Novel Binding Site for Src Homology 2-containing Protein-tyrosine Phosphatase-1 in CD22 Activated by B Lymphocyte Stimulation with Antigen*§

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CD22, a B lymphocyte membrane glycoprotein, contains immunoreceptor tyrosine-based inhibition motifs (ITIMs) in the cytoplasmic region and recruits Src homology 2-containing protein-tyrosine phosphatase-1 (SHP-1) to the phosphorylated ITIMs upon ligation of B lymphocyte antigen receptor (BCR), thereby negatively regulating BCR signaling. Among the three previously identified ITIMs, both ITIMs containing tyrosine residues at position 843 (Tyr843) and 863 (Tyr863), respectively, are shown to be required for CD22 to recruit SHP-1 and regulate BCR signaling upon BCR ligation with anti-Ig antibody (Ab), indicating that CD22 has the SHP-1-binding domain at the region containing Tyr843 and Tyr863. Here we address the requirement of CD22 for SHP-1 recruitment and BCR regulation upon BCR ligation by antigen, which induces much stronger CD22 phosphorylation than anti-Ig Ab does. We demonstrate that the CD22 mutant in which both Tyr843 and Tyr863 are replaced by phenylalanine (CD22F5/6) recruits SHP-1 and regulates BCR signaling upon stimulation with antigen but not anti-Ig Ab. This result strongly suggests that CD22 contains another SHP-1 binding domain that is specifically activated upon stimulation with antigen. Both of the flanking sequences of Tyr783 and Tyr817 fit the consensus sequence of ITIM, and the CD22F5/6 mutant requires these tyrosine residues for SHP-1 binding and BCR regulation. Thus, these ITIMs constitute a novel conditional SHP-1-binding site of CD22 that is activated upon BCR ligation by antigen but not by anti-Ig Ab.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1 and S2.

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2 The abbreviations used are: B cell, B lymphocyte; ITIM, immunoreceptor tyrosine-based inhibition motif; BCR, B cell antigen receptor; SH2, Src homology 2; SHP-1, SH2 domain-containing protein-tyrosine phosphatase; HA, hemagglutinin; NP, 4-hydroxy-3-nitrophenyl acetyl; ERK, extracellular signal-regulated kinase; Ab, antibody; mAb, monoclonal Ab; BSA, bovine serum albumin.
induced by antigen, because stimulation with anti-Ig Ab strongly phosphorylated various signaling molecules, including extracellular signal regulatory kinase (ERK), but only weakly phosphorylated CD22. Many anti-Ig Abs bind to the membrane-proximal part of BCR, whereas antigens bind to the antigen-binding site at the membrane-distal part of BCR. Therefore, we proposed that these anti-Ig Abs disrupt interaction between CD22 and BCR, thereby preventing phosphorylation of CD22 by BCR-associated kinase Lyn (19–22). Thus, SHP-1-binding sites in CD22 need to be examined in B cells after BCR ligation by antigens, because tyrosine phosphorylation of CD22 is crucial for its binding to SHP-1. We here demonstrate that, in B cells stimulated with antigen but not anti-Ig Ab, the CD22 mutant in which both Tyr843 and Tyr863 are replaced by phenylalanine is capable of recruiting SHP-1 and negatively regulating BCR signaling, suggesting that CD22 contains another SHP-1-binding domain specifically activated by antigen stimulation other than that containing Tyr843 and Tyr863. Further, we demonstrate that Tyr783 at a previously defined ITIM (15) does not regulate phosphorylation of other tyrosines, such as Tyr843 and Tyr863, but plays an essential role, together with Tyr817, in SHP-1 binding and BCR regulation of the mutant CD22 carrying Tyr → Phe mutations at both Tyr843 and Tyr863. These results clearly indicate that the region containing Tyr843 and Tyr863 constitutes a novel SHP-1-binding site that is activated upon BCR ligation by antigen but not anti-Ig Ab.

**EXPERIMENTAL PROCEDURES**

**Retrovirus Vectors**—The murine CD22 cDNA was obtained from pMX-CD22.2 REV (12) and was cloned into the bicistronic retrovirus vector pMXs-IG (23) (a gift of Dr. T. Kita
mura, University of Tokyo), allowing expression of both green fluorescent protein and CD22 (pMXs-CD22-IG). A tyrosine to phenylalanine mutation (Tyr → Phe) at Tyr783, Tyr843, Tyr863, or Tyr817 was introduced to the CD22 cDNA by PCR using specific primers (supplemental Table S1). A pair of synthetic oligonucleotides (5’-GGCCGCCTATCCTATGACGTGGCCGCATATGCCTAGCT-GA-3’ and 5’-GCCCTAGGCAAGCTGGGCACGCTATAGGATAGGC-3’) encoding both hemagglutinin (HA) tag and a stop codon was annealed and inserted at the NotI site of the packaging cell lines PLAT-E (a gift of Dr. T. Kitamura) (24) and 293G10 (Upstate Biotechnology, Inc., Lake Placid, NY), rat anti-CD22 mAb 4G10, rabbit anti-SHP-1 Ab, mouse anti-Grb2 Ab (BD Biosciences Pharmingen, San Diego, CA), Abs to phosphorylated ITIMs containing Tyr843 and Tyr863, respectively (26), anti-β-tubulin mAb TUB2.1 (Seikagaku Kogyo, Tokyo, Japan), and anti-phospho-ERK, anti-phospho-AKT Ab, followed(430,323),(572,406)

**Flow Cytometry**—Cells were stained with (4-hydroxy-3-nitrophenyl) acetyl (NP)-conjugated phycoerythrin or the combination of biotinylated anti-mouse CD22 monoclonal Ab (mAb) Cy34.1 (BD Biosciences) and Cy5-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). Cells were analyzed by flow cytometry using a fluorescence-activated cell sorter LSR (BD Biosciences) or a CyAn (DAKO, Glostrup, Denmark).

**RESULTS**

**CD22 ITIMs Containing Tyr843 and Tyr863 Are Dispensable for CD22-mediated BCR Regulation**—To investigate whether Tyr843 and Tyr863 are required for CD22-mediated signal regulation in antigen-stimulated B cells, we generated two retrovirus vectors expressing HA-tagged mutant CD22. One carries double Tyr → Phe mutations at both Tyr843 and Tyr863 (CD22F5/6), and the other carries triple Tyr → Phe mutations at Tyr783, Tyr843, and Tyr863 (CD22F2/5/6) (Fig. 1). Retrovirus expressing wild-type or mutant CD22 was transduced to the mouse B cell lines K46 and BAL17 IgM, both of which express membrane-bound IgM reactive to the hapten NP (12). Since K46 and not BAL17 IgM lacks expression of endogenous CD22 (12), we assessed expression of both transduced wild-type and mutant CD22 in K46 and BAL17 transfectants by flow cytometry using anti-CD22 Ab and Western blotting using anti-HA Ab, respectively. In these transfectants, the expression levels of mutant CD22 molecules were comparable with that of wild-type CD22, and the expression levels of NP-reactive BCR were similar to those in the cells transduced with...
the empty vector alone (K46μν-vector and BAL17lgM-vector) (supplemental Figs. S1 and S2).

We stimulated K46μν-vector, K46μνCD22, K46μνCD22F5/6, and K46μνCD22F2F5/6 transfectants with an antigen NP-BSA and examined phosphorylation of cellular substrates. Surprisingly, antigen stimulation-induced phosphorylation of various cellular substrates was reduced in K46μνCD22F5/6 cells compared with K46μν-vector, and the reduction of phosphorylation by CD22F5/6 was as strong as that by wild-type CD22 (Fig. 2A). Further, CD22F5/6 reduced antigen-induced phosphorylation of both ERK and AKT as efficiently as wild-type CD22 (Fig. 2, B and C). These results strongly suggested that both Tyr843 and Tyr863 of CD22 are dispensable for CD22-mediated signal inhibition in antigen-stimulated K46μν cells. In contrast, expression of CD22F2F5/6 failed to reduce the phosphorylation levels of various cellular substrates, including ERK and AKT, in antigen-stimulated K46μν transfectants (Fig. 2), suggesting that Tyr783 is required for BCR regulation by CD22F5/6.

We next examined association of mutant CD22 molecules with SHP-1 and, as a control, their association with Grb2. When K46μν transfectants were stimulated with NP-BSA, CD22F5/6 was phosphorylated and co-precipitated with both Grb2 and SHP-1 as efficiently as wild-type CD22 (5) (Fig. 3). Further, CD22F5/6 reduced antigen-induced phosphorylation of both ERK and AKT as efficiently as wild-type CD22 (Fig. 4). This result indicated that both Tyr843 and Tyr863 are dispensable for recruiting SHP-1 to CD22 upon antigen stimulation.

We could not determine the impact of mutant CD22 on BCR-mediated substrate phosphorylation in these cells due to expression of endogenous CD22. Taken together, CD22F5/6 carrying Y/F mutations at both Tyr843 and Tyr863 but not CD22F2F5/6 was able to regulate BCR signaling and recruit SHP-1 upon antigen stimulation. These Y/F mutants were expressed at similar levels on the cell surface and in Western blotting, indicating that Tyr783 is involved in CD22-mediated BCR regulation, probably by serving as a binding site for SHP-1.

Tyr783 is involved in CD22-mediated BCR Regulation without Influencing Phosphorylation of Other Tyrosine Residues in CD22—To address the role of Tyr783 in CD22-mediated signal regulation, we constructed the retrovirus vector encoding mutant CD22 containing a Tyr→Phe mutation at Tyr783 alone (CD22F2) (Fig. 1) and transduced the retrovirus vector to K46μν cells. The expression levels of CD22 and NP-reactive BCR on K46μνCD22F2 transfectants were similar to those on K46μνCD22 cells (supplemental Fig. S1). When the K46μνCD22F2 cells were stimulated with NP-BSA, CD22F2 was phosphorylated and associated with SHP-1 almost as efficiently as wild-type CD22 (Fig. 2, D). This result indicated that Tyr783 is dispensable for recruiting SHP-1 and suggested that Tyr783 is involved in the regulation of BCR signaling even in the presence of Tyr843 and Tyr863.
In contrast, CD22F2 down-modulated ERK phosphorylation almost as strongly as wild-type CD22 (Fig. 4B), suggesting that activation of ERK is more sensitive to SHP-1-mediated signal regulation than calcium signaling.

To address whether Tyr<sup>783</sup> regulates tyrosine phosphorylation of CD22, we examined phosphorylation of Tyr<sup>843</sup> and Tyr<sup>863</sup> using Abs that specifically recognize phosphorylated ITIMs containing Tyr<sup>843</sup> and Tyr<sup>863</sup>, respectively (26). Phosphorylation of both Tyr<sup>843</sup> and Tyr<sup>863</sup> in CD22F2 in antigen-stimulated K46<sub>v</sub> cells was comparable to that in wild-type CD22 (Fig. 4D), indicating that Tyr<sup>783</sup> does not regulate phosphorylation of other tyrosines in the cytoplasmic region of CD22. Taken together, Tyr<sup>783</sup> is involved in BCR regulation without influencing phosphorylation of other tyrosines in CD22.

Both Tyr<sup>783</sup> and Tyr<sup>817</sup> Are Required for CD22-mediated Signal Regulation in the Absence of Tyr<sup>843</sup> and Tyr<sup>863</sup>—We next asked whether the ITIM containing Tyr<sup>783</sup> regulates BCR signaling alone or in combination with yet uncharacterized ITIM. Besides tyrosine residues at the previously defined ITIMs, CD22 contains three tyrosine residues at positions 773, 817, and 828 in the cytoplasmic region. Among these tyrosine residues, the flanking sequence of Tyr<sup>783</sup> does not fit the consensus ITIM sequence at all. We thus constructed retrovirus vector encoding CD22 Tyr→Phe mutant containing only one tyrosine in the cytoplasmic region at Tyr<sup>783</sup> (CD22F1/3/4/5/6), that containing two tyrosine residues at Tyr<sup>783</sup> and Tyr<sup>817</sup> (CD22F1/4/5/6), and that containing Tyr<sup>783</sup> and Tyr<sup>828</sup> (CD22F1/3/5/6) (Fig. 1), and K46<sub>μ</sub>v cells were transduced with these vectors. The expression levels of both CD22 and NP-reactive BCR in these transfectants were similar to those of K46<sub>μ</sub>vCD22 and K46<sub>μ</sub>vCD22F5/6 cells (supplemental Fig. S1). When we stimulated the transfectants with an antigen NP-BSA, CD22F1/4/5/6 but not CD22F1/3/5/6 or CD22F1/3/4/5/6 negatively regulated both ERK phosphorylation (Fig. 5A) and calcium mobilization (Fig. 5B), although both CD22F5/6 and CD22F1/4/5/6 regulated calcium signaling less efficiently than wild-type CD22. CD22F1/4/5/6 and CD22F1/3/5/6 but not CD22F1/3/4/5/6 were phosphorylated as strongly as wild-type CD22 (Fig. 5C). However, SHP-1 was recruited by CD22F1/4/5/6 containing Tyr<sup>843</sup> and Tyr<sup>817</sup> but not CD22F1/3/5/6 containing Tyr<sup>783</sup> and Tyr<sup>828</sup> (Fig. 5C). These results indicated that Tyr<sup>783</sup> alone is not able to recruit SHP-1 or regulate BCR and requires co-preshence of Tyr<sup>817</sup> for recruiting SHP-1 and regulating BCR in the absence of Tyr<sup>843</sup> and Tyr<sup>863</sup>.

**DISCUSSION**

In this study, we demonstrate that the previously identified SHP-1-binding domain containing Tyr<sup>843</sup> and Tyr<sup>863</sup> is dispensable for CD22 to recruit SHP-1 and to regulate BCR signaling in the B cell lines K46<sub>μ</sub>v and BAL17igM and that the previously identified ITIM containing Tyr<sup>783</sup> is involved in both SHP-1 binding and BCR regulation. Some of the tyrosine residues in...
the molecules, such as HS1, augment their tyrosine phosphorylation by a mechanism known as processive phosphorylation (28), in which a phosphorylated tyrosine recruits the kinase that phosphorylates other tyrosine residues in the same molecule (29–31). However, Tyr783 does not regulate phosphorylation of other tyrosine residues of CD22. In contrast, the peptide containing Tyr783 was previously shown to bind to SHP-1 (14). Thus, Tyr783 does not augment binding of SHP-1 to other tyrosine residues of CD22 but rather serves as a SHP-1-binding site, thereby contributing to CD22-mediated signal regulation. We further demonstrated here that the flanking sequence of Tyr817 fits the consensus sequence of ITIM (27) and that both Tyr783 and Tyr817 are required for SHP-1 binding and BCR regulation of CD22F5/6 mutant carrying a Tyr→Phe mutation at Tyr843 and Tyr863. Both biochemical and structural studies previously demonstrated that efficient activation of SHP-1 requires engagement of both of the SH2 domains by peptides containing two phosphotyrosine residues (27, 32). Requirement of both Tyr783 and Tyr817 for SHP-1 recruitment in CD22F5/6 thereby contributes to SHP-1 binding and BCR regulation of CD22F5/6 mutant carrying a Tyr→Phe mutation at Tyr843 and Tyr863. Both biochemical and structural studies previously demonstrated that efficient activation of SHP-1 requires engagement of both of the SH2 domains by peptides containing two phosphotyrosine residues (27, 32). Requirement of both Tyr783 and Tyr817 for SHP-1 recruitment in CD22F5/6 there-

FIGURE 4. Tyr783 is involved in BCR regulation but does not regulate phosphorylation of Tyr843 or Tyr863. A, recruitment of SHP-1. The indicated K46μv transfectants were treated with 0.2 μg/ml NP-BSA for the indicated times. The indicated K46μv transfectants were treated with 0.2 μg/ml NP-BSA for the indicated times. Total cell lysates were separated by SDS-PAGE and analyzed for SHP phosphorylation by Western blotting using an anti-phosphotyrosine Ab. These blots were reprobed with anti-HA Ab to ensure equal loading. Representative data of more than three experiments are shown. B, regulation of ERK phosphorylation. The indicated K46μv transfectants were treated with 0.2 μg/ml NP-BSA for the indicated times. Total cell lysates were separated by SDS-PAGE and analyzed for ERK phosphorylation by Western blotting using an anti-phospho-ERK Ab. These blots were reprobed with anti-HA Ab to ensure equal loading. Representative data of more than three experiments are shown. C, regulation of calcium mobilization. The indicated K46μv transfectants were treated with 0.2 μg/ml NP-BSA for the indicated times. Total cell lysates were separated by SDS-PAGE and analyzed for ERK phosphorylation by Western blotting using an anti-phospho-ERK Ab. These blots were reprobed with anti-HA Ab to ensure equal loading. Representative data of more than three experiments are shown.

FIGURE 5. Both Tyr783 and Tyr817 are involved in BCR regulation and SHP-1 recruitment in antigen-stimulated B cells. A, regulation of ERK phosphorylation. The indicated K46μv transfectants were treated with 0.2 μg/ml NP-BSA for the indicated times. Total cell lysates were separated by SDS-PAGE and analyzed for ERK phosphorylation by Western blotting using an anti-phospho-ERK Ab. These blots were reprobed with anti-HA Ab to ensure equal loading. Representative data of more than three experiments are shown. B, regulation of calcium mobilization. The indicated K46μv transfectants were treated with 0.2 μg/ml NP-BSA for the indicated times. Total cell lysates were separated by SDS-PAGE and analyzed for ERK phosphorylation by Western blotting using an anti-phospho-ERK Ab. These blots were reprobed with anti-HA Ab to ensure equal loading. Representative data of more than three experiments are shown.
Novel SHP-1-binding Site in CD22

**A**

| K46μv | K48μv |
|-------|-------|
| CD22 | CD22F |
| NP-BSA anti-lg | NP-BSA anti-lg |
| 0 1 3 1 3 | 0 1 3 1 3 (min) |

**B**

| K46μv | K46μv | K46μv | K46μv | K46μv |
|-------|-------|-------|-------|-------|
| vector | CD22 | CD22F | CD22F | CD22F |
| 1/3/5/6 | 1/3/5/6 | 5/6 | 5/6 |
| (min) | | | |

**C**

![Graph](image)

**FIGURE 6.** Both Tyr^{543} and Tyr^{563} are required for CD22 to recruit SHP-1 and regulate BCR signaling in anti-Ig Ab-treated B cells. A, recruitment of SHP-1. The indicated K46μv transfectants were treated with 10 μg/ml anti-IgM for the indicated times. Cells were lysed, and HA-tagged CD22 was immunoprecipitated with anti-HA (3F10) Ab. Immunoprecipitates were analyzed by immunoblotting using anti-phospho-tyrosine mAb or anti-SHP-1 Ab. The same membranes were reprobed with anti-HA Ab to ensure equal loading. Representative data of more than three experiments are shown. B, regulation of ERK phosphorylation. The indicated K46μv transfectants were treated with 10 μg/ml anti-IgM for indicated times. Total cell lysates were separated by SDS-PAGE and analyzed by Western blotting using an anti-phospho-ERK Ab. The membranes were reprobed with anti-β-tubulin Ab to ensure equal loading. Representative data of more than three experiments are shown. C, regulation of calcium mobilization. The indicated K46μv transfectants were loaded with Fluo-4/AM, and intracellular free calcium was measured by flow cytometry using FACS LSR. Cells were treated with 10 μg/ml anti-IgM at 30 s (indicated by an arrow), and measurement of free calcium was continued for 300 s.

Therefore suggests that two ITIMs containing Tyr^{783} and Tyr^{817}, respectively, bind to the two SH2 domains of SHP-1, thereby recruiting it and regulating BCR signaling. Thus, Tyr^{783} plays a role together with Tyr^{817} in CD22-mediated BCR regulation by constituting a novel SHP-1-binding site.

Here we demonstrate that the ITIMs containing Tyr^{783} and Tyr^{817} recruit SHP-1 and regulate BCR signaling upon BCR ligation by antigens but not anti-Ig Ab. We previously demonstrated that antigen stimulation induces stronger CD22 phosphorylation than stimulation with anti-Ig Ab by generating a qualitatively distinct signaling (18). When B cells are stimulated with antigens, CD22 is translocated to the BCR-containing lipid rafts and is strongly phosphorylated by the tyrosine kinase Lyn, which is concentrated in lipid rafts (33). In contrast, BCR ligation with anti-Ig Ab induces weak phosphorylation of CD22 (18) probably because of exclusion of CD22 from BCR-containing lipid rafts. ITIMs containing Tyr^{543} and Tyr^{563} probably recruit SHP-1 with a lower phosphorylation level, whereas those containing Tyr^{783} and Tyr^{817} appear to require higher levels of phosphorylation for SHP-1 binding. Previously, Doody et al. (14) demonstrated that phosphorylated peptides containing Tyr^{783} and Tyr^{817}, respectively, but not that containing Tyr^{817} block binding of CD22 to SHP-1. This suggests that ITIM containing Tyr^{817} binds to SHP-1 less efficiently than the other ITIMs and may require strong phosphorylation for SHP-1 recruitment. However, the flanking sequence of Tyr^{817} completely fits the consensus sequence of ITIM (27) and does not show a particular difference from other ITIMs; therefore, how the ITIM containing Tyr^{817} binds to SHP-1 less efficiently is not yet known. Further studies are necessary to elucidate the mechanism and functional significance of stimulation-dependent activation of the novel inhibitory domain of CD22 containing Tyr^{783} and Tyr^{817}.

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