevation of intracellular calcium (18, 19). Previously we showed that both these signaling events were similar in B cells stimulated through CD72 and BCR (18). However, pathways used by CD72 and BCR were differentially regulated by PKA. Thus, cAMP-elevating agents like forskolin (18) or PGE₂ (25) inhibited BCR-mediated but not CD72-mediated B cell proliferation. cAMP inhibited BCR-induced activation of phospholipase C- γ and [Ca²⁺]_i mobilization indicating a “cross-talk” between cAMP and phosphatidylinositol pathways for BCR- but not CD72-mediated signaling pathways (18). These results suggested that the potential targets of PKA may be, at least in part, at the level of PTK activation and/or PLC activation. CD72-mediated signals partially overcome apoptosis in B lymphocytes caused by hyper-cross-linking the BCR in vitro (26). The PKA resistance of CD72 signaling, together with its ability to deliver antiapoptotic signals, strongly suggests that CD72 signals may have a bystander role to antagonize negative effects on B cell activation during immune responses.

As an initial step toward understanding the key differences in early signals generated through CD72 and BCR, we investigated the role of tyrosine phosphorylation and activation of different

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⁵ Abbreviations used in this paper: BCR, B cell Ag receptor; PKA, protein kinase A; PTK, protein tyrosine kinase; PLC, phospholipase C; CAB, control Ab; IB, immunoblot; CsA, cyclosporin A; PH, pleckstrin homology; PI, phosphatidylinositol.

PTKs following stimulation through these receptors. Ligation of CD72 resulted in activation of PTKs, lyn, and blk. Unlike BCR-ligated B cells, the non-src family kinase syk was not activated upon signaling through CD72. Surprisingly, tyrosine phosphorylation of PLC- γ 2 was apparently normal in CD72-stimulated B cells without activation of syk. Instead, btk, the tec family member, was found to be critical for CD72-mediated B cell activation.

Materials and Methods

Animals

Female DBA/2, BALB/c, CBA/Ca (non-xid), and CBA/N (xid) mice were obtained from the National Cancer Institute, Frederick, MD.

Reagents

Details have been previously described of monoclonal anti-CD72 Abs K10.6 (27, 28) and 10.1.D2 (17) and of isotype-matched control Ab (CAb) MOPC195 (M195) (18) used in this study. Goat anti-mouse IgM F(ab')₂ was purchased from Organon Teknika (Durham, NC). Genistein and herbimycin A were from Calbiochem-Behring (San Diego, CA). The stock solutions of these PTK inhibitors were prepared in DMSO and stored at -20°C until further use. Cyclosporin A was from Sandoz Research Institute (East Hanover, NJ). FK506 was a kind gift from Fujisawa GmbH (Munich, Germany). Abs to lyn, blk, syk, btk, and PLC- γ 2 were purchased from Santa Cruz Biotech (Santa Cruz, CA). IL-4 was purchased from Genzyme (Cambridge, MA) and recombinant horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine Ab (RC20H) was obtained from Transduction Laboratories (Lexington, KY).

B cell stimulation and Western blotting

Splenic B cells were prepared after T-depletion as described previously (18), and their purity exceeded 95% in most experiments. Splenic B cells in serum-free Iscove/F-12 (IF-12) medium were prewarmed at 37°C for 45 min. B cells were stimulated at 37°C with 10 μ g/ml of anti-IgM, 30 μ g/ml of anti-CD72, or M195. In some experiments, cells were pre-incubated with PTK inhibitors, genistein (100 μ M), or herbimycin A (7 μ g/ml) for 30 min at 37°C and later stimulated with the stimulatory Abs. Reactions were stopped with the addition of 1 \times cold PBS, pH 7.2, containing phosphatase inhibitors as described earlier (29, 30). After two to three washes with PBS, cells were lysed with 1% Triton-X 100 lysis buffer containing 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 1 mM EDTA, 1 mM PMSF, 2 μ g/ml aprotinin, and 2 μ g/ml of leupeptin for 30 min on ice. Lysates were cleared of nuclear debris by centrifugation at 12,000 \times g for 30 min at 4°C.

For immunoprecipitation studies, lysates prepared from 25 \times 10⁶ B cells were precleared with protein A beads (Repligen, Cambridge, MA) for 1 h at 4°C. Abs to lyn, blk, syk, PLC- γ 2, or btk were added and incubated at 4°C for 6 h. Immune complexes were collected by incubating with protein A beads for 2 h. Proteins were eluted by boiling with sample buffer for 3 to 5 min and were separated on 10% SDS-PAGE. Resolved proteins were transferred onto Immobilon-P membranes (Millipore, Bedford, MA), then blocked for 30 min with 1.5% BSA. Membranes were incubated with anti-phosphotyrosine Ab RC20 for 1 h at room temperature. After extensive washing, the blots were developed with an enhanced chemiluminescence kit (DuPont/NEN, Boston, MA) according to the manufacturer's instructions. For Western blotting of the individual enzymes, blots were incubated with their respective Abs for 1 h at room temperature followed by horseradish peroxidase-conjugated anti-rabbit secondary Ab (Santa Cruz Biotech, Santa Cruz, CA). The proteins were visualized as described above.

In vitro kinase assay

B cells (1 \times 10⁷) were stimulated for indicated time points, and immunoprecipitates from lysates were prepared as above. Protein A beads were incubated in 500 μ l of kinase buffer (20 mM HEPES, pH 7.5, 10 mM MnCl₂, 5 mM DDT, 1 mM Na₃VO₄, and 1 mM PMSF) with 5 μ Ci [γ -³²P]ATP for 30 min at 30°C. Reactions were stopped by adding kinase buffer containing 5 mM EDTA (stop buffer). Beads were extensively washed with stop buffer and proteins were eluted as described above. The proteins were resolved by SDS-PAGE and visualized by autoradiography. The autoradiograms were scanned with a UMAX flat-bed scanner (UMAX Data System, Taiwan, R.O.C.), and relative intensities expressed in arbitrary units (OD) of bands were quantified using the National Institutes of Health image program. Radioactivity of bands for syk experiments were quantified with a Molecular Dynamics (Sunnyvale, CA) computing densi-

tometer using a volume-integrated mode, and the results were expressed as fold increase.

Proliferation assay

DBA/2 B cells (2 \times 10⁵) were cultured in the presence or absence of 50 μ g/ml of Abs (anti-IgM or anti-CD72) or inhibitors (CsA or FK506) in a final volume of 200 μ l in IF-12 medium containing 10% FBS (Atlanta Biologicals, Norcross, GA). Bacterial LPS was used at a concentration of 10 μ g/ml. For studying proliferation of xid and non-xid B cells, 2 \times 10⁵ cells were cultured with increasing concentrations of anti-CD72 Abs in the presence or absence of 50 U/ml of IL-4. B cells were also stimulated with 50 μ g/ml of anti-IgM (in the presence or absence of IL-4) to make comparisons on the proliferative ability of cells to BCR engagement. Cells were cultured for 48 h in 5% CO₂ at 37°C and pulsed with 1 μ Ci of tritiated thymidine (sp. act. 2 Ci/mmol, New England Nuclear, Boston, MA) during the last 4 h. Cultures were harvested on glass fiber filters using a Packard automatic cell harvester, and thymidine incorporation was determined by a Matrix-96 β counter (Packard, Downers Grove, IL).

Results

Ligation of CD72 induces tyrosine phosphorylation of multiple cellular substrates via a PTK-dependent pathway

To analyze the initial signaling events after engaging CD72, B lymphocytes were stimulated with Abs to the CD72 receptor for different time periods. Following stimulation, lysates were prepared and analyzed using the anti-phosphotyrosine Ab RC20. As a control, cells were stimulated with an isotype-matched control Ab. Lysates from anti-IgM-stimulated cells were used to compare differences or similarities in the profile of induction of various proteins after BCR or CD72 engagement. Stimulation through CD72, but not the control Ab, resulted in tyrosine phosphorylation of several proteins of molecular masses (indicated by arrows in Fig. 1A) of 40, 45, 52 to 56, 60, 90 to 95, 110 to 120, and a prominent protein at 140 kDa. Proteins at 30 to 32 and 35 kDa were also phosphorylated but with lesser intensity. Induction was detected as early as 1 min (52-, 56-, and 140-kDa bands), reaching peak levels at 10 to 15 min and returned to basal levels of phosphorylation by 45 min, although the phosphorylation reached peak levels at 30 min for a 40-kDa protein. Similar results were obtained with another anti-CD72 Ab, 10.1.D2 (data not shown). When compared with anti-IgM-stimulated cells, there was an absence of tyrosine phosphorylation of a 72-kDa protein in CD72-stimulated cells. The reappearance of tyrosine-phosphorylated proteins at molecular mass < 28 kDa and 43 kDa at 30 min in CD72-stimulated cells was not seen in other similar experiments. Two PTK-specific inhibitors, genistein (Fig. 1B) and herbimycin A (data not shown) were used to determine whether the appearance of tyrosine-phosphorylated substrates required activation of tyrosine kinases. As expected, there was very little increase in protein tyrosine phosphorylation in both CD72- and BCR-stimulated cells pretreated with genistein (Fig. 1B). These results indicate that CD72 stimulation resulted in the appearance of several tyrosine-phosphorylated proteins due to PTK activation similar to BCR-stimulated cells.

Signaling through CD72 induces tyrosine phosphorylation of PTKs lyn and blk, but not syk

In B lymphocytes, two src-kinases, lyn and blk, were activated within seconds of BCR engagement (31), which is usually accompanied by an increase in the tyrosine phosphorylation status of PTKs. Hence it was interesting to identify candidate PTKs that are activated on CD72 stimulation. B cells were stimulated with anti-IgM Ab or anti-CD72 Ab for different time periods, and the phosphotyrosine content of immunoprecipitates of these src-kinases was analyzed. As shown in Figure 2A, stimulation through BCR for 10 min resulted in increased tyrosine phosphorylation of lyn (sixfold). An increase in the tyrosine phosphorylation of lyn was

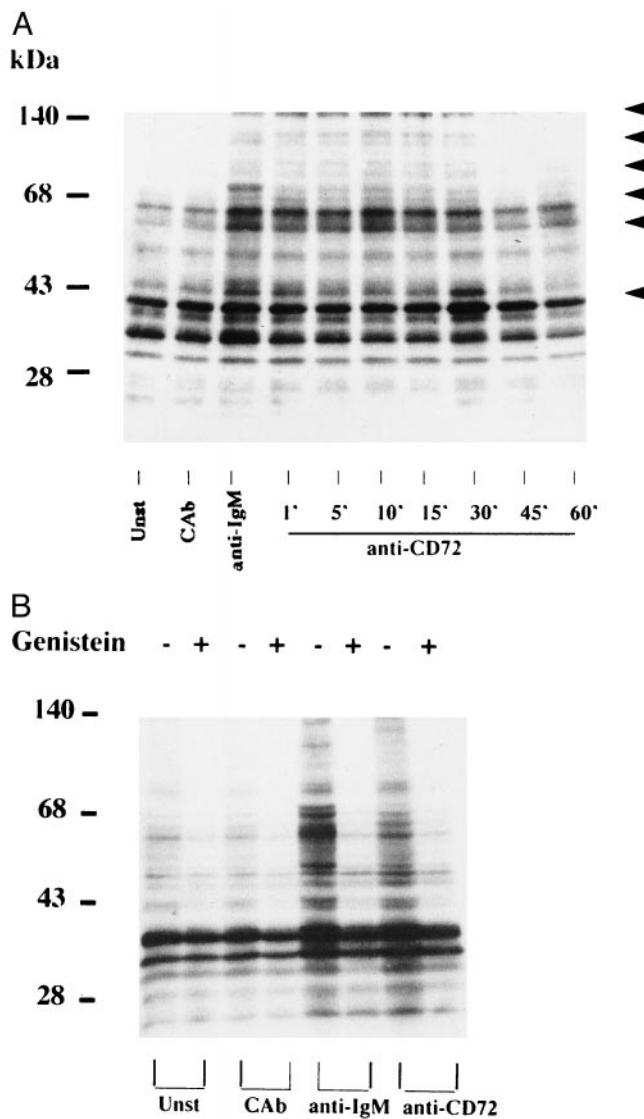


FIGURE 1. A, Induction of tyrosine phosphorylation of cellular substrates in CD72-stimulated cells. Splenic B cells were left unstimulated (unst) or stimulated with control Ab (CAb) or anti-IgM for 10 min, or anti-CD72 for indicated time points. Cell lysates were analyzed by immunoblotting with anti-phosphotyrosine Ab. Arrows indicate newly induced tyrosine phosphorylated proteins. Representative results from one of eight similar experiments are shown. B, Tyrosine phosphorylation induced in CD72- and BCR-stimulated cells is blocked by a PTK inhibitor, genistein. Splenic B cells were preincubated with medium (-) or 100 μ M genistein (+) for 30 min at 37°C before stimulation with CAb, anti-IgM, or anti-CD72 Abs for 10 min. Lysates were analyzed with anti-phosphotyrosine Ab. Results from one of two similar experiments are shown.

seen as early as 1 min of CD72 ligation, which was two- to three-fold over control after 30 to 45 min of stimulation. Immunoblot (IB) analysis of samples probed with anti-lyn Ab accounted for equivalent loading of proteins. On the other hand, there was a modest but highly reproducible increase in the tyrosine phosphorylation of blk in both BCR- and CD72-stimulated cells (Fig. 2B). Similar to lyn, maximal levels of tyrosine phosphorylation of blk were seen at 30 min of CD72 ligation. This pattern was highly reproducible in two other experiments.

Syk has been identified as one of the downstream targets of src kinases (11). Hence we asked whether syk was tyrosine phosphorylated on CD72 ligation. Lysates were prepared from

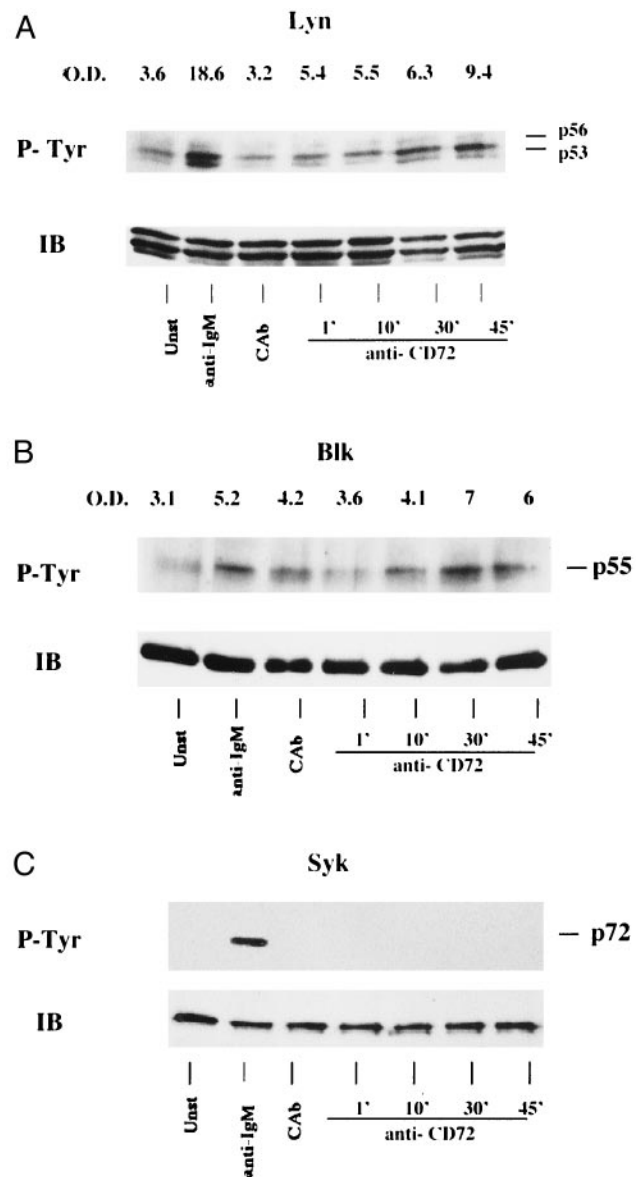


FIGURE 2. CD72 stimulation induces tyrosine phosphorylation of lyn and blk, but not syk. Splenic B cells were stimulated for 10 min with CAb or anti-IgM, or anti-CD72 for indicated time points. Lyn (A), blk (B), or syk (C) were immunoprecipitated from cell lysates as described in *Materials and Methods*. Immunoprecipitates were probed with anti-phosphotyrosine Ab (P-Tyr) or immunoblotted (IB) for the respective PTK to account for equivalent loading of proteins. The relative intensities (expressed in arbitrary OD units) of phosphorylated lyn (p56 and p53 isoforms) or blk proteins were quantified using a National Institutes of Health image program. Representative data from one of three experiments are shown.

anti-IgM or anti-CD72-stimulated cells, and the phosphotyrosine content of syk immunoprecipitates was analyzed. As expected, syk was inducibly tyrosine phosphorylated in BCR-ligated cells (Fig. 2C). On the other hand, there was no increase in the tyrosine phosphorylation of syk in CD72-stimulated B cells at any time point tested (Fig. 2C). Immunoblot analysis of syk immunoprecipitates showed that the changes were not due to differences in relative amounts of the protein loaded in each lane. These data suggest that PTKs lyn and blk, but not syk, may be activated in CD72-ligated B cells.

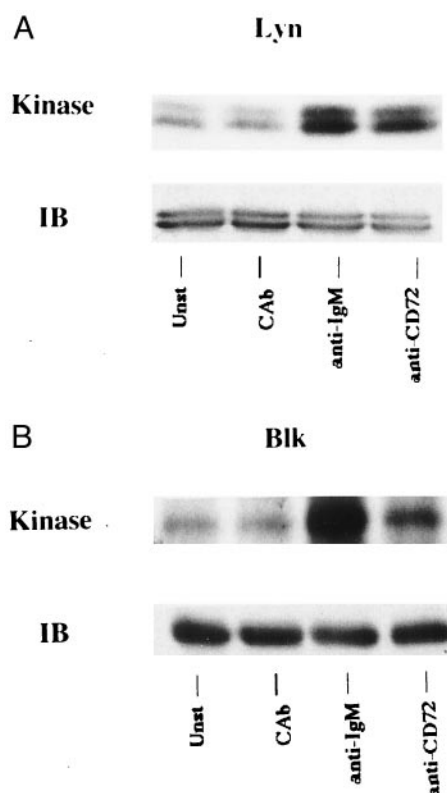


FIGURE 3. Increased autophosphorylation activities of lyn and blk in CD72-stimulated cells. Splenic B cells were stimulated with CAb, anti-IgM, or anti-CD72 for 10 min. Lyn (A) or blk (B) was immunoprecipitated and subjected to *in vitro* kinase assays. Duplicate samples were immunoblotted (IB) with Abs to lyn or blk. Results from one of two independent experiments are shown.

Kinase activities of PTKs lyn and blk, but not syk, are increased in CD72-ligated B cells

The lack of tyrosine phosphorylation of syk may be due to a failure by CD72 to stimulate an increase in kinase activities of the src kinases although increased tyrosine phosphorylation of these enzymes was evident. To confirm that the increase in phosphotyrosine content of lyn and blk in CD72-stimulated cells in fact reflected an increase in their kinase activities, *in vitro* autophosphorylation assays on these enzymes were performed. Stimulation through either CD72 or BCR increased kinase activities of both lyn (Fig. 3A) and blk (Fig. 3B). As expected, BCR ligation resulted in enhanced syk activity (Fig. 4A), in agreement with the previously reported data on syk autophosphorylation activity in such kinase assays (11, 14, 31). However, there was no detectable change in syk activity in CD72-stimulated cells (Fig. 4A). Longer exposures of autoradiograms did not reveal any increase in syk activity in CD72-ligated B cells (data not shown). To be certain that the failure to detect syk activity upon CD72 ligation is not due to limitations in the sensitivity of detection, we performed kinase assays with immunoprecipitates from five times more the number of cells (Fig. 4B) compared with that used in the experiment of Figure 4A. Phosphorimager analysis of the gel further confirmed the inability of CD72 to stimulate syk activity. Under the same conditions, BCR ligation induced a 2.8-fold increase in syk activity (Fig. 4B). These data clearly indicate that the src family kinases lyn and blk, but not the non-src kinase syk, were activated in CD72-stimulated cells, accounting for the first major difference in the initial signaling pathways between CD72 and BCR.

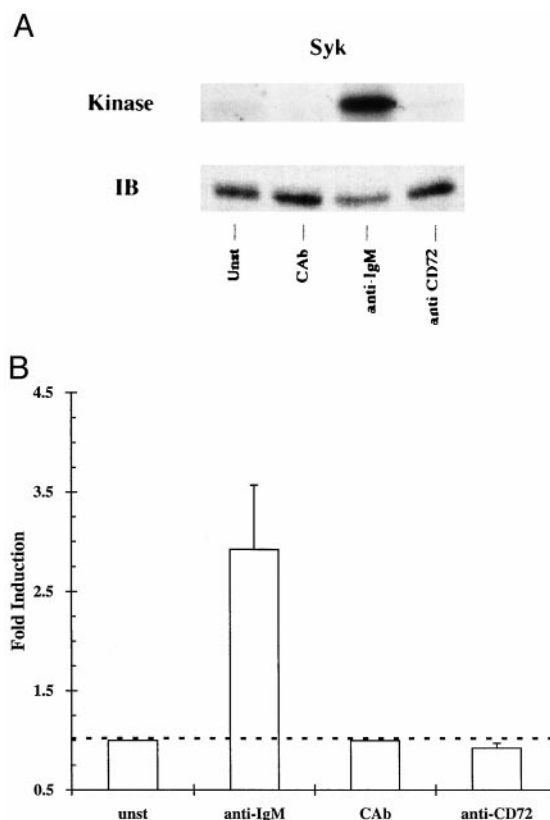


FIGURE 4. Lack of syk activation in CD72-ligated cells. Splenic B cells were stimulated with indicated stimulants for 10 min. Syk immunoprecipitates from 1×10^7 (A) or 5×10^7 (B) equivalent cell lysates were subjected to *in vitro* kinase assays or immunoblotted (IB) with anti-syk Ab. The radioactivity in the bands corresponding to syk were quantified by phosphorimager analysis and were plotted as fold induction (B).

Btk is activated in CD72-stimulated cells, and xid B cells are unresponsive to CD72-induced proliferation

Previous studies have indicated that the tec family kinase btk, whose function was defective in *xid* mice, was activated after src kinase(s) activation in cells triggered through the BCR (32–34). Furthermore, absence of wild-type btk function resulted in apoptosis of anti-IgM-activated splenic B cells (35), indicating its essential role in B cell activation. Btk was activated in *xid* B cells following BCR engagement, suggesting that a single mutation in the pleckstrin homology (PH) domain of the enzyme does not alter its catalytic activity (34). Since src kinases were activated upon CD72 triggering, we tested the possible involvement of btk in CD72-stimulated B cells. We could not detect any reproducible increase in the tyrosine phosphorylation status of btk on CD72 or BCR ligation. Hence, *in vitro* kinase assays were performed on btk immunoprecipitates prepared from B cell lysates from *xid* and non-*xid* control mice after stimulation through CD72 or BCR. Btk activity was measured at 1, 3, and 5 min of stimulation with anti-CD72 or anti-IgM Ab. Maximal kinase activity was demonstrated after 3 min of stimulation for both CD72- and BCR-ligated cells. As shown in Figures 5A (non-*xid*) and 5B (*xid*), btk activity was detectable in unstimulated cells, which was elevated in both CD72- and BCR-ligated B lymphocytes. Although the exaggerated increase in btk activity at 3 min of CD72 stimulation in CBA/N mice (Fig. 5B) was not reproducible in other experiments, there was a consistent increase in btk activity in all experiments. Btk activity increased to comparable levels in both CBA/N and CBA/Ca B

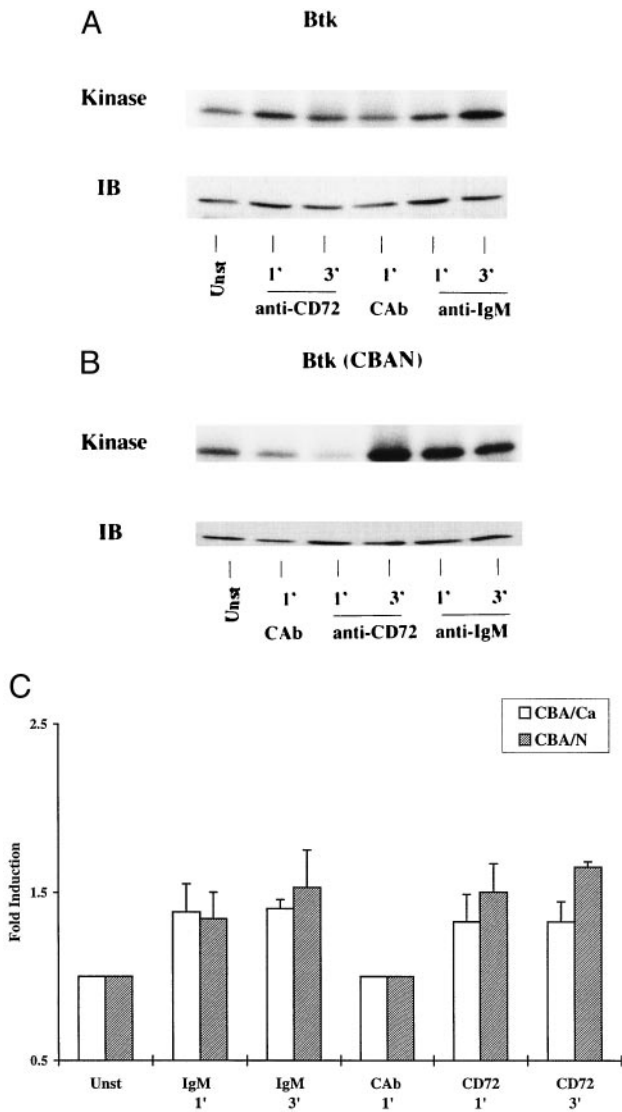


FIGURE 5. Activation of btk in xid and non-xid mice after ligation of CD72. Splenic B cells were stimulated with indicated stimulants. Btk immunoprecipitates from non-xid (A) and xid (B) mice were subjected to in vitro kinase assay or immunoblotted (IB) with anti-btk Ab. The intensities of kinase products were quantified using the National Institutes of Health image program and expressed as fold induction compared with the appropriate control (C). Mean \pm SE values of results from three independent experiments are shown. The differences between the control Ab and anti-CD72 or anti-IgM treatment groups were significant with a p value of < 0.05 in both strains. However, the differences between CBA/Ca and CBA/N mice for any of the treatments were not statistically significant.

cells on BCR ligation (Fig. 5C). Similarly, btk activity induced by CD72 was comparable in CBA/N and CBA/Ca B cells after 1 and 3 min of stimulation (Fig. 5C). Btk activity returned to basal levels by 5 min of BCR or CD72 stimulation (data not shown).

To further analyze the functional role of btk in CD72-induced B cell proliferation, B cells from xid and non-xid mice were stimulated in the presence of various concentrations of anti-CD72 Abs with or without the growth-promoting cytokine IL-4. K10.6, an Ab that recognizes the CD72^b allele (28) expressed by CBA/Ca and CBA/N mice, induced proliferation in wild-type but not xid B cells (Fig. 6, A and B). An allele-specific anti-CD72 Ab (10.1.D2) that does not recognize the CD72^b allele in CBA/N and CBA/Ca mice was used as a control for these experiments. IL-4 had minimal effects in enhancing CD72-induced B cell proliferation in CBA/Ca

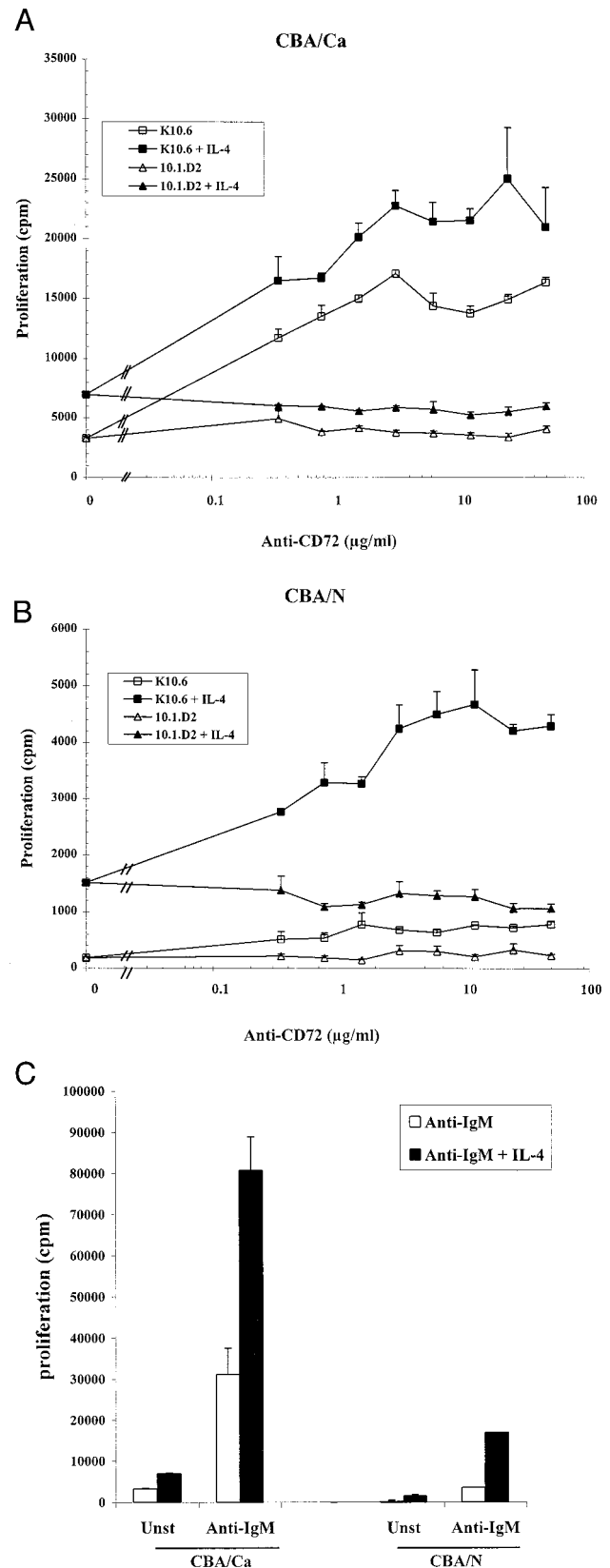


FIGURE 6. B cells from xid mice are defective in CD72 induced proliferation. B cells from non-xid mice (A) or xid mice (B) were stimulated with different concentrations of either K10.6 or 10.1.D2 anti-CD72 Ab in the presence or absence of 50 U/ml of IL-4. B cells stimulated with anti-IgM in the presence or absence of IL-4 were used to estimate their ability to proliferate in response to BCR engagement (C).

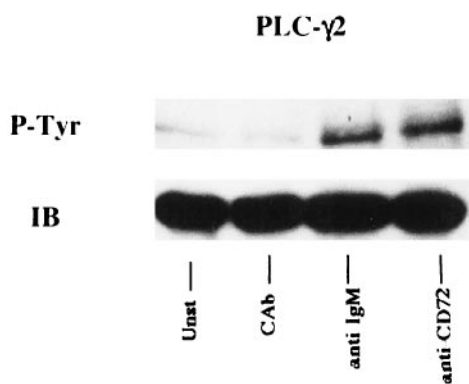


FIGURE 7. PLC- γ 2 is tyrosine phosphorylated in CD72-stimulated cells. PLC- γ 2 was immunoprecipitated and immunoblotted with anti-phosphotyrosine Ab (P-Tyr) or anti-PLC- γ 2 Ab (IB) from cells stimulated with anti-CD72 or anti-IgM Ab. Data from one of three similar experiments are shown.

mice (Fig. 6A). In *xid* mice, addition of IL-4 increased the level of CD72-induced B cell proliferation to two- to threefold when compared with cells stimulated with anti-CD72 alone (Fig. 6B). However, the magnitude of response of *xid* B cells to CD72 plus IL-4 was only 20% of the response of CBA/Ca B lymphocytes. As expected, *xid* B cells were unresponsive to anti-IgM-induced B cell proliferation (Fig. 6C). IL-4 enhanced the anti-IgM response almost sevenfold in *xid* mice as opposed to less than threefold in CBA/Ca mice. However, the absolute magnitude of the *xid* response to anti-IgM plus IL-4 was only 25% of the response of the wild-type mice. These results clearly show that *btk* is activated by CD72 ligation and that its intact PH domain-related function is essential for CD72-mediated B cell activation.

PLC- γ 2 is inducibly phosphorylated in CD72-stimulated cells

We have previously shown that CD72-mediated B cell activation resulted in PI hydrolysis (18, 24). Previous reports using the chicken DT40 B cell lymphoma showed the importance of both *syk* and *btk* in tyrosine phosphorylation and activation of PLC- γ 2 (13). Hence it was interesting to verify whether PLC- γ 2 was tyrosine phosphorylated in CD72-stimulated cells in which *btk*, but not *syk*, was activated. As shown in Figure 7, PLC- γ 2 was inducibly tyrosine phosphorylated to a similar extent in both CD72- and BCR-ligated cells.

CD72-mediated B cell proliferation is CsA and FK506 sensitive

Since we previously showed that stimulation through CD72 resulted in activation of phospholipase- γ and mobilization of $[Ca^{2+}]_i$, we asked whether Ca^{2+} -dependent activation events were similar in CD72- and BCR-ligated cells. Two immunosuppressive agents CsA and FK506 have been previously shown to block Ca^{2+} -dependent activation of the ser/thr phosphatase, calcineurin, which is required for the translocation of NF-AT to the nucleus (36). B cells were cultured with anti-IgM or two different anti-CD72 Abs (K10.6 and 10.1.D2) in the presence of various concentrations of CsA. There was a dose-dependent inhibition of CD72-stimulated proliferation in the presence of CsA similar to that of BCR-ligated cells (Fig. 8A). Similar results were obtained in experiments conducted in the presence of FK506 (Fig. 8B). The growth response of B cells to bacterial LPS was minimally inhibited by both these agents.

Discussion

In the present report we have investigated the signaling events that are activated after triggering through the CD72 receptor. CD72

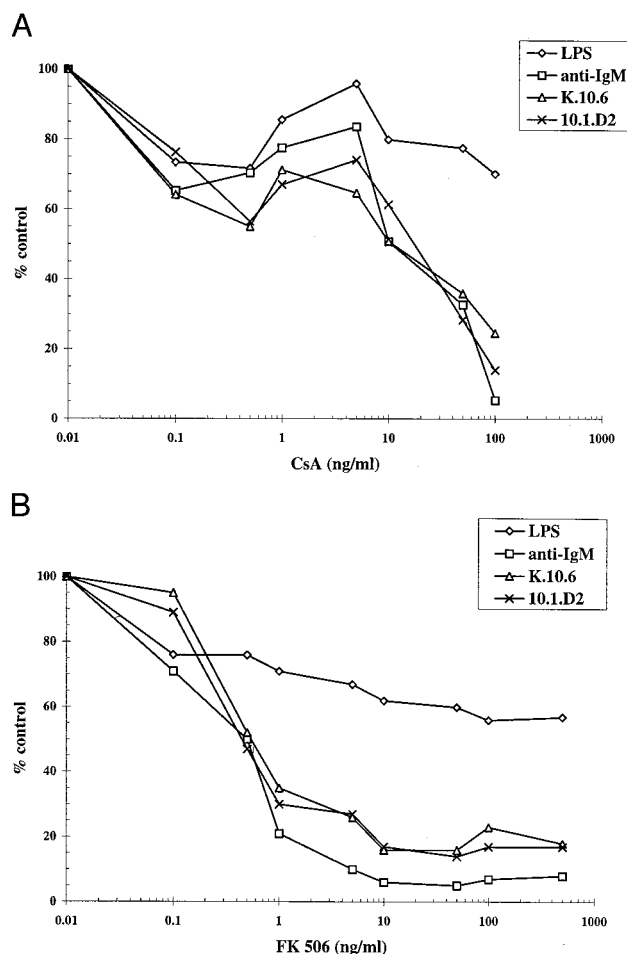


FIGURE 8. CD72-induced B cell proliferation is sensitive to CsA and FK506. DBA/2 B cells were stimulated with 50 μ g/ml of anti-IgM or anti-CD72 (K10.6 or 10.1.D2) Abs in the presence of increasing concentrations of CsA (A) or FK506 (B). Results were expressed as percent of proliferation induced by the stimulants in the absence of these inhibitors.

ligation resulted in the tyrosine phosphorylation of several proteins through a PTK-dependent pathway. Although the profile of most of the tyrosine-phosphorylated proteins is similar in CD72- and BCR-stimulated cells, there was an absence of induction of a 72-kDa protein in the former. Our results are in contrast to those of Brunswick et al., who did not observe any newly tyrosine-phosphorylated proteins in anti-CD72-stimulated B cells (37) but are in agreement with the studies on human B cells stimulated via CD72 (38). The difference between the earlier studies and the present work with murine CD72 may be due to the use of a more potent anti-CD72 Ab (K10.6) to stimulate B cells as well as in the use of a phosphotyrosine detection system that gives a lower background.

Several reports have suggested that tyrosine phosphorylation of protein substrates was mediated by the src-family PTKs. Activation of PTKs occur in a temporal fashion following BCR engagement in a murine B cell lymphoma (31). Src family kinases *lyn* and *blk* were activated as early as 5 sec, followed by activation of *btk* and *syk* at 5 to 10 min after receptor ligation. Stimulation through CD72 caused a time-dependent increase in the phosphotyrosine content of src kinases *lyn* and *blk*, but to a lesser extent when compared with BCR-stimulated cells. The increase in phosphorylation closely correlated with the activation of these enzymes. The lesser strength of activation may be due to the absence of a canonical immunoreceptor-based activation motif in the cytoplasmic

tail of CD72. However, the cytoplasmic domain of CD72 contains three tyrosine residues that could be potential phosphorylation sites (39). We could not detect any evidence of tyrosine phosphorylation of CD72, based on a detailed kinetic analysis of anti-CD72, anti-IgM, or pervanadate-treated cells (C. Venkataraman and S. Bondada, unpublished observations). Also under these detergent conditions, we could not find any CD72-associated kinase activity (data not shown). It is also possible that CD72 physically associates with another protein that may recruit and activate various PTKs. Use of a milder detergent to maintain protein-protein interactions may help resolve this issue. Using 1% Brij-96 lysis conditions, we could not demonstrate coimmunoprecipitation of Ig α with CD72. However, under these treatments, the physical association between membrane IgM and Ig α remained intact (Venkataraman et al., manuscript under preparation). Several downstream substrates of lyn have been identified, which include syk (11), HS1 (40, 41), and the CD19/CD21 complex (42, 43). Syk was neither inducibly tyrosine phosphorylated nor activated in CD72-stimulated B cells. This is in contrast to other surface molecules on B cells (e.g., BCR, CD40, and CD38) that activate syk on receptor ligation (3, 5, 31).

Several receptors on B cells, including the BCR, IL-5R, and CD38, involve activation of btk (6). B cells from xid mice do not respond to signaling through these receptors, indicating the important role of btk in various signal transduction pathways. Additionally, btk has been shown to be essential for tyrosine phosphorylation of PLC- γ 2 in a chicken B cell lymphoma (14). We have identified btk as an important PTK in CD72-mediated signal transduction. Btk was activated in both xid and non-xid mice after CD72 or BCR ligation. There are very few reports on the activation of btk in normal B cells in response to BCR stimulation. Studies using the murine immature B cell line WEHI-231 (31), human tonsillar B cells, and Daudi B cells (44) showed a four- to fivefold increase in btk kinase activity that remained elevated until 10 min of stimulation. However the extent of btk activation seen in our studies in response to CD72 or BCR ligation is similar to that reported in the chicken DT40 cell line (14) stimulated with anti-IgM. To our knowledge, this is the first demonstration of an increase in btk activity in response to BCR or CD72 signaling in normal mature splenic B cells.

The importance of btk in CD72 signaling is underscored by the findings that B lymphocytes from xid mice do not proliferate in response to CD72 signaling. This is similar to our earlier report, which showed that xid mice heterozygous for CD72^a expression did not respond to stimulation with CD72^a-specific mAb (10.1.D2) (17). This is not due to expression of reduced levels of CD72 on xid B cells since B lymphocytes from both xid and non-xid mice express similar levels of CD72 as determined by flow cytometry (data not shown). These results suggest that, similar to other receptors, interactions mediated through the PH domain of btk were essential for complete signal transduction through the CD72 receptor. Our preliminary data and an earlier report (45) demonstrate normal levels of inducible protein tyrosine phosphorylation in xid B cells in response to BCR ligation. Moreover, levels of PLC- γ 2 tyrosine phosphorylation and calcium mobilization in response to anti-IgM stimulation are not altered in xid mice when compared with wild-type control (data not shown). These results argue that the xid B cells are not defective in inducing src tyrosine kinase activity in response to BCR engagement.

Previous studies have shown that tyrosine phosphorylation of PLC- γ 2 was required for its activity (46). A study using chicken DT40 B cell lymphoma showed an absolute requirement of syk activity for PLC- γ 2 tyrosine phosphorylation after BCR stimulation (13). PLC- γ 2 was inducibly tyrosine phosphorylated to a

lower extent in the btk-deficient DT40 cell line, suggesting that syk can still phosphorylate PLC- γ 2 in the absence of btk involvement (14). In contrast to these findings, normal levels of inducible phosphorylation of PLC- γ 2 were observed in CD72-stimulated cells, in spite of lack of syk activation. The discrepancy in these results may be due to three possible reasons: 1) There may be fundamental species differences in the requirement of syk for PLC activation in murine and chicken B cells. 2) There may also be differences based on the stage of B cell development (immature vs mature); i.e., the chicken DT40 cell line is an immature B cell lymphoma whereas our study used normal mature splenic B cells. 3) Since btk has been shown to activate PLC- γ 2 (14), it might replace the need for syk in CD72-signaling pathway.

To our knowledge, this is the first demonstration of syk-independent tyrosine phosphorylation of PLC- γ 2 in mature B lymphocytes. The absence of syk activation with normal PI hydrolysis (18, 24), calcium mobilization (18, 19), and cell proliferation (16–18) in CD72-stimulated B cells has at least two major implications. First, this may explain the existence of “cross-talk” between cAMP and PI pathways in BCR-, but not CD72-, mediated signaling (18, 25). Thus, in neutrophils, the basis of “cross-talk” between the two pathways lies in the inhibition of syk activity by cAMP-elevating agents (47). Furthermore, the inhibition of syk is associated with serine phosphorylation, supporting the involvement of cAMP-dependent PKA (47). Whether such a mechanism can account for cAMP-mediated inhibition of BCR signaling is currently being tested. Second, hyper-cross-linking of surface Ig on mature B cells induces apoptosis (48), which can be rescued by the simultaneous addition of antibodies to CD40 (49) or CD72 (26) to B cell cultures. This led to a model suggesting the need for a second costimulatory signal to prevent BCR-mediated death (26). Although the exact regulatory mechanisms that rescue these cells are unknown, syk-independent activation of the PLC- γ 2 pathway and subsequent signaling cascade by CD72 stimulation may have a role in rescue from apoptosis and induction of growth in B cells.

Calcium-dependent activation events were required for BCR-induced proliferation that could be specifically blocked by immunosuppressive agents CsA and FK506 (50, 51). Previously we showed that CD72 engagement caused increased elevation of [Ca²⁺]_i (18, 19). Consistent with this finding, both CsA and FK506 effectively inhibited CD72-induced B cell proliferation, indicating an important role for Ca²⁺-regulated activation pathways for CD72 signaling similar to that observed for BCR-stimulated cells.

In summary, our previous studies and this report have shown that CD72 stimulation results in differential activation of signaling events when compared with BCR-stimulated cells. Similar to anti-IgM-stimulated cells, CD72 induces activation of PTKs lyn, blk, and btk, activation of phospholipase- γ (18, 24), calcium mobilization (18, 19), and Ca²⁺-dependent signaling events required for growth responses of B lymphocytes. Unlike BCR stimulation, CD72 ligation induced tyrosine phosphorylation of PLC- γ 2 in the apparent absence of syk activation.

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