CD22 Regulates B Cell Receptor-mediated Signals via Two Domains That Independently Recruit Grb2 and SHP-1*

Received for publication, June 12, 2001, and in revised form, July 19, 2001
Published, JBC Papers in Press, September 10, 2001, DOI 10.1074/jbc.M105446200

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The binding of antigenic determinants by the BCR (BCR) determines the subsequent fate of a B cell and is regulated in part by the involvement of other surface molecules, termed coreceptors. CD22 is a B cell-restricted coreceptor that gets rapidly tyrosyl-phosphorylated and recruits various signaling molecules to the membrane following BCR ligation. Although CD22 contains three immunoreceptor tyrosine-based inhibitory motifs (ITIMs), only the two carboxyl-terminal ITIM tyrosines are required for efficient recruitment of the SHP-1 phosphatase after BCR ligation. Furthermore, Grb2 is inducibly recruited to CD22 in human and murine B cells. Unlike SHP-1, Grb2 recruitment to CD22 is not inhibited by specific doses of the Src family kinase-specific inhibitor PP1. The tyrosine residue in CD22 required for Grb2 recruitment to CD22 is distinct and independent from the two ITIM tyrosines required for efficient SHP-1 recruitment (Tyr-828) and that require for efficient SHP-1 recruitment (Tyr-843 and Tyr-863). Individually both Lyn and Syk are required for maximal phosphorylation of CD22 following ligation of the BCR, and together Lyn and Syk are required for all of the constitutive and induced tyrosine phosphorylation of CD22. We propose that the cytoplasmic tail of CD22 contains two domains that regulate signal transduction pathways initiated by the BCR and B cell fate.

The hypothesis is supported by evidence from studies of CD22-deficient mice. Although cd22−/− mice develop relatively normal numbers of peripheral B cells, cd22−/− B cells have an abnormal surface phenotype: they have decreased expression of surface IgM (slgM) and elevated expression of major histocompatibility complex class II molecules, indicative of B cells that have encountered antigen in vivo (22–25). In agreement with a so-called “hyper-responsive” phenotype, engagement of the BCR on cd22−/− B cells leads to greater increases in levels of intracellular free calcium ([Ca2+]i) compared with wild-type B cells.
cells (22–25). Based on these data it has been proposed that CD22 functions primarily as a negative regulator of BCR signal transduction (18, 26). However, B cells from CD22-deficient mice proliferate less well compared with wild-type B cells when treated with the same reagent that leads to augmented release of [Ca\(^{2+}\)]\(_i\) (22, 24). Furthermore, cd22\(^{-/-}\) mice generate decreased antibody responses when immunized with a thymus-independent type II antigen (22, 25). These data suggest that CD22 can also function as a positive regulator of BCR-mediated signals. How CD22 might regulate B cell responses in both a positive and a negative manner has remained an enigma. We have looked at the role that single tyrosine residues play in the recruitment of signaling proteins and the role that CD22 plays in activating mitogen-activated protein kinase (MAPK) pathways. Here we present data suggesting that the cytoplasmic tail of CD22 contains two signaling domains that regulate signal transduction pathways initiated by the BCR. These findings may explain the paradoxical phenotype of CD22-deficient mice.

**EXPERIMENTAL PROCEDURES**

**Mice**—cd22\(^{-/-}\) mice, which had been backcrossed to C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME) greater than seven generations, were crossed to generate cd22\(^{+/+}\) and cd22\(^{-/-}\) mice (22). Litter-and gender-matched, 7–12-week-old cd22\(^{+/+}\) and cd22\(^{-/-}\) mice were used for all of the mouse studies. All mice were raised in the specific pathogen-free facility at the University of Washington. Mice were genotyped for the wild-type and mutant alleles of cd22 by subjecting tail DNA to three PCRs. Briefly, one forward primer that annealed in intron 2 (27) (5′-GCC AGC CTG GCC TCA GAC TGA CAA TTC-3′) was used in three separate reactions with different reverse primers that annealed in intron 3 (5′-TCT CCA GAC GCA GCT ACC CTG GGC CTC-3′), intron 4 (5′-GGT CCC TTC TCT TCT GGG TCA GAA CAG TTG-3′), or the neomycin cassette (5′-GGG CTT GGG GTT GGG GCG GGC TAT CAG-3′). The PCRs generated a 207-base pair fragment from the wild-type allele with primers that annealed in intron 2\(\rightarrow\)intron 3, a 342-base pair fragment from the mutant allele with primers that annealed in intron 2\(\rightarrow\)neomycin, and 829- and 1309-base pair fragments from wild-type and mutant alleles, respectively, with primers that annealed in intron 2\(\rightarrow\)intron 4.

**Cells**—The EBV–Burkitt’s lymphoma line BJAB was obtained from ATCC and cultured in RPMI 1640 medium (Fisher Scientific) supplemented with 10% fetal calf serum (BioWhittaker, Walkersville, MD), 1 mM sodium pyruvate, and 1× nonessential amino acids (Invirone Scientific, Santa Ana, CA), 2 mM l-glutamine (Gemini Bioproducts, Woodland, CA), and 100 units of penicillin-streptomycin (Life Technologies, Inc.). Wild-type, Lyn\(^{-/-}\), and c-myc tag/stop codon (generated by annealing 5′-TCG AGT AGT TTG ACC GAT GAC ACA GAG ACC TCA TTT GCA GTC C-3′) were cloned into the chicken expression vector pApuro-2 (28), the full-length murine cd22 cDNA (22, 24) was cloned into pcDNA3(+) (22). Furthermore, B cells from CD22-deficient mice generate decreased antibody responses when immunized with a thymus-independent type II antigen (22, 25). These data suggest that CD22 contains two signaling domains that regulate signal transduction pathways initiated by the BCR. These findings may explain the paradoxical phenotype of CD22-deficient mice.

**Reagents**—Purified mouse B cells were prepared as described previously (22). mAbs to murine CD22 (2D6), human CD22 (HD39), c-Myc, and c-myc tag/stop codon (generated by annealing 5′-TCG AGT AGT TTG ACC GAT GAC ACA GAG ACC TCA TTT GCA GTC C-3′) were cloned into the chicken expression vector pApuro-2 (28), the full-length murine cd22 cDNA (22, 24) was cloned into pcDNA3(+) (22). Furthermore, B cells from CD22-deficient mice generate decreased antibody responses when immunized with a thymus-independent type II antigen (22, 25). These data suggest that CD22 contains two signaling domains that regulate signal transduction pathways initiated by the BCR. These findings may explain the paradoxical phenotype of CD22-deficient mice.

**CD22 Expression Constructs for Human and Chicken Cells—**For transient transfection in BJAB cells, the cytoplasmic tail of murine CD22 contains two signaling domains that regulate signal transduction pathways initiated by the BCR. These findings may explain the paradoxical phenotype of CD22-deficient mice.

**Biochemical Methods—**Cells in exponential phase were cultured in electroporation medium (RPMI 1640 medium supplemented with 15% fetal calf serum, 1 mM sodium pyruvate, 1× nonessential amino acids, and 2 mM l-glutamine) and resuspended at 3 \times 10^6 ml\(^{-1}\). Next, 350 \(\mu\)l of the cell suspension was placed in a 0.4-cm cuvette with 50 \(\mu\)l of linearized plasmid DNA containing c-myc-tagged wild-type or mutant cd22 inserts in pcDNA3 for 10 min on ice. Cells were then electroporated (Bio-Rad Gene Pulser with Capacitance Extender) at 230 V and 960 microfarads. After electroporation, cells were placed on ice for 10 min, resuspended in 20 ml of electroporation medium, and cultured at 37°C. After 2 days, Geneticin (Life Technologies, Inc.) was added to final concentration of 3 mg ml\(^{-1}\). After 7 days, cells were cloned by limiting dilution. Clones were screened for similar expression levels of wild-type or mutant murine CD22 by surface staining with biotinylated mAb 2D6 and flow cytometry (FACScan, Becton Dickinson, Mountain View, CA). Stable transfected clones were then cultured in 1 mg ml\(^{-1}\) Geneticin. Wild-type and mutant DT40 cells in exponential phase were washed once in RPMI 1640 medium. DT40 cells were electroporated with 10 \(\mu\)g of linearized plasmid DNA containing pApuro-2 and cultured at 290 V and 960 microfarads and cultured as described above for transfection in BJAB cells. After 2 days, puromycin (2 \(\mu\)g ml\(^{-1}\)) was added to the medium. Five days later, clones were removed by centrifugation over IsoPrep (Robbins Scientific, Sunnyvale, CA). Cells at the interface were washed twice and cultured at 37°C. Cells were later stained for surface expression of murine CD22 with biotinylated cd22 mAb, sorted by a FACS (FACS Vantage Turbo S.E., Becton Dickinson) for high expression of CD22, and cloned by limiting dilution. Clones were screened and selected for similar expression levels of CD22.

**CD22 Expression Constructs for Human and Chicken Cells—**Six mutant constructs that contained single tyrosine to phenylalanine mutations from pcDNA3(+)cd22-ΔHA (22) were used for our screening of murine cd22 cDNA in pcDNA3 with a 3′ EcoR I site, HA tag/stop codon, and XhoI site replacing the natural stop codon. Forward primers with the indicated mutation (Y773F, 5′-CAG GGA TGC TAC AAT GCG CCA-3′; Y783F, 5′-ACT GTT AGT TTG GCC ATC TTG-3′; Y817F, 5′-AGC GTC ATC TTC TCG GTA-3′; Y828F, 5′-ATG GGG GTT TAT GAT GAA-3′; Y843F, 5′-AGC ATC CAT TTC TCG GAT-3′; and Y863F, 5′-GAT GAC TAC TTG TTG ACC CTC-3′) were used with the commercially available Sp6 reverse primer to generate six mutant fragments by PCR mutagenesis. After purifying the PCR products corresponding to the six mutated 3′-regions of cd22, another set of PCRs with pcDNA3 cd22-ΔHA as the template was set up using the indicated primers and a forward primer to generate the pcDNA3 cd22-ΔHA (5′-GAT CCG ATC GCC GGC CTG GGA GAA AAA TAG CAG-3′, and the Sp6 reverse primer. Amplified full-length, HA-tagged wild-type or mutant murine cd22 cDNA products were then ligated into pcDNA3 after digesting both the PCR fragments and the vector with BamHI and EcoRI digested wild-type or mutant HA-tagged cd22, EcoRI- and XhoI-digested c-myc tag/stop codon (generated by annealing 5′-TG CAG AGT AAC TGG TTG ATG AAA ATG CTA AAC TTT GCA GTC C-3′) were cloned into pcDNA3 for 10 min on ice. Cells were then electroporated (Bio-Rad Gene Pulser with Capacitance Extender) at 230 V and 960 microfarads. After electroporation, cells were placed on ice for 10 min, resuspended in 20 ml of electroporation medium, and cultured at 37°C. After 2 days, Geneticin (Life Technologies, Inc.) was added to final concentration of 3 mg ml\(^{-1}\). After ~2 weeks, cells were cloned by limiting dilution. Clones were screened for similar expression levels of wild-type or mutant murine CD22 by surface staining with biotinylated mAb 2D6 and flow cytometry (FACScan, Becton Dickinson, Mountain View, CA). Stable transfected clones were then cultured in 1 mg ml\(^{-1}\) Geneticin. Wild-type and mutant DT40 cells in exponential phase were washed once in RPMI 1640 medium. DT40 cells were electroporated with 10 \(\mu\)g of linearized plasmid DNA containing pApuro-2 and cultured at 290 V and 960 microfarads and cultured as described above for transfection in BJAB cells. After 2 days, puromycin (2 \(\mu\)g ml\(^{-1}\)) was added to the medium. Five days later, clones were removed by centrifugation over IsoPrep (Robbins Scientific, Sunnyvale, CA). Cells at the interface were washed twice and cultured at 37°C. Cells were later stained for surface expression of murine CD22 with biotinylated cd22 mAb, sorted by a FACS (FACS Vantage Turbo S.E., Becton Dickinson) for high expression of CD22, and cloned by limiting dilution. Clones were screened and selected for similar expression levels of CD22.
The Two Carboxyl-terminal ITIM Tyrosines Are Required for Recruitment of SHP-1—The cytoplasmic tail of CD22 contains six conserved tyrosine residues and becomes rapidly tyrosine phosphorylated following BCR engagement (11–14). Three of these tyrosines, Tyr-783, Tyr-843, and Tyr-863 (numbering is based on the unprocessed murine CD22 protein), are located within ITIM motifs as defined by the consensus motif (V/L/I)XX(X/I/V) (where X is any amino acid) (32). When the tyrosine residue within an ITIM motif is phosphorylated, the ITIM motif can bind molecules such as the SHP-1 protein tyrosine phosphatase and potentially play a role in activating SHP-1 (16, 18). Using phosphopeptides equivalent to the six tyrosine-containing regions in CD22, it was previously reported that phosphopeptides corresponding to the three ITIM motifs of CD22 can compete with CD22 for the binding of SHP-1, whereas the analogous nonphosphorylated peptides do not (18). These data suggested that Tyr-783, Tyr-843, and Tyr-863 were involved in the binding and activation of SHP-1 but did not clarify which of these tyrosines was required in vivo for recruitment of SHP-1 to CD22 following engagement of the BCR.

To address this question, we used the sIgM-positive human B cell line BJAB to generate a stable transfectant expressing c-Myc-tagged wild-type murine CD22 and six stable transfectants expressing mutant forms of c-Myc-tagged murine CD22 with single tyrosine to phenylalanine mutations. All seven stable lines were selected for similar expression levels of the transfected wild-type or mutant murine CD22 molecules (Fig. 1A). This system had several advantages relative to other transfection systems. Unlike non-B cell lines where CD22 has been studied, e.g. HeLa cells (33), BJAB contains the same or very similar signal transduction machinery present in normal B cells. Also, the upstream and downstream components of the signal transduction machinery are present and at unmanipulated levels. Furthermore, BCR cross-linking, a relatively physiological stimulus, was used instead of pharmacological agents to activate the B cells. Finally, the transfected BJAB lines express endogenous human CD22, which was used as an internal positive control for recruitment of SHP-1 and was easily distinguishable from the transfected wild-type or mutant murine CD22 by specific mAbs and antisera.

Stimulation through the BCR on transfectants expressing wild-type murine CD22 led to efficient association of SHP-1 with both the transfected murine CD22 and endogenous human CD22 (Fig. 1B). This was also observed for four of the six transfectants expressing different mutant forms of murine CD22. Transfectants with single tyrosine to phenylalanine mutations at position 773, 783, 817, or 828 (Y773F, Y783F, Y817F, or Y828F, respectively) efficiently recruited SHP-1 to both the mutant murine CD22 molecules and the endogenous human CD22 in response to BCR cross-linking (Fig. 1B and data not shown). Thus, although a phosphopeptide of the “ITIM tyrosine” Tyr-783 blocked SHP-1 binding (18), phosphorylation of Tyr-783 was not required for BCR-induced SHP-1 recruitment in vivo. However, after normalizing for the amount of immunoprecipitated CD22, we found that single tyrosine to phenylalanine mutations at position 843 (Y843F) or 863 (Y863F) decreased the amount of SHP-1 recruited to CD22 by 90 and 97%, respectively, compared with the amount bound by wild-type CD22 (Fig. 1B). The endogenous human CD22 in these same cells maintained its ability to recruit SHP-1 (Fig. 1B). Thus, the upstream kinases required for phosphorylation of CD22 and recruitment of SHP-1 were not altered during the transfection and cloning process. Similar results were seen in two sets of clones generated by independent transfections.
These data demonstrate that the ITIM tyrosines Tyr-843 and Tyr-863, but not Tyr-783, in murine CD22 are individually required for efficient recruitment of SHP-1 upon BCR stimulation.

CD22 Influences ERK and JNK Activation but Not p38 MAPK Activation—Members of the MAPK family of serine/threonine protein kinases are activated after BCR ligation (26, 31, 34). Since it can recruit and activate SHP-1, CD22 has been suggested to negatively regulate BCR-induced Erk2 and JNK but not p38 MAPK activation (26). To test whether CD22 may regulate BCR-mediated activation of the MAPKs, we compared Erk1/2, JNK, and p38 MAPK phosphorylation in purified B cells from wild-type or CD22-deficient mice (22). Western blot analysis of whole B cell lysates with mAbs specific for the phosphorylated forms of the various MAPKs showed that the absence of CD22 led to only a 1.5-fold increase in Erk1/2 phosphorylation after a 1-min stimulation and 1.6-fold increase in JNK phosphorylation after a 10-min stimulation through the antigen receptor. The absence of CD22 led to a 1.3-fold decrease in the phosphorylation of p38 MAPK after a 3-min stimulation (Fig. 2A). Furthermore, in vitro kinase assays of either JNK or p38 MAPK immunoprecipitates confirmed that JNK activity was elevated only 1.6-fold, and p38 MAPK activity was decreased 1.3-fold after BCR cross-linking of cd22−/− B cells versus wild-type B cells (Fig. 2B). These results suggest that CD22 does not play a major role in regulating BCR-induced Erk2 or JNK activation as proposed (26). Poe et al. (35) did not detect JNK phosphorylation after BCR ligation of cd22−/− B cells; they did measure JNK kinase activity, and the B cells they used had detectable JNK phosphorylation prior to stimulation, which might account for differences from our results.

CD22 Recruits Grb2 to the Membrane following BCR Stimulation—We have previously noted that the amino acids surrounding one of the non-ITIM tyrosines in CD22, Tyr-828, has sequence homology to two regions in the T cell- and natural killer cell-restricted molecule LAT (2). LAT is rapidly tyrosyl-phosphorylated by either Zap70 or Syk following ligation of the T cell antigen receptor and recruits the adaptor molecule Grb2 (36). Furthermore, the tyrosine residues within LAT with homology to CD22 are required for Grb2 recruitment by LAT (36, 37). Thus, we hypothesized that the cytoplasmic tail of CD22 recruits Grb2 after it is tyrosine-phosphorylated. To test this hypothesis, we immunoprecipitated human CD22 from BJAB cells after cross-linking the BCR; by Western blotting we found that Grb2 is indeed inducibly recruited to bind CD22 (Fig. 3A). A small amount of Grb2 was associated with CD22 prior to BCR ligation but is increased −2.2-fold within 3 min following...
with 61 and 37% inhibition at 10 μM PP1, respectively (Fig. 5). Further analysis showed that 50% inhibition occurred at 5 μM for SHP-1 recruitment and 18 μM for induced tyrosine phosphorylation. Interestingly, Grb2 recruitment showed only 0 and 2% inhibition at 2 and 10 μM PP1 (Fig. 5). Densitometric analysis revealed that at 50 μM PP1, Grb2 binding was decreased 23% versus the amount seen without PP1, demonstrating that Grb2 binding can be blocked in this system. This effect at 50 μM may be due to decreased specificity of the Src family kinase-specific inhibitor at high doses. Nonetheless, these data suggest that SHP-1 and Grb2 recruitment to CD22 are differentially regulated by upstream kinases and further support the hypothesis that CD22 regulates at least two independent signaling pathways initiated by the BCR.

Both Lyn and Syk Contribute to the Constitutive and Induced Tyrosine Phosphorylation of CD22—Since BCR-induced tyrosine phosphorylation of CD22 in B cells from Lyn-deficient mice is reduced but not completely absent (39–42), we hypothesized that another PTK can phosphorylate the cytoplasmic tail of CD22. Furthermore, since the Syk family PTK Zap70 phosphorylates LAT (30) and Syk physically associates with CD22 (16), we tested whether Syk could affect CD22 tyrosine phosphorylation. To address this question, we used the chicken B cell line DT40 and three mutant lines derived from DT40 that are Lyn-deficient (Lyn−/−), Syk-deficient (Syk−/−), and Lyn/Syk double-deficient (Lyn/Syk−/−) mutant cells (28, 29). Transfectants were selected for similar expression of c-Myc-tagged murine CD22 (Fig. 6A). CD22 was constitutively phosphorylated in wild-type DT40 cells, and phosphorylation increased significantly after BCR cross-linking (Fig. 6B). BCR-induced phosphorylation of CD22 increased 70% in Lyn−/− and 25% in Syk−/− DT40 cells relative to the induced phosphorylation in wild-type cells, showing that both PTKs are required for maximal phosphorylation of CD22. In the absence of both Lyn and Syk, tyrosine phosphorylation of CD22 was not observed even when the blot was intentionally overexposed (Fig. 6B). This demonstrates that together Lyn and Syk are responsible for all of the constitutive and induced tyrosine phosphorylation of CD22. These data also suggest that the small amount of BCR-induced tyrosine phosphorylation of CD22 after purified splenic B cells were signaled through the antigen receptor (Fig. 3B). These results confirm and extend those of others (35, 38).

Different Phosphotyrosines Recruit Grb2 and SHP-1 to CD22 following BCR Engagement—Since CD22 appears to regulate signals in both positive and negative manners, we hypothesized that the tyrosine requirements for Grb2 and SHP-1 recruitment to CD22 were independent of each other. To test this, we analyzed our panel of stable BJAB transfectants expressing single tyrosine to phenylalanine mutants of murine CD22 for association of SHP-1 and Grb2 before and after BCR ligation. The Tyr-817 residue was not required for recruitment of either Grb2 or SHP-1 (Fig. 4). However, SHP-1 was efficiently recruited to the Y828F mutant, while Grb2 was not; thus, Tyr-828 is required for Grb2 recruitment as suggested by previous in vitro studies with phosphopeptides (35, 38). Furthermore, Grb2 recruitment remained intact in the Y843F or Y863F murine CD22 mutants, whereas SHP-1 recruitment was abolished (Fig. 4). These data suggest that CD22 contains two independent signaling domains within its cytoplasmic tail, one functioning to recruit SHP-1 and one functioning to recruit Grb2.

Effect of the Src Family Kinase-specific Inhibitor PP1 on Grb2 and SHP-1 Recruitment—Based on both genetic and biochemical data, a signaling pathway involving Lyn, CD22, and SHP-1 has been established. For instance, the BCR-induced phosphorylation of CD22 is decreased in B cells from Lyn-deficient mice, and SHP-1 recruitment is abolished (39–42). This probably explains why lyn−/−, cd22−/−, and shp-1−/− mice have similar defects, e.g. decreased sIgM expression, increased major histocompatibility complex class II expression, and increased sensitivity to the BCR-induced increase in [Ca2+]i, release (2). To test whether Grb2 recruitment to CD22 also requires Src family kinase activity, we preincubated BJAB cells in varying concentrations of the Src family kinase-specific inhibitor PP1 (43). Then we activated the cells by BCR cross-linking and analyzed the phosphorylation status of CD22 and its ability to recruit Grb2 and SHP-1 by Western blotting. Consistent with published data (39–42), we found that PP1 inhibited the induced recruitment of SHP-1 and induced tyrosine phosphorylation of CD22 in a dose-dependent manner stimulated. Similarly, Grb2 was recruited to murine CD22 after purified splenic B cells were signaled through the antigen receptor (Fig. 3B). These results confirm and extend those of others (35, 38).

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Tyrosine phosphorylation of CD22 is dependent on upstream of CD22 tyrosine phosphorylation. Three of the six tyrosine residues within the cytoplasmic tail of CD22 correspond to classic ITIM motifs (18, 32), but only two of these ITIM tyrosines, Tyr-843 and Tyr-863, are required for efficient SHP-1 recruitment. Although a phosphopeptide containing Tyr-783, like Tyr-843- and Tyr-863-containing phosphopeptides, could compete for SHP-1 binding to CD22 and increase the phosphatase activity in vitro (18), Tyr-783 was not required for BCR-induced recruitment of SHP-1 in vivo (Fig. 1B). Recent studies suggest that CD22 is also a substrate for SHP-1 phosphatase activity and that these three ITIM tyrosines may be the sites of dephosphorylation (33, 44). By overexpressing substrate-trapping and catalytically inactive SHP-1 with a chimeric CD22 molecule in HeLa cells, Blasioli et al. (33) have suggested that phosphorylation of Tyr-783 may stabilize the binding of SHP-1 to CD22. Whether or not Tyr-783 is normally tyrosine-phosphorylated and -dephosphorylated in vivo after BCR ligation remains to be determined. Since a small amount of SHP-1 is recruited to CD22 when, Tyr-843 is mutated to phenylalanine, phosphorylated Tyr-843 may also function to stabilize the binding of SHP-1 to CD22. Our results suggest that CD22, Tyr-783, Tyr-843, and Tyr-817 may be required for either SHP-1 or Grb2 recruitment. Interestingly, some defects observed in cd22−/− mice are not observed in lyn−/− or shp-1mut/mut mice (2). For instance, the antibody responses to thymus-independent type II antigens are relatively normal in lyn−/− mice (45, 46) but decreased in cd22−/− mice (22, 25). Even more telling is the observation that when stimulated through the antigen receptor in vitro, cd22−/− B cells proliferate less well than wild-type B cells (22, 24), whereas B cells purified from lyn−/− or shp-1mut/mut mice are hyper-responsive to similar stimuli (47–49). These data suggest that CD22 may regulate BCR-mediated signals in a Lyn− and SHP-1-independent manner. Other studies also have suggested that CD22 may signal independently of Lyn and SHP-1. Chan et al. (39) have found that cross-linking CD22 leads to low level Erk2 and JNK activation in the absence of Lyn.

We have found that a region in CD22 is functionally similar to both Lyn and Syk. A, wild-type (WT), Lyn-deficient (Lyn−), Syk-deficient (Syk−) and Lyn/Syk double-deficient (Lyn-Syk−) chicken DT40 cells were transfected with c-Myc-tagged murine CD22. Stable transfectants were sorted by a FACS for similar surface expression and cloned by limiting dilution. Cells were stained with murine CD22 mAb (solid histogram) or isotype control (thin lines). B, stable transfectants were stimulated for 3 min with chicken IgM mAb (+) or mouse IgM (−) and lysed. Anti-CD22 mAb or control rat IgG was used for immunoprecipitation. Samples were split, and replicate gels were run and transferred to nylon membranes. Blots were probed with either phosphotyrosine (P-Y) or c-Myc mAb. The anti-phosphotyrosine blot was intentionally overexposed to show the complete lack of CD22 tyrosine phosphorylation in Lyn-Syk− cells. IP, immunoprecipitation; Stim., stimulation; Wt, wild type.

DISCUSSION

Many factors determine the fate of a B cell upon encounter with antigen (1), including the participation or lack of participation of coreceptors such as CD22 (2). Previous evidence suggests that CD22 can negatively regulate signals initiated by the BCR by recruiting and activating the protein tyrosine phosphatase SHP-1 (18, 26). The tyrosine kinase Lyn is required for this recruitment, thereby defining a signaling pathway in which Lyn directly or indirectly phosphorylates CD22 and promotes SHP-1 recruitment (39–42). Mice homozygous for mutations at each of these three loci have similar phenotypes with respect to slG down-regulation, major histocompatibility complex class II up-regulation, and increased sensitivity to BCR-mediated increase in [Ca2+]i (2). Furthermore, Lyn, CD22, and SHP-1 regulate tolerance induction in a polygenic manner (40). These data suggest that a Lyn/CD22/SHP-1 “inhibitory pathway” regulates some B cell responses (10). However, data from this study suggest that CD22 may also regulate other signaling pathways.

We have defined which of the six tyrosines in the cytoplasmic tail of CD22 are required for SHP-1 recruitment following BCR ligation. Three of the six tyrosine residues within the cytoplasmic tail of CD22 correspond to classic ITIM motifs (18, 32), but only two of these ITIM tyrosines, Tyr-843 and Tyr-863, are required for efficient SHP-1 recruitment. Although a phosphopeptide containing Tyr-783, like Tyr-843- and Tyr-863-containing phosphopeptides, could compete for SHP-1 binding to CD22 and increase the phosphatase activity in vitro (18), Tyr-783 was not required for BCR-induced recruitment of SHP-1 in vivo (Fig. 1B). Recent studies suggest that CD22 is also a substrate for SHP-1 phosphatase activity and that these three ITIM tyrosines may be the sites of dephosphorylation (33, 44). By overexpressing substrate-trapping and catalytically inactive SHP-1 with a chimeric CD22 molecule in HeLa cells, Blasioli et al. (33) have suggested that phosphorylation of Tyr-783 may stabilize the binding of SHP-1 to CD22. Whether or not Tyr-783 is normally tyrosine-phosphorylated and -dephosphorylated in vivo after BCR ligation remains to be determined. Since a small amount of SHP-1 is recruited to CD22 when Tyr-843 is mutated to phenylalanine, phosphorylated Tyr-843 may also function to stabilize the binding of SHP-1 to CD22. Our results suggest that CD22, Tyr-783, Tyr-843, and Tyr-817 may be required for either SHP-1 or Grb2 recruitment. Interestingly, some defects observed in cd22−/− mice are not observed in lyn−/− or shp-1mut/mut mice (2). For instance, the antibody responses to thymus-independent type II antigens are relatively normal in lyn−/− mice (45, 46) but decreased in cd22−/− mice (22, 25). Even more telling is the observation that when stimulated through the antigen receptor in vitro, cd22−/− B cells proliferate less well than wild-type B cells (22, 24), whereas B cells purified from lyn−/− or shp-1mut/mut mice are hyper-responsive to similar stimuli (47–49). These data suggest that CD22 may regulate BCR-mediated signals in a Lyn− and SHP-1-independent manner. Other studies also have suggested that CD22 may signal independently of Lyn and SHP-1. Chan et al. (39) have found that cross-linking CD22 leads to low level Erk2 and JNK activation in the absence of Lyn.

We have found that a region in CD22 is functionally similar to two regions in LAT (2). This region, containing Tyr-828, recruits the adaptor molecule Grb2 (Fig. 4). In contrast to SHP-1, Grb2 recruitment to CD22 is not inhibited by 2 and 10 μM PP1 (Fig. 5), doses that specifically inhibit Src family PTKs (43). Grb2 recruitment is slightly inhibited at 50 μM PP1, which may be due to decreased specificity of the inhibitor. These data suggest that Grb2 recruitment may not require a Src family kinase. Alternatively, Lyn and a non-Src family PTK together may influence the localization and/or activity of each other upstream of CD22 phosphorylation. Nevertheless, we cannot formally rule out a role for Lyn or other Src family PTKs in the recruitment of Grb2 to CD22. However, our data are most consistent with the model that SHP-1 and Grb2 recruitment to CD22 are differentially regulated by upstream PTKs. Furthermore, since Syk and Zap70 phosphorylate the analogous regions in LAT leading to Grb2 recruitment in T cells (36, 37) and Grb2 binds to CD22 in a phosphorylation-dependent manner (35, 38), Syk is a likely candidate for phosphorylating Tyr-828 in CD22 and facilitating the binding of Grb2. Moreover, Syk associates with CD22 (16) and is required for maximal phosphorylation of CD22 (Fig. 6B). However, preincubation of B cells with the Syk-selective inhibitor piceatannol (50) over a range of doses did not inhibit the induced recruitment of Grb2 to CD22 (data not shown). Furthermore, analysis of the ability of CD22 to associate with endogenous SHP-1 and Grb2 in.
B cell receptor (BCR)-activated wild-type and mutant chicken DT40 lines proved inconclusive due to the lack of specific reagents that reliably detect chicken signaling molecules (data not shown). Therefore, we have not definitively implicated Syk in the phosphorylation of Tyr-828 and Grb2 recruitment. Further studies will be required to clarify the role of Syk in recruiting Grb2 to CD22.

CD22 apparently contains at least two signaling domains that regulate BCR-mediated signaling pathways. These tyrosine-based domains are defined by the molecules they recruit to the membrane following BCR ligation. One domain, containing Tyr-843 and Tyr-863, is most likely phosphorylated by Lyn and is required for efficient SHP-1 recruitment. Another domain, containing Tyr-872, is required for Grb2 recruitment and may promote a proliferative pathway. This model explains the various defects observed in cd22−/− mice compared with Lyn−/− and sph-1mer/mov mice; it also predicts that these two signaling pathways regulate different aspects of B cell responses. The Lyn/CD22/SHP-1 pathway almost certainly regulates sIgM expression, major histocompatibility complex II expression, and probably the BCR-induced increase in [Ca^{2+}], (2). The proposed CD22/Grb2 pathway may regulate proliferative responses to anti-IgM and antibody responses to thymus-independent type II antigens, perhaps with Src and Src homology 2-containing inositol 5'-phosphatase (35). In the absence of cd22, we propose that both signaling pathways are disrupted, whereas in the absence of either Lyn or SHP-1 only one of the two-regulated pathways is completely disrupted. We are currently testing this hypothesis by creating mice that express mutant forms of CD22 with single tyrosine to phenylalanine substitutions within each signaling domain. We predict that each of these lines of mice will show a subset of the defects observed in CD22-deficient mice.

Pezzuto et al. (51, 52) have shown that preincubation of human B cells with CD22 mAb lowered the threshold for BCR-induced proliferation and increase in [Ca^{2+}], signaling through the BCR. This provided initial functional data on the role of CD40, CD95, and the BCR itself (2), CD22 too has more than one signaling domain and it is required for efficient SHP-1 recruitment. Another do-
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51. Pezzuto, A., Dorken, B., Moldenhauer, G., and Clark, E. A. (1987) *J. Immunol.* 138, 98–103
52. Pezzuto, A., Rabinovitch, P. S., Dorken, B., Moldenhauer, G., and Clark, E. A. (1988) *J. Immunol.* 140, 1791–1795
53. Tuscano, J., Engel, P., Tedder, T. F., and Kehrl, J. H. (1996) *Blood* 87, 4723–4730
54. Tuscano, J. M., Riva, A., Toscano, S. N., Tedder, T. F., and Kehrl, J. H. (1999) *Blood* 94, 1382–1392
55. Graves, J. D., Gotoh, Y., Draves, K. E., Ambrose, D., Han, D. K., Wright, M., Chernoff, J., Clark, E. A., and Krebs, E. G. (1998) *EMBO J.* 17, 2224–2234
56. Graves, J. D., Draves, K. E., Craxton, A., Saklatvala, J., Krebs, E. G., and Clark, E. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 13814–13818