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CD72 Negatively Regulates Signaling Through the Antigen Receptor of B Cells

Takahiro Adachi,* Chisato Wakabayashi,* Toshinori Nakayama,† Hidetaka Yakura,‡ and Takeshi Tsubata*‡

The immunoreceptor tyrosine-based inhibition motif (ITIM) is found in various membrane molecules such as CD22 and the low-affinity Fc receptor for IgG in B cells and the killer cell-inhibitory receptor and Ly-49 in NK cells. Upon tyrosine phosphorylation at the ITIMs, these molecules recruit SH2 domain-containing phosphatases such as SH2-containing tyrosine phosphatase-1 and negatively regulate cell activity. The B cell surface molecule CD72 carries an ITIM and an ITIM-like sequence. We have previously shown that CD72 is phosphorylated and recruits SH2-containing tyrosine phosphatase-1 upon cross-linking of the Ag receptor of B cells (BCR). However, whether CD72 modulates BCR signaling has not yet been elucidated. In this paper, we demonstrate that expression of CD72 down-modulates both extracellular signal-related kinase (ERK) activation and Ca\(^{2+}\) mobilization induced by BCR ligation in the mouse B lymphoma line K46, whereas BCR-mediated ERK activation was not reduced by the ITIM-mutated form of CD72. Moreover, coligation with CD72 with BCR reduces BCR-mediated ERK activation in spleen B cells of normal mice. These results indicate that CD72 negatively regulates BCR signaling. CD72 may play a regulatory role in B cell activation, probably by setting a threshold for BCR signaling.}

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homodimer (25–27). CD72 carries an ITIM and an ITIM-like sequence in the cytoplasmic tail. We (28) and Wu et al. (29) have previously demonstrated that CD72 is tyrosine-phosphorylated and recruits SHP-1 upon BCR ligation, as is the case for CD22. However, it has not yet been known whether CD72 negatively regulates BCR signaling. In this paper we demonstrate that expression of CD72 inhibits ERK activation and Ca2+ mobilization by BCR cross-linking even in the absence of coligation of CD72 with BCR. This result strongly suggests that CD72 constitutively down-regulates BCR signaling and sets a signaling threshold for B cell activation, as is the case for CD22.

Materials and Methods

Mice and cells

DBA/2 and BALB/c mice were purchased from Sankyo (Tokyo, Japan). Spleen B cells were purified as described previously (30). K46μm, a transfectant of the mouse B lymphoma line K46 expressing both the H and L chains of IgM specific for hapten (4-hydroxy-3-nitrophenyl) acetyl (NP), was kindly provided by Drs. M. Reth and J. Wienands (31). The mouse B lymphoma line WEHI-231.5 was described previously (28). Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-ME, and 1 mM glutamine. cDNA encoding a mutated CD72, in which tyrosine residues at 7 and 39 were replaced by phenylalanine (SHP-1Y/F), was generated by site-directed mutagenesis using mouse pBSCD72 carrying the CD72 cDNA (28) as a template and was inserted into the expression vector pMJKITneo (a gift from Dr. K. Maruyama, Tokyo Medical and Dental University) (pMJKITCD72Y/F). The expression plasmids pMikCD72 (28) and pMKITCD72Y/F were transfected by electroporation. K46μm and its transfectants were stimulated with NP-BSA, and 1 mg/ml D-glucose. Fluo-3 fluorescence of cells was measured in Hanks’ solution and incubated at 37°C for 10 min, and the ratio of Fluo-3/AM (Molecular Probes, Eugene, OR) and 0.02% (w/v) pluronic F-127 at 37°C for 30 min. After washing three times, cells were incubated in Hanks’ solution containing either 10 μg/ml of anti-mouse CD72 mAb CT72.2 (mouse IgM; Cedarlane Laboratories, Burlington, MA), rabbit anti-ERK2 Ab (Santa Cruz Biotechnology) and 30 μg/ml of [γ-32P]ATP (Amersham, Amersham, U.K.) at room temperature for 20 min. The reaction was terminated by adding SDS-PAGE sample buffer, and proteins were separated by SDS-PAGE before autoradiography. Phosphorylation of MBP was quantitated by a BAS-2500 (Fuji Photo Film, Tokyo, Japan).

Results

CD72 negatively regulates both ERK activation and Ca2+ mobilization induced by BCR ligation in the K46μmλ B lymphoma cells

To investigate the signaling function of CD72, we assessed CD72 expression on the surface of B cell lines by flow cytometry. The B lymphoma line K46μmλ was not stained by Ab to CD72 (Fig. 1), although this line is derived from a BALB/c mouse carrying the CD72 allelotype. This indicated that K46μmλ does not express CD72 on the surface. Moreover, Western blot analysis using rabbit
K46 transfectants with a similar time course to that in the parent K46. Representative data of three experiments are shown. Numbers under each lane indicate relative intensities of phospho-ERK2 with different exposure times because the intensity of the phospho-ERK1 that the data on ERK1 and ERK2 were taken from the same membrane treated with medium alone. Cells were subsequently lysed and subjected to Western blot analysis using anti-phospho-ERK Ab. Please note that the parent cells regardless of the duration of Ag stimulation. Taken together, expression of CD72 most probably down-modulates phosphorylation of ERK induced by BCR signaling. Because phosphorylation of ERK correlates with its activity, CD72 may negatively regulate BCR-mediated Ca2+ mobilization in K46 cells. The reduced calcium response to BCR ligation was not due to clonal variation in calcium channel function, as evidenced by the fact that the CD72 transfectants showed a comparable response to a calcium ionophore A23187 (Fig. 4D). Thus, expression of CD72 appears to negatively regulate BCR-mediated Ca2+ mobilization in K46 cells. Taken together, CD72 down-modulates both ERK activation and Ca2+ mobilization induced by BCR ligation, strongly suggesting that CD72 negatively regulates BCR signaling in K46 cells.

To assess whether CD72 regulates BCR signaling, we treated K46μmα and its CD72 transfectants with various amounts of NP-BSA because surface IgM on K46μmα is specific to NP. Phosphorylation of ERK was assessed by Western blotting of total cell lysates using anti-phospho-ERK Ab. When treated with Ag, phosphorylation of both ERK1 and ERK2 was enhanced in both K46μmα and its CD72 transfectants (Fig. 2A). This result indicated that Ag stimulation induces phosphorylation of both ERK1 and ERK2 in K46μmα and its CD72 transfectants. However, Ag-induced phosphorylation of ERK in both of the K46μmα CD72 transfectants was weaker than that in the parent K46μmα cells, regardless of the amount of Ag (Fig. 2A). Moreover, treatment with NP-BSA induced phosphorylation of ERK in K46μmα CD72 transfectants with a similar time course to that in the parent K46μmα cells (Fig. 2B). However, both of the CD72 transfectants showed reduced phosphorylation of ERK1 and ERK2 compared to that of the parent cells.
were replaced by phenylalanine. Although the CD72Y/F transfec-
tant expressed an even higher level of CD72 on the surface than
the K46\textsuperscript{mml}l CD72-4 transfectant did, the transfectant showed sim-
ilar BCR-mediated phosphorylation of ERK1 and ERK2 to that of
the parent K46\textsuperscript{mml}l cells (Fig. 5). Essentially the same results
were obtained in three independent transfectants (data not shown).
This indicated that CD72 requires its ITIM, ITIM-like sequence, or
both for negative regulation of BCR signaling.

\textbf{Coligation of CD72 with BCR negatively regulates both ERK
activation and Ca\textsuperscript{2+} mobilization in normal spleen B cells.

To investigate whether CD72 negatively regulates BCR signaling
in normal B cells, we isolated spleen B cells from 10-wk-old
BALB/c mice carrying the CD72\textsuperscript{b} allotype and cross-linked BCR
together with CD72 to enhance the regulatory effect of CD72 on
BCR signaling. We treated B cells with either anti-CD72\textsuperscript{b} mAb
CT72.2 (mouse IgM) or an isotype-matched control mAb B1-8 on
ice before addition of F(ab\textsuperscript{'})\textsubscript{2} fragments of anti-mouse
IgM Ab. The treatment with the combination of CT72.2 and anti-IgM Ab
coligated CD72 with BCR as anti-IgM Ab reacted to both CT72.2 and
BCR (surface IgM) of B cells. In contrast, treatment with the combination of the control Ab and anti-IgM Ab ligated BCR alone.
Because CT72.2, B1-8, and F(ab\textsuperscript{'})\textsubscript{2} fragments of anti-IgM Ab do
not contain Fcy, these treatments did not coligate FcyR by BCR.
After treatment with anti-IgM Ab at 37°C for 5 min, we collected
B cells as BCR ligation induced the maximal ERK2 phosphory-
lation at this time point (Fig. 6A). Western blotting of total cell
lysates with anti-phospho-ERK Ab showed that both ERK1 and
\textbf{FIGURE 4.} CD72 reduces Ca\textsuperscript{2+} mobilization induced by BCR ligation.
Cells (1 \times 10\textsuperscript{5}) of K46\textsuperscript{mml}, K46\textsuperscript{mml}CD72-4, and K46\textsuperscript{mml}CD72-6 were
loaded with 5 \mu M Fluo-3 in the presence of 0.02% pluronic F-127 at 37°C
for 30 min. After washing, cells were suspended in HEPES buffer. Intra-
cellular free Ca\textsuperscript{2+} concentration was measured by a FACS Calibur. Cells
were added with the indicated concentrations of NP-BSA (A–C) or with 5
mM A23187 (D) at 30 sec (indicated by arrows), and measurement of free
Ca\textsuperscript{2+} concentration was continued for 360 s. Representative data of five
experiments are shown.

\textbf{FIGURE 5.} Expression of an ITIM-mutated form of CD72 (CD72Y/F)
fails to reduce BCR-mediated phosphorylation of ERK in K46\textsuperscript{mml} cells.
A and B, CD72 expression on the cell surface. Cells of a K46\textsuperscript{mml} CD72Y/F transfectant (B) were reacted with biotinylated anti-mouse
CD72\textsuperscript{a} mAb 9.6.1 before staining with FITC-labeled streptavidin. As a
control, K46\textsuperscript{mml}CD72-4 cells (A) were stained as parallel. Cells reacted
without Abs were used as negative controls (gray histograms). Cells were
analyzed by flow cytometry using a FACSCalibur (Becton Dickinson).
C and D, Phosphorylation of ERK. Cells (5 \times 10\textsuperscript{5}) of K46\textsuperscript{mml} and its
CD72Y/F transfectant were treated with the indicated amounts of NP-BSA
for 3 min (C) or with 10 \mu g/ml of NP-BSA for the indicated times (D) at
37°C. As negative controls, cells were treated with medium alone. Cells
were subsequently lysed and subjected to Western blot analysis using anti-
phospho-ERK Ab. Please note that the data on ERK1 and ERK2 were
taken from the same membrane with different exposure times because the
intensity of the phospho-ERK1 band in each lane was much less than that
of the phospho-ERK2 band. Numbers under each lane indicate the relative
intensities of the phospho-ERK2 bands. The same blots were reprobed with
anti-ERK2 Ab to ensure equal loading. Representative data of three ex-
periments are shown.
cells were incubated with or without 20 μg/ml of rabbit anti-mouse κ-chain Ab on ice for 5 min. After washing, cells were incubated with or without 20 μg/ml of goat anti-mouse IgM Ab at 37°C for 5 min. Cells were then lysed and subjected to Western blot analysis using anti-phospho-ERK Ab. Numbers under each lane indicate the relative intensities of the phospho-ERK2 bands. The results strongly suggest that CD72 negatively regulates BCR signaling in B cell lines and normal mature B cells and that the ITIM and/or the ITIM-like sequence in CD72 are crucial for its negative regulatory effect on BCR signaling. Because induced coligation of CD72 with BCR is not required for negative regulation of BCR signaling by CD72 in K46μmα cells, CD72 may interact with BCR and down-modulate its signaling constitutively. This observation is in agreement with the previous finding that BCR ligation alone induces both phosphorylation of CD72 and recruitment of SHP-1 to CD72 in K46μmα cells, and the function of CD72 in normal spleen B cells. However, ERK2 were phosphorylated by either BCR ligation alone or coligation of BCR and CD72 (Fig. 6B). However, BCR ligation reduced stronger ERK phosphorylation than coligation of CD72 with BCR did, indicating that BCR ligation-induced phosphorylation of ERK is down-modulated when CD72 is coligated with BCR. To confirm this observation, we coligated CD72 with BCR on spleen B cells from DBA/2 mice carrying CD72α using anti-CD72α and anti-mouse κ mAb. Because both anti-CD72α mAb and anti-κ mAb contain Fcγ, we blocked FcγR by pretreating B cells with anti-FcγR mAb 2.4G2. Phosphorylation of both ERK1 and ERK2 induced by coligation of BCR and CD72 was weaker than that induced by BCR ligation alone (Fig. 6C), indicating that coligation with CD72 reduced BCR ligation-mediated phosphorylation of ERK in DBA/2 spleen cells.

Finally, we stimulated spleen B cells from BALB/c mice by BCR ligation alone or by coligation of CD72 and BCR, and then we measured intracellular Ca2+ concentration by flow cytometry using Indo-1. Coligation of CD72 with BCR showed a reduced Ca2+ flux compared to that with BCR ligation alone in spleen B cells (Fig. 6D). Taken together, these results indicate that coligation with CD72 negatively regulates BCR-induced ERK activation and Ca2+ mobilization in normal spleen B cells.

Discussion
By taking advantage of the finding that the B lymphoma line K46μmα expresses no detectable CD72, we have established K46μmα transfectants expressing either wild-type CD72 or ITIM-mutated CD72 and demonstrated that expression of CD72 diminishes both ERK activation and Ca2+ mobilization induced by BCR ligation, whereas the ITIM-mutated form of CD72 does not reduce BCR-mediated ERK activation. Moreover, coligation of CD72 with BCR down-modulates both BCR-mediated ERK activation and Ca2+ mobilization in normal spleen B cells. These results strongly suggest that CD72 negatively regulates BCR signaling in B cell lines and normal mature B cells and that the ITIM and/or the ITIM-like sequence in CD72 are crucial for its negative regulatory effect on BCR signaling. Because induced coligation of CD72 with BCR is not required for negative regulation of BCR signaling by CD72 in K46μmα cells, CD72 may interact with BCR and down-modulate its signaling constitutively. This observation is in agreement with the previous finding that BCR ligation alone induces both phosphorylation of CD72 and recruitment of SHP-1 to CD72 (28, 29), indicating that CD72 functionally interacts with BCR even in the absence of coligation of CD72 with BCR.

cells from 10-wk-old DBA/2 mice were preincubated with 10 μg/ml of anti-mouse FcγRII mAb 2.4G2 on ice for 5 min. Cells were then added with 10 μg/ml of anti-mouse κ-chain mAb 187.1 at 37°C for 5 min. Cells were then lysed and subjected to Western blot analysis using anti-phospho-ERK Ab. Numbers under each lane indicate the relative intensities of phospho-ERK2 bands. The results strongly suggest that CD72 negatively regulates BCR signaling in B cell lines and normal mature B cells and that the ITIM and/or the ITIM-like sequence in CD72 are crucial for its negative regulatory effect on BCR signaling. Because induced coligation of CD72 with BCR is not required for negative regulation of BCR signaling by CD72 in K46μmα cells, CD72 may interact with BCR and down-modulate its signaling constitutively. This observation is in agreement with the previous finding that BCR ligation alone induces both phosphorylation of CD72 and recruitment of SHP-1 to CD72 (28, 29), indicating that CD72 functionally interacts with BCR even in the absence of coligation of CD72 with BCR. However,
induced coligation of CD72 with BCR by using Abs to those molecules diminishes BCR signaling in spleen B cells (Fig. 6). Coligation of CD72 with BCR may enhance interaction of CD72 with BCR, resulting in further down-modulation of BCR signaling. This observation is in agreement with the finding on CD22 that coligation of CD22 with BCR further reduces BCR signaling (21), although several lines of evidence indicate that CD22 negatively regulates BCR signaling constitutively (22–24). Taken together, CD72 appears to constitutively down-modulate BCR signaling, but its negative regulatory effect is further enhanced by coligation of CD72 with BCR.

Treatment of B cells with anti-CD72 Abs has been shown to enhance activation and proliferation of normal mature B cells induced by BCR ligation (32, 35). However, this observation may not contradict the idea of a negative regulatory role of CD72 on BCR signaling. Indeed, BCR-mediated B cell activation is enhanced by treatment with Abs to CD22 (36, 37), whose inhibitory role on BCR signaling has already been established by lines of evidence including that on CD22-deficient mice (17–20). Anti-CD72 Abs may disrupt interaction between CD72 and BCR, resulting in enhancement of BCR signaling in the absence of the negative regulatory effect of CD72 on BCR signaling. Alternatively, CD72 transmits a stimulatory signal independent of BCR when CD72 is ligated by anti-CD72. This is consistent with the recent finding that CD72 ligation activates Src-family kinases Lyn and Blk in the absence of activation of Syk, which is essential for BCR signaling (38).

Both motheaten mice deficient in SHP-1 and Lyn-deficient mice show a marked increase in the number of plasma cells and development of autoimmune disease associated with autoantibody production (39–43). Thus, SHP-1 and Lyn may prevent development of autoimmune disease, probably by inhibiting B cell hyperactivity. This inhibitory role of SHP-1 and Lyn appears to involve CD22. Indeed, CD22 is a substrate of Lyn and induces activation of SHP-1 (12, 44), suggesting that CD22 is a component of a signaling pathway including Lyn and SHP-1. This notion is also supported by the genetic evidence obtained using mice with heterozygous deficiency in SHP-1, Lyn, or CD22 (45). Although CD22-deficient mice show B cell hyperresponsiveness, the severity of the defects in CD22-deficient mice is much milder than that of SHP-1-deficient motheaten mice. Thus, other ITIM-containing molecules in B cells may play a role in maintaining the normal immune response together with CD22 by activating SHP-1. FcγRII may not be involved in this pathway, as shown by the fact that the inhibitory function of FcγRII is mostly ascribed to SHIP and not SHP-1 (5, 46). In contrast, CD72 negatively regulates BCR signaling in a manner similar to that of CD22. Indeed, both CD22 and CD72 constitutively associate with BCR (15, 16, 47), are substrates of Lyn (12, 28, 44), recruit SHP-1 upon BCR ligation, and negatively regulate BCR signaling such as Ca2+ mobilization even in the absence of coligation with BCR (Figs. 2–4 and 6 and Refs. 17–21). Moreover, the cytoplasmic tails of both CD22 and CD72 carry ITIMs essential for recruitment of SHP-1 and negative regulation of BCR signaling (Fig. 5 and Refs. 6 and 28). Thus, CD72 may carry a function redundant with CD22 and, together with CD22, may maintain normal humoral immunity by activating an inhibitory signaling pathway involving Lyn and SHP-1. As defects in this pathway cause autoimmune disease with autoantibody production, defects in CD72 may be involved in development of autoimmune diseases.

CD72 may interact with its natural ligands through the extracellular region containing a C-type lectin-like domain. Interaction with the ligands probably modulates B cell activation induced by BCR ligation and may be involved in activation of B cells in certain humoral immune responses. CD5 expressed on T cells and B cells has been shown to be a ligand for CD72 (48). However, this is controversial because Biancone et al. (49) and Bikah et al. (50) have recently demonstrated that CD5 fails to bind to CD72. Further elucidation of the role of CD72-mediated regulation of BCR signaling and its modification by CD72 ligands may be crucial for understanding the molecular mechanisms for normal and abnormal humoral immune responses.

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