B Cell Antigen Receptor and CD40 Differentially Regulate CD22 Tyrosine Phosphorylation

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Cell surface molecules on lymphocytes positively or negatively modulate the Ag receptor signaling, and thus regulate the fate of the cell. CD22 is a B cell-specific cell surface protein that contains multiple ITIMs in the cytoplasmic tail, and critically regulates B cell activation and survival. CD22 regulation on B cell signaling is complex because CD22 can have both positive and negative roles in various contexts. We generated phosphospecific polyclonal Abs reacting four major CD22 tyrosine motifs (Y762, Y807, Y822, and Y842) and analyzed the pattern and intensity of phosphorylation of these tyrosine residues. The tyrosine motifs, Y762, Y807, Y822, and Y842, are considered as ITIM, whereas the other, Y807, is suggested to be important for Grb2 recruitment. Approximately 10% of the four tyrosine residues were constitutively phosphorylated. Upon anti-IgM ligation, CD22 Y762 underwent most rapid phosphorylation, whereas all four tyrosine residues were eventually phosphorylated equally at ~35% of all CD22 molecules in the cell. By contrast, anti-CD40 stimulation specifically up-regulated anti-IgM-induced phosphorylation of tyrosines within two ITIM motifs, Y762 and Y842, which was consistent with in vivo finding of the negative role of CD22 in CD40 signaling. Thus, CD22 phosphorylation is not only quantitatively but also qualitatively regulated by different stimulations, which may determine the outcome of B cell signaling. The Journal of Immunology, 2006, 176: 873–879.

Lymphocytes respond to extracellular microenvironment for development, activation, proliferation, differentiation, and survival. BCR signaling is critical in determining B cell fate (1, 2). BCR signals are amplified, fine-tuned, or dampened by multiple other cell surface signaling molecules that provide essential links between the cell surface and intracellular signaling (3, 4). These molecules include CD22, a B cell-specific lectin-like member of the Ig superfamily that binds sialylated cell surface and soluble ligands (5–8). Although ligand binding with CD22 has important roles, CD22 also regulates B cell signaling independent of ligand engagement (5–10). Thus, CD22 serves as a “response regulator” to modulate multiple aspects of B cell functions.

Although CD22 does not have intrinsic enzymatic activity, CD22 has six highly conserved tyrosine residues within the ~140 aa cytoplasmic domain (11). Several of these tyrosine residues are contained within consensus ITIM sequences. After BCR ligation, tyrosine residues of CD22 are targeted for rapid phosphorylation (12), which in turn recruit signaling molecules that contain Src homology 2 (SH2)3 domains. Best appreciated is a potent protein tyrosine phosphatase SH2-containing protein-tyrosine phosphatase (SHP)-1 that binds to ITIM motifs within CD22 (13). Furthermore, CD22 has also been shown to recruit other signaling molecules including Lyn, Syk, Grb2, Shc, SHIP, PI3K, and phospholipase Cγ (14–17). Thus, how these molecules are recruited determines the net effect of CD22 signaling in each context. Therefore, assessing which tyrosine residues are phosphorylated in each situation is critical to clarify CD22 functions.

The importance of CD22 in B cell signal transduction in vivo has been demonstrated by CD22-deficient (CD22−/−) mice (18–21). Importantly, CD22 functions appear complex because it has both negative and positive roles depending on circumstances. CD22−/− B cells have a “hyperresponsive” phenotype characterized by decreased expression of surface IgM and increased expression of MHC class II. BCR ligation leads to exaggerated intracellular calcium concentration ([Ca2+]i) responses. These suggest negative regulatory roles of CD22. Despite this, CD22−/− B cells are hypoproliferative to BCR-induced stimulation. In addition, naive B cell apoptosis is enhanced in CD22−/− mice, resulting in reduced circulating B cell numbers by ~66% in CD22−/− mice. Remarkably, CD40 stimulation rescues CD22−/− B cell apoptosis and results in enhanced BCR-induced proliferation of CD22−/− B cells (22).

These complex CD22 roles may be caused by differential regulation of multiple tyrosines on its cytoplasmic domain. However, a detailed phosphorylation pattern of each tyrosine residue has not been investigated. In this study, we generated phosphospecific Abs against four major tyrosines and examined their phosphorylation status upon BCR and CD40 stimulations.
Materials and Methods

Animals and cells
C57BL/6 mice were purchased from Clea Japan. CD22−/− mice were generated as described previously (18) and backcrossed eight times on a C57BL/6 background. Mice were housed in a specific pathogen-free barrier facility. All procedures were approved by the institutional review board. Human B lymphoblastoid cell lines, BJAB and RAMOS cells, were cultured in RPMI 1640 medium supplemented with 10% FCS and 2-ME.

Generation of phosphospecific Abs
Phosphospecific polyclonal Abs to four CD22 tyrosines (Y762, Y807, Y822, and Y842) were generated as follows. Tyrosine-phosphorylated and unphosphorylated peptides corresponding to mouse CD22 amino acid sequences Y762 (aa 757–767, DDTVSYAILRF), Y807 (aa 802–812, RPMGQDYENVNPN), Y822 (aa 817–827, DESIHYSHELVQ), and Y842 (aa 837–847, KEDVDYVTLKH) were synthesized. Rabbits were immunized five times with the keyhole limpet hemocyanin-phosphopeptide conjugates mixed with Freund’s complete adjuvant, and were bled 7 days after the last immunization. After IgG fraction was collected from the immunized sera using a protein A-Sepharose column, phosphopeptide-reactive Abs were captured by a column containing phosphopeptide-conjugated Sepharose. Abs were then eluted, and those reactive to sequences other than phospho-tyrosines were removed using a column containing unphosphorylated peptides. To minimize nonspecific reactions, columns containing phosphopeptides of the other three motifs (for example, pY807, pY822, and pY842 peptides against pY762 Abs) were also used to deplete Abs that showed nonspecific binding to other phosphotyrosines. Specific reactivity with the targeted phosphotyrosine sequences was confirmed by ELISAs in which phosphorylated and unphosphorylated peptides were coated.

Reagents
Antisera used in this study included the following: anti-ERK2 (Santa Cruz Biotechnology) and anti-Vav (Upstate Biotechnology). mAbs used in this study included the following: anti-Lyn (Wako Pure Chemicals), anti-B220 (RA3-6B2; eBioscience), and anti-CD22 (Cy34 (BD Pharmingen); MB22-1 (Ref. 15)). Piceatannol and 4-amino-5-(4-chlorophenyl)-7-(4-butyryl)pyrazolo[3,4-d]pyrimidine (FP2) (Calbiochem) were used to inhibit activities of Syk and Src-family protein tyrosine kinases.

Dot blot analysis
Phosphopeptides and non-phosphopeptides were spotted onto a nitrocellulose membrane in two concentrations (5 μg/ml and 0.5 μg/ml). The membrane was probed by the four anti-phospho-CD22 Abs, followed by incubation with HRP-conjugated anti-rabbit IgG Ab (Jackson ImmunoResearch Laboratories). The membranes were developed using an ECL kit (Pierce).

B cell activation, immunoprecipitations, and Western blot analysis
Splenic B cells were purified by removing T cells with anti-Thy1.2 Ab-coated magnetic beads (Dynabeads). Splenic B cells were stimulated with goat anti-mouse IgM Ab F(ab′)2 fragments (Cappel) or together with anti-CD40 Ab (1C10; eBioscience; 10 μg/ml) at 37°C in RPMI 1640 as described elsewhere (23). BJAB and Ramos cells were stimulated with anti-human IgM Ab F(ab′)2 fragments (Cappel). The cells were subsequently lysed in buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM Na orthovanadate, 2 mM EDTA, 50 mM NaF, and protease inhibitors. The lysates were either analyzed by SDS-PAGE or subjected to immunoprecipitation. Immunoprecipitations were performed by incubating the cell lysates with protein G-Sepharose beads (Amersham Pharmacia Biotech) plus anti-mouse CD22 Ab (Cy34) for 3 h at 4°C. To achieve full phosphorylation of CD22, immunoprecipitants were incubated with active Lyn (Upstate Biotechnology) and ATP in the presence of phosphatase inhibitors. The lysates were either analyzed by SDS-PAGE or subjected to immunoprecipitation. Immunoprecipitations were performed by incubating the cell lysates with protein G-Sepharose beads (Amersham Pharmacia Biotech) plus anti-mouse CD22 Ab (Cy34) for 3 h at 4°C. To achieve full phosphorylation of CD22, immunoprecipitants were incubated with active Lyn (Upstate Biotechnology) and ATP in the presence of phosphatase inhibitors for 45 min at 30°C. Samples were separated by SDS-PAGE, and electrotransferred to nitrocellulose membranes. These membranes were incubated with anti-phospho-CD22 Abs or other primary Abs, followed by incubation with HRP-conjugated anti-rabbit or anti-rat IgG Abs (Jackson ImmunoResearch Laboratories). The membranes were developed using an ECL kit (Pierce). To verify equivalent amounts of protein in each lane, the blots were probed with Abs against the proteins of interest. Band intensity was quantified using Quantity One software (Bio-Rad).

Preparation of lipid raft fraction
After cells were incubated with anti-IgM Ab for 3 min, 1 × 10⁶ cells per sample were lysed for 30 min on ice in 0.05% Triton X-100 in TNEV buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA; 1 mM Na orthovanadate, and protease inhibitors). Nuclei and cellular debris were pelleted by centrifugation at 900 × g for 10 min, and 1 ml of cleared supernatant was mixed with 1 ml of 35% sucrose in a 14 × 89-mm centrifuge tube (Beckman Coulter). The sample was overlaid with 6 ml of 35% sucrose and 3.5 ml of 5% sucrose in TNEV buffer. The samples were centrifuged in a SW41 rotor at 200,000 × g for 16–20 h at 4°C, and 1-ml fractions were collected from the top of the gradient (fractions 1–12) for subsequent SDS-PAGE. Fractions 4–5 and 10–12 represented the raft-containing fractions and the soluble fractions, respectively. Isolation of lipid raft membranes was confirmed by immunoblotting for Lyn, and CD45R.

Statistical analysis
All data are shown as mean values ± SEM. Comparisons between groups were made using the Student t test.

Results

Generation of phosphospecific Abs to four tyrosines of CD22
There are six tyrosine residues on CD22 cytoplasmic domain (Fig. 1A). Among them, three tyrosine motifs (Y762, Y822, and Y842) are considered as ITIM. Another (Y807) is suggested to be important for Grb2 recruitment (15, 24, 25). The functional relevance of the other two (Y752 and Y796) remains less characterized. Therefore, to assess phosphorylation of individual CD22 tyrosine residues, polyclonal phosphospecific Abs against these four major tyrosine motifs (Y762, Y807, Y822, and Y842) were generated. Dot blot analyses using phosphorylated and unphosphorylated peptides demonstrated that each Ab showed strong reaction to phosphorylated forms of the corresponding tyrosine motif, but showed minimal reaction, if any, to unphosphorylated forms or other phosphorylated or unphosphorylated motifs (Fig. 1B). Additionally, by ELISA, each Ab reacted specifically with the corresponding phosphopeptidet but not with non-phosphopeptide in a concentration-dependent manner (data not shown). Therefore, these Abs specifically reacted with corresponding phosphorylated motifs, and appeared useful for detecting phosphorylation of individual tyrosine residues of CD22.

Phosphorylation kinetics of each tyrosine residues of CD22 after anti-IgM ligation
IgM ligation induces rapid and intense CD22 tyrosine phosphorylation. Therefore, we first examined whether all of the four tyrosine residues were phosphorylated by IgM ligation. Splenic B cells from wild-type and CD22−/− mice were stimulated with F(ab′)2 anti-IgM Abs, and whole cell lysates were blotted by phosphospecific Abs. All of the four tyrosine residues underwent intense phosphorylation by 3- to 3.5-fold of basal phosphorylation following BCR ligation (Fig. 2A). The Abs also cross-reacted with several other proteins, although CD22−/− B cells showed no phosphorylated proteins around 140 kDa, and thus the band in each blot was considered to represent CD22 phosphorylation. Y807, Y822, and Y842 showed similar kinetics with maximal phosphorylation at 3 min, whereas Y762 were phosphorylated slightly faster than the other three (Fig. 2, A and B).

Tyrosine phosphorylation of the four residues was also examined in human B cell lines, BJAB and RAMOS. Similar to mouse splenic B cells, all of the four tyrosines were phosphorylated following anti-IgM ligation in the both human cell lines (Fig. 2C and data not shown). Therefore, BCR ligation induced phosphorylation of all four major tyrosines within CD22.

Phosphorylated CD22 localizes outside of lipid rafts following BCR ligation
Detergent-insoluble cholesterol- and sphingolipid-rich microdomains of the plasma membrane are called “lipid rafts,” which have
been considered to play critical roles in mediating signal transduction (26). Lyn constitutively localizes to the rafts, whereas SHP-1 is excluded from the lipid rafts. CD22 has been reported to be mainly outside the rafts (27), whereas the localization of its phosphorylated form remains unknown. We assessed whether phosphorylation affects the localization of CD22. After BCR ligation, cells were lysed and subjected to separation. CD22 phosphorylation was detected only in soluble fractions and not in detergent-insoluble fractions (Fig. 3). Thus, the result confirmed that CD22 is excluded from the lipid rafts whether it is phosphorylated or unphosphorylated.

Anti-IgM-induced phosphorylation of the four tyrosines on CD22 is dependent on Src-family tyrosine kinases

Lyn is considered to be responsible for CD22 phosphorylation (28–31), although whether each CD22 tyrosine is directly phosphorylated by Lyn is unknown. Therefore, we examined whether Syk and Src-family tyrosine kinase inhibitors affected intrinsic and BCR-induced phosphorylation status of each tyrosine residue within CD22. Mouse splenic B cells were pretreated with optimal concentrations of piceatannol or PP2 for 20 min before anti-IgM engagement. These pretreatment completely abrogated Syk or Lyn kinase activity assessed by in vitro kinase assays. Syk inhibitor piceatannol did not inhibit BCR-induced phosphorylation of any tyrosine residues, whereas PP2 pretreatment resulted in nearly complete blockade of all four tyrosine phosphorylations (Fig. 4). Additionally, PP2-treated B cells showed lower basal phosphorylation, compared with B cells treated with DMSO alone, whereas piceatannol pretreatment resulted in slightly higher basal phosphorylation of all tyrosines relative to pretreatment with DMSO alone or PP2. Pretreatment with both PP2 and piceatannol resulted in inhibition levels similar to the pretreatment with PP2 alone (data not shown). As a negative control, PI3K inhibitor LY294002 did not affect basal or BCR-induced tyrosine phosphorylation of CD22 (data not shown). Thus, Src-family tyrosine kinases, presumably Lyn, are likely to phosphorylate all four tyrosine residues directly, whereas Syk activity may also affect basal phosphorylation of CD22.

Tyrosine phosphorylation intensity of the four residues of CD22

BCR engagement induced phosphorylation of all four tyrosines (Fig. 2), whereas the relative intensity of each tyrosine phosphorylation remained unknown because the differences were not directly comparable with different Abs used. To solve this, we estimated tyrosine phosphorylation percentage as a ratio in comparison to fully phosphorylated status for each anti-phosphotyrosine Ab. After CD22 immunoprecipitation, active Lyn and ATP were added to phosphorylate remaining unphosphorylated sites of CD22. After ~30 min, the tyrosine phosphorylation intensity of all the residues in this sample reached plateau (data not shown), indicating that nearly all CD22 was phosphorylated. Then, based on the assumption that CD22 was phosphorylated at 100% in these samples, the relative intensity of CD22 phosphorylation in unstimulated or anti-IgM-stimulated samples was estimated. In unstimulated samples ~10% of tyrosine residues were phosphorylated, whereas optimal BCR ligation increased the phosphorylation ratio to ~35% (Fig. 5, A and B). Furthermore, when B cells were stimulated with various concentrations of anti-CD40 Abs, the phosphorylation intensity of four tyrosines decreased similarly in parallel with decreasing concentrations (Fig. 5C). Collectively, despite the slight kinetic difference, all of these four tyrosines undergo almost equal phosphorylation upon BCR engagement.

Anti-CD40 costimulation selectively increases BCR-induced phosphorylation of two ITIM motifs

Because CD40 engagement remarkably enhances BCR-induced proliferation in CD22−/− B cells, tyrosine phosphorylation following anti-CD40 ligation was examined. However, anti-CD40 stimulation alone did not result in significant increase of phosphorylation in any sites (data not shown). Then, B cells were simultaneously stimulated with anti-IgM and anti-CD40 Abs. Remarkably, anti-CD40 ligation increased Y762 and Y842 phosphorylation by 2-fold, but did not alter Y807 or Y822 phosphorylation significantly (Fig. 6, A and B). The same result was obtained using a different anti-CD40 mAb (clone, HM40-3; data...
not shown). Lyn appeared to be also responsible for this up-regulation, because PP2 pretreatment abrogated them, whereas piccateannol did not (Fig. 6B). Therefore, CD40 engagement selectively enhanced Y762 and Y842 phosphorylation by Lyn.

**Discussion**

Although tyrosine phosphorylation is essentially required for CD22 function, each tyrosine’s role is still controversial. The current study focused on phosphorylation mechanism of four major tyrosine residues within CD22 cytoplasmic domain during B cell activation, whereas the other two tyrosines (Y752 and Y796) may also have some roles. All of the four tyrosine residues examined were equally phosphorylated following BCR ligation over a range of dose (Figs. 2 and 4), suggesting that these tyrosine residues are functionally relevant in BCR-mediated signal transduction. The four tyrosine residues were constitutively phosphorylated at \( \approx 10\% \) of all CD22 molecules in the cell (Fig. 5). Upon anti-IgM ligation, CD22 Y762 was phosphorylated most quickly, although all of the four tyrosine residues eventually reached phosphorylation at \( \approx 35\% \) of total CD22 molecules (Fig. 5). By contrast, whereas anti-CD40 stimulation alone did not increase any of CD22 tyrosine phosphorylation significantly, simultaneous stimulation of
anti-IgM and anti-CD40 Abs specifically up-regulated phosphorylation of tyrosines within two ITIM motifs, Y762 and Y842, compared with anti-IgM ligation alone (Fig. 6). The selectively increased phosphorylation of ITIM motifs may support in vivo finding of negative regulation by CD22 in CD40 signaling. Collectively, CD22 phosphorylation is not only quantitatively but also qualitatively regulated by different stimulations, which may determine the outcome of B cell signaling.

Although loss of Lyn expression results in nearly complete abrogation of CD22 phosphorylation (28–31), mechanisms for tyrosine phosphorylation on individual residues have remained unclear. In Lyn-deficient and Syk-deficient DT40 cells, total CD22 phosphorylation is decreased by 70 and 25%, respectively (25), which may suggest that individual CD22 tyrosines are differentially phosphorylated by either Lyn or Syk. Alternatively, all tyrosine residues are phosphorylated by Lyn, although Syk may be required for optimal phosphorylation. The results presented in this study supported the latter; all of the four tyrosines underwent a similar pattern of phosphorylation (Fig. 4), suggesting that Src-family protein tyrosine kinases, presumably Lyn, are the dominant kinases that directly phosphorylate all of the four tyrosines. Interestingly, Src-family inhibitor lowered the basal phosphorylation levels of all the CD22 tyrosines (Fig. 4), suggesting that Lyn is also required for maintaining intrinsic phosphorylation of CD22. By contrast, Syk inhibitor increased the basal phosphorylation levels of CD22 tyrosines (Fig. 4). Therefore, Syk is likely to play an inhibitory role for constitutive phosphorylation of CD22. Syk may be critical for activation of SHP-1, which in turn dephosphorylates CD22 tyrosines. Thus, SHP-1 has double-edged roles because it acts as an effector that executes negative regulation when recruited to CD22 and also limits negative regulation by dephosphorylating CD22. Collectively, Src-family tyrosine kinases and Syk reciprocally regulate basal CD22 phosphorylation levels. Phosphorylation of CD22 tyrosines induced by anti-IgM stimulation was slightly present when Src-family protein tyrosine kinase activity was inhibited, which may support the finding that Syk is also required for optimal phosphorylation.

Y762 showed slightly faster kinetics in phosphorylation when compared with the other three (Fig. 2). Constitutive phosphorylation of Y762 was also slightly higher. These may indicate that Y762 is the initial phosphorylation site on CD22. SHP-1 recruitment has been shown to require Y822 and Y842 by in vitro experiments that substituted tyrosine(s) with phenylalanine(s),
Therefore, Y762 may not be necessary for SHP-1 recruitment but may be required for the optimal phosphorylation of the other tyrosines.

Remarkably, CD40 specifically regulates two CD22 tyrosines that are contained in ITIM motifs, Y762 and Y842 (Fig. 6). This may be consistent with the finding that B cells from CD22+/− mice exhibit augmented proliferation induced by anti-CD40 ligation or anti-CD20 and anti-IgM coligation. These two motifs are considered to recruit SHP-1. Thus, enhancing phosphorylation of these two sites can result in enhancing negative regulation by CD22/SHP-1. Alternatively, because Y762 has been previously shown to interact with SHIP directly (32), and is also important to form the Shc/Grb2/SHIP complex (15), augmented phosphorylation of the two tyrosines may contribute to enhancing negative regulation via SHIP. Collectively, simultaneous stimulation of anti-IgM and anti-CD40 Abs may lead to dominant signaling using the two ITIMs, Y762 and Y842, which may suppress or dampen the net effect of the signaling using the other two tyrosines, Y807 and Y822. This may also explain apparently opposite outcomes between BCR ligation alone and simultaneous ligation of BCR and CD40. Lyn is also responsible for the augmentation of these two tyrosines phosphorylation. The mechanism of how Lyn differentially regulates tyrosine phosphorylation for different stimulation remains unknown. Nonetheless, CD40 ligation induces Lyn activation (33). Therefore, difference in membrane localization or signaling intensity may result in qualitative difference of CD22 phosphorylation.

In summary, the current study demonstrates that BCR and CD40 differentially regulate CD22 tyrosine phosphorylation. CD22 plays an important role in B cell apoptosis and activation. Defects in CD22 or its pathways are likely to contribute to autoimmunity. CD22+/− mice have increased autoantibody production with age, including anti-DNA, anti-cardiolipin, and anti-myeloperoxidase Abs (34). Also, a recent study has revealed that the loss of CD22 inhibitory regulation is a hallmark of IgG signaling when compared with IgM signaling (35). Thus, precise understanding of CD22 phosphorylation and function will be important in modulating B cell signaling pathways since CD22 is a potential therapeutic target in B cell-mediated diseases.

Disclosures
The authors have no financial conflict of interest.

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