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*J Immunol* 2003; 171:1835-1843; doi: 10.4049/jimmunol.171.4.1835

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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Inhibitory Coreceptors Activated by Antigens But Not by Anti-Ig Heavy Chain Antibodies Install Requirement of Costimulation Through CD40 for Survival and Proliferation of B Cells

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Ag-induced B cell proliferation in vivo requires a costimulatory signal through CD40, whereas B cell Ag receptor (BCR) ligation by anti-Ig H chain Abs, such as anti-Ig μ H chain Ab and anti-Ig δ H chain Ab, alone induces proliferation of B cells in vitro, even in the absence of CD40 ligation. In this study, we demonstrate that CD40 signaling is required for survival and proliferation of B cells stimulated by protein Ags in vitro as well as in vivo. This indicates that the in vitro system represents B cell activation in vivo, and that protein Ags generate BCR signaling distinct from that by anti-Ig H chain Abs. Indeed, BCR ligation by Ags, but not by anti-Ig H chain Abs, efficiently phosphorylates the inhibitory coreceptors CD22 and CD72. When these coreceptors are activated, anti-Ig H chain Ab-stimulated B cells can survive and proliferate only in the presence of CD40 signaling. Conversely, treatment of Ag-stimulated B cells with anti-CD72 mAb blocks CD72 phosphorylation and induces proliferation, even in the absence of CD40 signaling. These results strongly suggest that activation of B cells by anti-Ig H chain Abs involves their ability to silence the inhibitory coreceptors, and that the inhibitory coreceptors install requirement of CD40 signaling for survival and proliferation of Ag-stimulated B cells. The Journal of Immunology, 2003, 171: 1835–1843.

E ncounter with pathogens induces humoral immune responses through activation and differentiation of Ag-specific B cells. Activation of B cells depends on various factors, including nature of Ags, duration of Ag stimulation, and presence or absence of T cell help (1–4). The B cell Ab responses normally require T cell help. Besides B cell Ag receptor (BCR) signaling generated by interaction with Ags, B cell activation appears to require activation signals derived from T cells. Using hen egg lysozyme-specific B cells, Jenkins and colleagues (5) clearly demonstrated in vivo that proliferation of Ag-stimulated B cells requires T cell help, especially signaling through CD40. In contrast, ligation of BCR by anti-Ig H chain Abs, such as anti-Ig μ H chain Ab and anti-Ig δ H chain Ab, alone induces proliferation of B cells in vitro (6), indicating that BCR ligation alone can induce B cell proliferation, at least in vitro. One possible explanation for this contradiction is that an in vitro culture system may not represent B cell activation in vivo. Alternatively, BCR ligation by anti-Ig H chain Abs may generate signaling distinct from that induced by Ags, and there may be a mechanism that makes activation of Ag-stimulated B cells dependent on a costimulatory signal via CD40.

BCR ligation activates the cytoplasmic kinase Syk and Src-family kinases such as Lyn (7, 8). These kinases then activate various signaling cascades, including Ca2+ mobilization and mitogen-activated protein kinases, which are crucial for activation and proliferation of B cells (9–12). Lines of evidence suggest that Lyn is also involved in negative regulation of BCR signaling (13–18). When BCR is ligated, Lyn phosphorylates the inhibitory BCR coreceptors CD22 and CD72, probably because these molecules constitute a signal with BCR (16–20). Both CD22 and CD72 contain the immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their cytoplasmic regions (18, 21). When phosphorylated, the ITIMs in these coreceptors recruit and activate the Src-homology-domain-2-containing protein tyrosine phosphatase-1 (SHP-1) (18, 21, 22). Activated SHP-1 then induces dephosphorylation of various signaling molecules involved in BCR signaling, such as Syk, B cell linker protein, and phospholipase C-γ (23–25), thereby down-modulating BCR signaling. The inhibitory coreceptors CD22 and CD72 thus serve as membrane-bound adapters that link BCR-associated kinases with SHP-1, which inactivates signaling molecules involved in BCR signaling. By negatively regulating BCR signaling, these inhibitory coreceptors are implicated in setting a BCR signal threshold (26–29).

The quasi-monoclonal (QM) mice contain a VH1DJ14 exon inserted in the IgH locus and targeted deletions at both κ L chain alleles, thereby expressing the IgH chain with the inserted VH1 and L chain in almost all of the B cells (30). Because the IgH chain carrying the inserted VH1 generates Abs reactive to the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) by pairing with the Ig L chain, almost all of the B cells in QM mice are reactive to NP. By treating QM B cells with various Ags with different epitope densities and with

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different affinities in vitro, we demonstrated that Ags do not activate B cells, but rather induce apoptosis of B cells by themselves and require coexistence of costimulation through CD40 for survival and proliferation of B cells in agreement with previous in vivo findings (5). This result indicated that BCR ligation by Ags generates signaling distinct from that by anti-Ig H chain Abs, which induces B cell proliferation by themselves. Indeed, we further demonstrated that Ags, but not anti-Ig H chain Abs, efficiently induce phosphorylation of the BCR coreceptors CD22 and CD72. Activation of both CD22 and CD72 prevented survival and proliferation of anti-Ig H chain Ab-stimulated B cells in the absence of CD40 signaling, and CD40 signaling became essential for survival and proliferation of B cells. Conversely, inactivation of CD72 induced survival and proliferation of Ag-stimulated B cells, even in the absence of CD40 signaling, suggesting that the inhibitory coreceptors are essential for CD40-dependent survival and proliferation of Ag-stimulated B cells. These results strongly suggest that Ags generate BCR signaling distinct from that by anti-Ig H chain Abs in that Ags, but not anti-Ig H chain Abs, activate the inhibitory coreceptors CD22 and CD72, which install requirement of costimulatory signals for B cell survival and activation.

Materials and Methods

Mice

C57BL/6 mice were purchased from Sankyo Labo (Tokyo, Japan). QM mice were described previously (30) and were maintained in our animal facility.

Plasmids

The cDNA encoding porcine Syk was obtained by PCR with a set of primers (5′-AAGAATTCCTGGGACAGTGGCAACACCAC and 5′-AAGGGGGCCGTTAATTACACATCGTCTA-3′) using the plasmid pA-puro-Syk (a gift of Dr. H. Yamamura, Kobe University, Kobe, Japan) as a template (31). The DNA fragment was then digested with EcoRI and NotI and subcloned into the pMIK vector (pMIK-Syk0). The DNA fragment encoding hemagglutinin (HA)-tag was prepared by annealing a pair of synthetic oligonucleotides (5′-AAGAATTCATGCCAGGGGTCTA and 5′-AAGGGGGCCGTTAATTACACATCGTCTA-3′) and inserting them into the EcoRI site of pMIK-Syk0. The resulting expression plasmid encoding HA-tagged Syk was designated pMIK-Syk.

BCR ligation

Cells were treated with the following Ags or anti-Ig Abs to ligate BCR at 37 °C: (i) NP-BSA (NP-NP, NP-NP, NP-NP, NP-NP, (4-hydroxy-5-ido-3-nitrophenyl)acetyl (NP)-BSA (NP-NP, NP-NP) (a gift of Dr. T. Takemori, National Institute of Infectious Diseases, Tokyo, Japan), 2,4-dinitrophenyl (DNP)-coupled chicken γ globulin (CGG) (DNP-CGG), (4-hydroxy-3,5-dinitrophenyl)acetyl (NP)-coupled CGG (NP-NP, CGG) (gifts of Dr. T. Azuma, Science University of Tokyo, Chiba, Japan), (ii) F(ab′)2 of goat anti-mouse Ig H chain Ab (ICN Pharmaceuticals, Aurora, OH), F(ab′)2 of goat anti-mouse Ig G chain Ab (Nordic Immunology Laboratories, Tilburg, The Netherlands), rat anti-mouse Ig H chain mAb B7-6, rat anti-mouse Ig δ H chain mAb AbO-MD6 (a gift of Dr. H. Bazin, University of Louvain, Brussels, Belgium) (32), (iii) anti-Id Ab Ac146 reactive to K46 cells (a gift of Dr. K. Rajewsky, University of Paris, France), (iv) a pair of synthetic oligonucleotides (5′-GGAATTCATGCCAGGGGTCTA and 5′-AAGAATTCATGCCAGGGGTCTA-3′) to generate the human IgG fusion protein containing CD22 (a gift of Drs. P. R. Crocker, University of Dundee, Dundee, U.K., and L. Nitschke, University of Würzburg, Würzburg, Germany), respectively. Rabbit anti-C72Ab was described previously (18). Total cell lysates or immunoprecipitates were separated by SDS-PAGE and transferred to nylon membranes. Membranes were incubated with peroxidase-conjugated anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY) or rabbit anti-mouse CD72Ab, rabbit anti-mouse C72Ab, rabbit anti-mouse Syk Ab, or anti-SHP-1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA), followed by reaction with peroxidase-conjugated anti-rabbit IgG Ab (New England Biolabs) or anti-mouse IgG Ab (Southern Biotechnology Associates), respectively. Proteins were then visualized by an ECL system (Amersham Pharmacia Biotech).

Results

BCR ligation by protein Ags, but not anti-Ig H chain Abs, requires coexistence of CD40 signaling for survival and cell cycle entry of spleen B cells

We first asked whether BCR ligation by anti-Ig H chain Abs activates mature B cells from QM mice, as is the case for those from normal mice (6). We prepared purified spleen B cells from QM mice and normal C57BL/6 mice and cultured B cells with polyclonal anti-Ig H chain Ab or anti-Ig δ H chain Ab, as almost all spleen B cells express both BCR containing IgM (IgM-BCR) and IgD-BCR. After 48 h, cell cycle status was analyzed by flow cytometry. The >40% of QM B cells, as well as normal B cells treated with either anti-μ or anti-δ Ab, showed cell cycle progression into the S phase (Fig. 1A). Essentially the same results were obtained with anti-μ Ab B7-6 and anti-δ Ab AbO-MD6 (data not shown). In contrast, almost all of the untreated B cells stayed in the G0/G1 phase of the cell cycle, regardless of whether they are from normal mice or QM mice. This indicates that BCR ligation by anti-Ig H chain Ab induces cell cycle progression in B cells from QM mice as well as normal mice.

Next, we assessed whether BCR ligation by Ags activates QM B cells. Because almost all the QM B cells are reactive to the
hapten NP (30), we cultured QM B cells with various concentrations of BSA coupled at various ratios with NP (NP–BSA). In 48 h, <5% of the cells entered into the S phase by any of the Ag tested, demonstrating that NP–BSA fails to induce cell cycle progression in NP-reactive B cells by itself (Fig. 1B). However, treatment with NP15–BSA or NP18–BSA together with agonistic anti-CD40 mAb induced cell cycle progression to the S phase in >30% of the cells, whereas <10% of the cells entered to the S phase by treatment with anti-CD40 mAb alone (Fig. 1B). These results indicated that NP15–BSA or NP18–BSA does not induce cell cycle progression of specific B cells by itself, but appears to trigger activation signaling that induces cell cycle progression in cooperation with CD40 signaling. When we treated QM B cells with NP15–BSA or NP18–BSA alone for 24 h, percentages of both dead cells and apoptotic cells were increased compared with untreated cells (Fig. 1B). The increase in the percentages of dead cells and apoptotic cells were reversed in the presence of CD40 signaling. This indicates that treatment with NP15–BSA or NP18–BSA generates BCR signaling, which by itself induces apoptosis of B cells and requires anti-apoptotic signaling through CD40 for activating B cells. In contrast, NP2–BSA fails to induce apoptosis or cell cycle progression, regardless of presence or absence of anti-CD40 mAb, demonstrating a threshold of epitope density on Ags for both B cell apoptosis and activation (Fig. 1B).

We then stimulated QM B cells with Ags containing another carrier, CGG, or those with different affinities to QM B cells. QM BCR shows 10 times higher affinity to the NP derivatives NIP and NNP than NP, whereas it binds to DNP only weakly (33). QM B cells did not undergo cell cycle progression by any of the Ags tested (Fig. 1C), indicating that Ags with different carriers or those with various affinities fail to induce cell cycle progression in B cells. When QM B cells were stimulated with high affinity Ags such as NIP22–BSA or NNP15–CGG, QM B cells underwent apoptosis in the absence of anti-CD40 mAb, and in the presence of anti-CD40 mAb showed cell cycle progression, as is the case for NP–BSA, with high epitope density. In contrast, treatment with DNP7–CGG regulated neither apoptosis nor cell cycle progression, as is the case for NP2–BSA. These results suggest that high affinity Ags induce apoptosis of B cells and require coexistence of CD40 signaling for B cell survival and activation, whereas low affinity Ags fail to induce BCR signaling. Taken together, BSA or CGG coupled with NP or its derivatives at various ratios fail to induce cell cycle progression by itself in QM B cells, although high affinity Ags with high epitope density induce activation or apoptosis depending on the presence or absence of CD40 signaling. Because treatment with anti-Ig H chain Abs alone induces proliferation of QM B cells, BCR appears to transmit distinct signaling upon interaction with Ags from that induced by anti-Ig H chain Abs.

Ags, but not anti-Ig H chain Abs, induce efficient phosphorylation and activation of the inhibitory coreceptors CD22 and CD72

To assess the molecular basis for the distinct BCR signaling induced by Ags, we stimulated QM B cells with either NP15–BSA or polyclonal anti-μ Ab and examined tyrosine phosphorylation of cellular substrates by phosphotyrosine blotting of total cell lysates. Phosphorylation of various cellular substrates in NP15–BSA-treated cells was similar to that of anti-μ Ab-treated cells, except that phosphorylation of a 160-kDa protein was much stronger in NP15–BSA-treated cells (Fig. 2A). NP–BSA ligated both IgM–BCR and IgD–BCR on QM B cells, whereas only IgM–BCR was ligated by anti-μ Ab. However, the differential phosphorylation of the 160-kDa protein was not caused by a difference in Ig isotypes of

![FIGURE 1. BCR ligation by Ags, but not by anti-Ig H chain Ab, requires CD40 signaling for survival and cell cycle progression of B cells. Purified spleen B cells were obtained from 8- to 12-wk-old C57BL/6 mice and QM mice. Cells were cultured either with 10 μg/ml F(ab')2 of goat anti-mouse μ or δ Ab (A) or with indicated amounts of BSA or CGG coupled with NP or its derivatives in the presence or absence of 10 μg/ml anti-CD40 mAb (B and C). After 24 h cells were collected. Percentages of dead cells (left panels) and apoptotic cells (middle panels) were measured by trypan blue exclusion and flow cytometric measurement of cells with hypodiploid DNA, respectively. Thin lines in the left panels indicate SD of triplicates. Alternatively, cells were cultured for 48 h. Cells were pulsed with BrdU for the last 10 min of the culture. Percentages of BrdU-incorporated cells (% S phase) were measured by flow cytometry (right panels). The results of Ag-stimulated cells were corrected by the fraction of NP-reactive B cells in total B cells. The data are representative of at least three experiments.](http://www.jimmunol.org/)

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ligated BCR, because treatment with anti-δ Ab also failed to induce strong phosphorylation of this protein (data not shown). Essentially the same results were obtained in K46μμλ (Fig. 2B) and K46δμλ (data not shown) transfectants of the B cell line K46 expressing NP-specific IgM and IgD, respectively (38). Because CD22 is a protein of 160 kDa prominently phosphorylated upon BCR ligation (21, 39), it appears that Ag stimulation induces phosphorylation of CD22 more strongly than anti-Ig H chain Abs.

Both CD22 and CD72 are inhibitory BCR coreceptors phosphorylated upon BCR ligation, and they negatively regulate BCR signaling by recruiting SHP-1 (18, 21, 22). Treatment of K46μμλ cells expressing HA-tagged Syk (K46μμλSyk) with various concentrations of NP-BSA induced marked phosphorylation of both CD22 and CD72 and their recruitment of SHP-1 (Fig. 3). In contrast, both phosphorylation of these molecules and their association with SHP-1 were only marginal when K46μμλSyk cells were treated with polyclonal anti-Ig μ H chain Ab (Fig. 3) or anti-μ mAb B7-6 (data not shown). This indicated that BCR ligation by NP-BSA induces phosphorylation of the inhibitory BCR coreceptors CD22 and CD72 and their recruitment of SHP-1, essential for signal inhibition, much more strongly than BCR ligation by anti-μ Abs. Differential phosphorylation of CD72 was not detectable by Western blot analysis of total cell lysates (Fig. 2), probably because of less prominent phosphorylation of this protein. Weak phosphorylation of CD22 and CD72 by anti-Ig H chain Abs was not caused by weak BCR signaling generated by this treatment. Indeed, anti-μ Abs induced rather enhanced phosphorylation of cytoplasmic kinases Syk and ERK compared to treatment with

**FIGURE 2.** Tyrosine phosphorylation of cellular substrates in B cells treated with Ag or anti-μ Ab. Spleen B cells (2 × 10^9) from QM mice (A) or 1 × 10^9 K46μμλ cells (B) were stimulated with either 0.2 μg/ml NP15-BSA or 10 μg/ml F(ab')_2 of goat anti-mouse μ Ab for 3 min at 37°C. Untreated cells were used as negative controls. Total cell lysates were separated by SDS-PAGE and immunblotted with anti-phosphotyrosine mAb 4G10, and the same blot was reprobed with anti-β-tubulin mAb to ensure equal loading. A 160-kDa protein phosphorylated more strongly in Ag-treated cells is indicated by an arrow. The data are representative of at least three experiments.

**FIGURE 3.** NP-BSA, but not anti-Ig H chain Abs, induces strong phosphorylation of CD22 and CD72 and their association with SHP-1 in the NP-specific B cell line K46μμλSyk. Cells (1 × 10^9) of K46μμλSyk were treated either with 0.2 μg/ml NP15-BSA or 10 μg/ml F(ab')_2 of goat anti-mouse μ Ab for the indicated time (A), or with indicated amounts of NP15-BSA or F(ab')_2 of goat anti-mouse μ Ab for 3 min (B) at 37°C. Cell lysates were immunoprecipitated (IP) with indicated Abs. Immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine mAb 4G10. Blots were reprobed with Abs to indicated molecules to ensure equal loading. Alternatively, total cell lysates were immunoblotted with anti-phospho-ERK Ab, and the same blot was reprobed with anti-β-tubulin mAb to ensure equal loading. The data are representative of at least three experiments.
NP-BSA. Essentially the same observation was obtained by ligation of IgD-BCR on K46μmAsyk cells (data not shown). Further, efficient phosphorylation of CD22 and CD72 was induced by NP-CGG as well as NP-BSA (data not shown). Taken together, BCR ligation by NP-coupled protein Ags generates qualitatively distinct signaling from that by anti-Ig H chain Abs, in that Ags efficiently phosphorylate and activate the inhibitory coreceptors CD22 and CD72.

To ask whether NP-coupled protein Ags induce distinct signaling in primary B cells as well as K46 cells, we stimulated spleen B cells from QM mice with NP-BSA, anti-Ig μ H chain Ab, or anti-Ig δ H chain Ab. Treatment with NP-BSA induced both enhanced phosphorylation of and SHP-1 recruitment to CD22 and CD72, compared with anti-μ or anti-δ Ab (Fig. 4), although these anti-Ig H chain Abs induced stronger ERK phosphorylation than NP-BSA, excluding the possibility that weak phosphorylation of CD22 and CD72 by anti-Ig H chain Abs is caused by weak BCR signaling. Thus, the inhibitory coreceptors CD22 and CD72 are efficiently phosphorylated and activated upon BCR ligation by Ags, but not by anti-Ig H chain Abs, in primary B cells as well as the B cell line K46.

Inhibitory coreceptors are responsible for setting the requirement of CD40 signaling for survival and proliferation of Ag-stimulated spleen B cells

We asked whether activation of CD22 and CD72 alters the response of B cells stimulated with anti-Ig H chain Abs. Because coligation with BCR activates the inhibitory coreceptors CD22 and CD72 (40, 41), we cross-linked BCR by anti-μ Abs, and at the same time coligated the inhibitory coreceptors with BCR by treating B cells with both biotinylated anti-μ Abs and biotinylated mAbs to the coreceptors, followed by treatment with streptavidin (Fig. 5A). Coligation of CD22 and CD72 with BCR enhanced both their phosphorylation and SHP-1 recruitment, and reduced ERK phosphorylation, indicating that signal inhibition mediated by these coreceptors is activated by coligation with BCR in anti-μ-stimulated B cells (Fig. 5, B and C). Essentially the same results were obtained with K46μmAsyk cells (data not shown). When we cultured QM B cells under this coligation condition in the presence of CD40 signaling, B cells survived and proliferated, whereas CD40 signaling alone induced marginal proliferation of B cells (Fig. 5D). This suggests that BCR is efficiently ligated by anti-μ Abs, even in this coligation condition. However, survival and cell cycle entry of B cells were reduced in the absence of CD40 signaling, indicating that CD40 signaling becomes essential for B cell activation when these coreceptors are activated. Coligation of CD72 alone regulated B cell activation more effectively than that of CD22 alone, probably because coligation with BCR phosphorylates CD72 more efficiently than CD22 (Fig. 5, B and C). Nonetheless, coligation of CD72 blocked B cell survival and proliferation only partially (Fig. 5D), although this treatment phosphorylated CD72 as strongly as Ag stimulation did (Fig. 5B). Coligation of both CD22 and CD72 with BCR further reduced survival and cell cycle progression in the absence of CD40 signaling (Fig. 5D). Apoptosis induced by this treatment was comparable to that induced by Ag stimulation. Thus, activation of both of the coreceptors efficiently blocks survival and proliferation of anti-Ig H chain Ab-stimulated B cells in the absence of CD40 signaling, and CD40 signaling becomes essential for both survival and proliferation of B cells, as is the case for Ag-stimulated B cells.

Next, we did the converse experiment in which we inactivated inhibitory coreceptors in Ag-stimulated B cells. When we treated K46μmAsyk cells and spleen B cells of QM mice with anti-CD22 mAb, both phosphorylation of these coreceptors and their association with SHP-1 were not altered (data not shown). However, treatment with anti-CD72 mAb reduced phosphorylation of CD72 and its association with SHP-1 almost completely (Fig. 6A), indicating that this treatment silences CD72, probably because of dissociation of CD72 from BCR by anti-CD72 mAb. When we cultured QM B cells with BSA or CGG coupled with NP or its derivatives together with anti-CD72 mAb, apoptosis induced by high affinity Ags with high epitope density (such as NP15-BSA, FIGURE 4. NP-BSA, but not anti-Ig H chain Abs, induces phosphorylation of CD22 and CD72 and their association with SHP-1 in spleen B cells from QM mice. Spleen B cells from QM mice were treated either with 0.2 μg/ml NP15-BSA, 10 μg/ml F(aβ)2 of goat anti-mouse μ Ab, or 10 μg/ml F(aβ)2 of goat anti-mouse δ Ab for the indicated time (A), or with indicated amounts of NP15-BSA, F(aβ)2, of goat anti-mouse δ Ab or anti-μ Ab for 3 min (B and C) at 37°C. Cell lysates were immunoprecipitated (IP) with indicated Abs. Immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine mAb (A and B) or anti-SHP-1 Ab (C). Alternatively, total cell lysates were immunoblotted with anti-phospho-ERK Ab. The same blots were reprobed with Abs to indicated molecules to ensure equal loading. The data are representative of at least three experiments.
NP 18 -BSA, NIP 22 -BSA, NP 15 -CGG, and NNP 45 -CGG) was abrogated almost completely (Fig. 6B). Moreover, these B cells efficiently entered into the S phase even in the absence of CD40 incubated with 3 μg/ml of streptavidin at 37°C for 3 min. As a positive control of phosphorylation of CD72 and its association with SHP-1, Spleen B cells (2 × 10^5) from QM mice were incubated with 10 μg/ml anti-CD72 or isotype-matched control mAb (cont. Ab) on ice for 5 min. After washing, cells were incubated with or without 0.2 μg/ml NP 15 -BSA at 37°C for 3 min. Cells were then lysed, immunoprecipitated, and subjected to Western blot analysis as in Fig. 5. The data are representative of at least three experiments.

FIGURE 5. Activation of the inhibitory coreceptors CD22 and CD72 installs requirement of CD40 signaling for survival and cell cycle entry in anti-μ-treated spleen B cells. A, Coligation of CD22 and CD72 with BCR using biotin-labeled Abs. B and C, Activation of CD22 and CD72 by coligation with BCR. Spleen B cells (2 × 10^5) from QM mice were incubated with 10 μg/ml biotin-conjugated anti-mouse CD72 mAb (B), anti-CD22 mAb (C), or isotype-matched control Abs together with biotin-conjugated F(ab')2 of goat anti-mouse αAb on ice for 5 min. After washing, cells were incubated with 10 μg/ml anti-CD72 or isotype-matched control mAb (cont. Ab) on ice for 5 min. After washing, cells were then lysed, immunoprecipitated, and subjected to Western blot analysis as in Fig. 5. The data are representative of at least three experiments.

FIGURE 6. Treatment with anti-CD72 mAb silences CD72 and induces survival and cell cycle progression in Ag-stimulated spleen B cells. A, Phosphorylation of CD72 and its association with SHP-1. Spleen B cells (2 × 10^5) from QM mice were incubated with 10 μg/ml anti-CD72 or isotype-matched control mAb (cont. Ab) on ice for 5 min. After washing, cells were incubated with or without 0.2 μg/ml NP 15 -BSA at 37°C for 3 min. Cells were then lysed, immunoprecipitated, and subjected to Western blot analysis as in Fig. 5. The data are representative of at least three experiments. B, Survival and cell cycle entry of Ag-stimulated spleen B cells. Purified B cells from QM mice were cultured with indicated amounts of Ags or F(ab')2 of goat anti-mouse αAb in the presence or absence of 10 μg/ml anti-CD72 mAb. Cell death, apoptosis, and cell cycle progression were examined as in Fig. 1. The data are representative of at least three experiments.

NP 18 -BSA, NIP 22 -BSA, NP 15 -CGG, and NNP 45 -CGG) was abrogated almost completely (Fig. 6B). Moreover, these B cells efficiently entered into the S phase even in the absence of CD40 incubated with 3 μg/ml of streptavidin at 37°C for 3 min. As a positive control of phosphorylation of CD72 and CD72 and their SHP-1 recruitment, cells were treated with 0.2 μg/ml NP 15 -BSA in parallel. Cells were then lysed, immunoprecipitated, and subjected to Western blot analysis as in Fig. 5. The data are representative of at least three experiments. D, Survival and cell cycle entry of anti-Ig-treated spleen B cells. B cells from QM mice were cultured with indicated Abs at the concentration of 10 μg/ml in the presence or absence of 3 μg/ml of streptavidin. Cell death, apoptosis, and cell cycle progression were examined as in Fig. 1. The data are representative of at least three experiments.
were treated with 0.2 µg/ml of phosphorylation of CD22 and CD72 and their SHP-1 recruitment, cells from QM mice were cultured with 1 µg/ml NP 15 -BSA or indicated Abs at the concentration of 10 µg/ml NP 15 -BSA or indicated Abs. Cells were then lysed, immunoprecipitated, and subjected to Western blot analysis as in Fig. 5. The data are representative of at least three experiments.

**FIGURE 7.** Anti-Ig L chain Ab and anti-Id Ab partially activate inhibitory coreceptors. A. Phosphorylation of CD22 and CD72 and their association with SHP-1. Spleen B cells from QM mice were treated either with 10 µg/ml anti-Id mAb or with the same amount of anti-Ig L chain (anti-µ) Ab for 3 min at 37°C. As positive and negative controls of phosphorylation of CD22 and CD72 and their SHP-1 recruitment, cells were treated with 0.2 µg/ml NP 15 -BSA or 10 µg/ml F(ab')2 of goat anti-mouse µ Ab, respectively. Cells were then lysed, immunoprecipitated, and subjected to Western blot analysis as in Fig. 5. The data are representative of at least three experiments. B. Survival and cell cycle progression of QM B cells stimulated with anti-Id Ab or anti-µ Ab. Purified spleen B cells from QM mice were cultured with 1 µg/ml NP 15 -BSA or indicated Abs at the concentration of 10 µg/ml. Cell death, apoptosis, and cell cycle progression were examined as in Fig. 1. The data are representative of at least three experiments.

Signaling, as is the case for B cells stimulated with anti-Ig H chain Abs. These results indicated that inactivation of the inhibitory coreceptor CD72 alone abrogates the requirement of CD40 signaling for survival and cell cycle progression in Ag-stimulated B cells, supporting the notion that activation of both CD22 and CD72 is essential for setting a requirement of CD40 signaling for survival and proliferation of Ag-stimulated B cells. Taken together, activation or inactivation of these inhibitory coreceptors plays a crucial role in the distinct responses of B cells to Ags and B cells to anti-Ig H chain Abs, respectively.

**Anti-Id Ab and anti-Ig L chain Ab partially activate inhibitory coreceptors**

Because most of the B cells in QM mice express anti-NP Ig containing V_H 17.2.25 and Ig λ L chain (30), we next stimulated QM B cells with anti-µ Ab or anti-Id Ab that recognizes V_H 17.2.25 together with Ig L chain. Treatment with anti-µ Ab phosphorylated CD72 but not CD22, whereas anti-Id Ab induced phosphorylation of CD22 alone (Fig. 7A). SHP-1 was recruited only to the phosphorylated coreceptors. Essentially the same results were obtained when we stimulated K46µmASyK cells with anti-Ig L chain Ab or anti-Id Ab (data not shown). These results indicated that anti-Ig Ab that recognizes the membrane-distal part of Ig activate inhibitory coreceptors only partially. Treatment with anti-µ or anti-Id Ab induced survival and cell cycle progression of QM B cells only weakly compared with that of anti-µ Ab (Fig. 7B), probably because of partial activation of inhibitory coreceptors. This notion was supported by the finding that anti-µ Ab induced survival and cell cycle progression of QM B cells as efficiently as anti-µ Ab when CD72 was inactivated by anti-CD72 Ab (Fig. 7B). Taken together, BCR ligation by Ab to the membrane-distal part of Ig weakly activates B cells because of partial activation of inhibitory coreceptors.

**Discussion**

Using NP-specific spleen B cells from QM mice, we demonstrate in this study that B cells proliferate upon BCR ligation by anti-Ig H chain Abs, but not by any of various forms of Ags tested. Whereas Ags with high affinity and high epitope density induce phosphorylation of cellular substrates as much as anti-Ig H chain Abs, these Ags induce B cell proliferation only in the presence of CD40 ligation. This result is in agreement with the in vivo findings by Jenkins and colleagues (5) that Ag-induced B cell proliferation requires CD40 signaling. We further demonstrate that the inhibitory coreceptors CD22 and CD72 are efficiently activated upon BCR ligation by Ags, but not by anti-Ig H chain Abs. Activation of both of the inhibitory coreceptors CD22 and CD72 efficiently prevents survival and proliferation of anti-Ig H chain Ab-stimulated B cells in the absence, but not in the presence, of CD40 signaling. Activation of either one of the coreceptors shows partial prevention. Thus, both CD22 and CD72 are essential for installing requirement of CD40 signaling for survival and proliferation in BCR-ligated B cells. This is further supported by the finding that inactivation of CD72 alone can induce survival and proliferation of Ag-stimulated B cells, even in the absence of CD40 signaling, although CD22 cannot be inactivated using anti-CD22 Ab. Taken together, Ag stimulation, but not treatment with anti-Ig H chain Abs, efficiently activates the inhibitory coreceptors CD22 and CD72, thereby installing requirement of CD40 signaling for B cell survival and proliferation, probably because Ag-induced BCR signaling is not sufficient to activate B cells in the presence of coreceptor-mediated signal inhibition.

It has been proposed that the inhibitory coreceptors CD22 and CD72 set BCR signal threshold by negatively regulating BCR signaling (26–29). Enhanced B cell response in mice deficient in either CD22 or CD72 (42–46) has been attributed to the decrease in signaling threshold in the absence of these molecules. In this study we demonstrate that Ags with low affinity or low epitope density fail to induce B cell proliferation, even in the presence of CD40 signaling, suggesting the presence of a BCR signal threshold. However, abrogation of the coreceptor-mediated signal inhibition by mAbs to the coreceptors does not generate a response to
Ags with low affinity or low epitope density (Fig. 6B), although closer examination might reveal a modest decrease in the signaling threshold. Thus, CD22 and CD72 do not appear to strongly regulate signaling threshold. Enhanced B cell activation in mice deficient in CD22 or CD72 may be caused by a loss of requirement of CD40 signaling for B cell activation in these mice, although a modest decrease in signaling threshold for B cell activation might also be involved.

Both CD22 and CD72 have been reported to possess functions other than inhibition of BCR signaling (42, 47, 48). CD22-deficient B cells have a shortened life span in vivo and enhanced apoptosis in vitro, even in the absence of BCR ligation (43), suggesting that CD22 regulates homeostasis of B cells by blocking “spontaneous” apoptosis. Because the number of mature B cells is reduced in CD72-deficient mice (46), spontaneous apoptosis may be enhanced in CD72-deficient B cells as well as CD22-deficient B cells. Such enhanced spontaneous apoptosis of B cells makes it impossible to assess BCR ligation-induced apoptosis or proliferation in B cells deficient in CD22 or CD72. In contrast, regulation of BCR signaling by CD22 and CD72 is enhanced or inhibited using mAbs to these coreceptors without modulating spontaneous apoptosis of B cells (Figs. 5D and 6B). Thus, the roles of BCR coreceptors in apoptosis and proliferation of BCR-ligated B cells can be assessed using mAbs to CD22 and CD72, but not by using B cells deficient in these molecules. Indeed, we inactivated and activated CD72 by treatment with anti-CD72 mAb and coligation of CD72 with BCR using anti-CD72 mAb, respectively, and demonstrated that inactivation and activation of CD72 modulate both apoptosis and proliferation of B cells. However, anti-CD22 mAb modulated CD22 activation less efficiently, and we could not inactivate CD22 using this Ab. To ask whether CD22 inactivation modulates B cell fate, further studies are required to establish a system in which CD22-mediated regulation on BCR signaling is completely abrogated without modulating spontaneous apoptosis.

CD22, a member of the siglec family, contains a lectin domain in the extracellular portion and specifically recognizes α,2,6 sialic acid (49). CD22 is suggested to interact with sialic acid residues on molecules, including BCR, in the same membrane (50). This interaction appears to be essential for activation of CD22-mediated signal inhibition, because BCR-induced CD22 phosphorylation and SHP-1 recruitment are inhibited when recognition of α,2,6 sialic acid is abrogated by mutation of the lectin domain or use of a specific inhibitor (51, 52). In this study we demonstrate that CD22 and CD72 are marked by Ags, but only marginally by anti-Ig H chain Abs. Anti-Ig H chain Abs, but not Ags, bind to BCR at the membrane-proximal region of BCR, including the Fe portion of Ig, and may perturb interaction between BCR and those associating with BCR extracellularly, such as CD22. Because CD72 contains a lectin-like domain in the extracellular part (53), CD72 may also interact with glycans on the extracellular part of the BCR complex, and this interaction may be blocked by anti-Ig H chain Abs. Similarly, treatment with anti-CD72 Ab may block interaction between CD72 and BCR, leading to inactivation of CD72. The notion that the inhibitory coreceptors interact with the membrane-proximal part of BCR is supported by the finding that Abs binding to the membrane-distal part of Ig, such as anti- Ig L chain Ab and anti-Id Ab, silence the coreceptors only partially (Fig. 7). By blocking the interaction of the inhibitory coreceptors with the membrane-proximal part of BCR, anti-Ig H chain Abs ligate BCR without activating the inhibitory coreceptors.

Anti-Ig H chain Abs have been proposed to be a model of T cell-independent type 2 (TI-2) Ags such as Ficoll, bacterial flagellin, and dsRNA because of their ability to activate B cells by BCR ligation alone (6). However, recent findings suggest that TI-2 Ags activate B cells by BCR ligation together with T cell-independent costimulatory signals. B cell response to Ficoll requires transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) (54), a member of the TNF receptor family recognizing B cell activating factor belonging to the TNF family (also known as B lymphocyte stimulator), expressed on both non-T cells as well as activated T cells (55), suggesting that signaling via TACI is generated when B cells are activated by interaction with Ficoll. Bacterial flagellin and double-stranded RNA activate B cells through Toll-like receptor (TLR)5 and TLR3, respectively, together with ligation of specific BCR (56, 57). As is the case for CD40L, ligands for TACI and various TLRs block BCR-mediated apoptosis and activate B cells (58–61), although it is not yet proven that such a signal is transmitted by these particular receptors. These findings suggest that B cells stimulated by TI-2 Ags are activated in the presence of costimulatory signals through TACI or TLRs together with BCR ligation. Thus, TI-2 Ag-induced B cell activation may be distinct from that by anti-Ig H chain Abs, which activate B cells by suppressing signal inhibition by CD22 and CD72. Both CD22 and CD72 have endogenous ligands that appear to reverse their signal inhibition (62, 63). B cells that interact with Ags together with these ligands may be activated even in the absence of costimulatory signals through CD40, TACI, or TLRs. B cell activation in the presence of the ligands for inhibitory coreceptors might be involved in yet unknown immune responses, and B cell activation by anti-Ig H chain Abs provides a model for such B cell responses.

In summary, in this study we demonstrate that Ag-induced B cell activation in vitro requires a costimulatory signal such as that through CD40. This is inconsistent with the in vivo finding (5), and thus our experimental system may provide a useful tool to analyze mechanisms for Ag-induced activation and proliferation of B cells. Moreover, we demonstrate that the inhibitory coreceptors CD22 and CD72 install a requirement of CD40 signaling for B cell survival and activation. These inhibitory coreceptors thus appear to be involved in determining the fate of Ag-stimulated B cells, i.e., apoptotic or proliferation, and may be good target molecules in immune manipulation. Further studies on signaling functions and immunological roles of these molecules are required for developing novel strategies to control immune responses by manipulating these inhibitory coreceptors.

Acknowledgments

We thank Drs. M. Reth and J. Wienands for cells; Dr. H. Ohnori (Okayama University, Okayama, Japan) for mice; Drs. H. Yamamura, T. Takemori, T. Azuma, H. Bazin, H. Yagita, H. Yakura, P. R. Crocker, L. Nitschke, T. Imanishi-Kari, K. Rajewsky, and J. Wienands for reagents; Dr. Y. Yamanashi (Tokyo Medical and Dental University, Tokyo, Japan) for mice; Drs. H. Ohmori for critical reading of the manuscript; and S. Irie and K. Mizuno for technical help.

References


