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The B Cell Coreceptor CD22 Associates with AP50, a Clathrin-Coated Pit Adapter Protein, Via Tyrosine-Dependent Interaction[1]

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The B cell coreceptor CD22 plays an important role in regulating signal transduction via the B cell Ag receptor. Studies have shown that surface expression of CD22 can be modulated in response to binding of ligand (i.e., mAb). Thus, it is possible that alterations in the level of CD22 expression following binding of natural ligand(s) may affect its ability to modulate the Ag receptor signaling threshold at specific points during B cell development and differentiation. Therefore, it is important to delineate the physiologic mechanism by which CD22 expression is controlled. In the current study, yeast two-hybrid analysis was used to demonstrate that CD22 interacts with AP50, the medium chain subunit of the AP-2 complex, via tyrosine-based internalization motifs in its cytoplasmic domain. This interaction was further characterized using yeast two-hybrid analysis revealing that Tyr^{843} and surrounding amino acids in the cytoplasmic tail of CD22 comprise the primary binding site for AP50. Subsequent studies using transfectant Jurkat cell lines expressing wild-type or mutant forms of CD22 demonstrated that either Tyr^{843} or Tyr^{863} is sufficient for mAb-mediated internalization of CD22 and that these motifs are involved in its interaction with the AP-2 complex, as determined by coprecipitation of α-adaptin. Finally, experiments were performed demonstrating that treatment of B cells with either intact anti-Ig Ab or $F(ab')_2$ blocks ligand-mediated internalization of CD22. In conclusion, these studies demonstrate that internalization of CD22 is dependent on its association with the AP-2 complex via tyrosine-based internalization motifs. ***The Journal of Immunology*****, 2003, 170: 3534–3543.**

CD22 is a B cell-restricted transmembrane glycoprotein that exhibits lectin-like properties and mediates homotypic as well as heterotypic cell:cell adhesion by binding to α-2,6 sialic acid-containing glycoproteins (1, 2). Besides its role as a sialoadhesin, CD22 has been shown using both cell lines and whole animal models to function as a coreceptor that modulates signaling via the B cell Ag receptor (BCR)[4] complex (3, 4). Studies have demonstrated that CD22 physically interacts with the BCR at low stoichiometry and is rapidly phosphorylated on tyrosine residues in response to cross-linking of the BCR (5, 6). Tyrosine phosphorylation of CD22 leads to the recruitment of multiple intracellular Src homology 2 (SH2) domain-containing effector molecules, including the protein tyrosine phosphatase SHP-1, phospholipase C γ, phosphoinositide 3-kinase, Grb2, and the protein tyrosine kinase Syk (7–10). Thus, both stimulatory as well as inhibitory effector proteins are recruited to CD22.

The physiologic relevance underlying the recruitment of multiple effector proteins to CD22 has yet to be completely elucidated. Recent studies have demonstrated that CD22 transduces BCR-independent signals leading to activation of the c-Jun N-terminal kinase/stress-activated protein kinase signaling cascade, suggesting that it may positively affect B cell activation (11, 12). Alternatively, numerous studies support the conclusion that recruitment of SHP-1 to the CD22/BCR complex results in attenuation of signal transduction (13). Independent ligation of CD22 using immobilized anti-CD22 mAb was observed to potentiate B cell proliferation in response to anti-Ig and IL-4, and actually decreased the threshold of stimulus required for optimal activation by more than 10-fold (21). This finding was interpreted as providing evidence that sequestration of CD22, and thus SHP-1, away from the BCR leads to enhanced signal transduction. Additional studies have demonstrated that B cells from CD22-deficient mice ($CD22^{-/-}$) are hyperresponsive to acute cross-linking of the BCR and exhibit many characteristics common to chronically stimulated anergic cells (14–17). Moreover, $CD22^{-/-}$ mice exhibit increased autoantibody production (16, 18). Therefore, alterations in CD22 expression or function are likely to contribute to the development of autoimmune disease. This is further supported by studies in Lyn-deficient mice that develop autoimmune glomerulonephritis (19, 20). In these mice, CD22 is not phosphorylated and does not recruit SHP-1 to the CD22/BCR complex, presumably resulting in hyperstimulation of B cells leading to autoantibody production (21–24).

The results of physiologic studies with $CD22^{-/-}$ mice are consistent with the hypothesis that CD22 is involved in regulating the threshold of signaling via the BCR. It is logical to predict that the ability of CD22 to modulate BCR-dependent signaling may be regulated by the spatial relationship between these receptors in the

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[4] Abbreviations used in this paper: BCR, B cell Ag receptor; AD, activation domain; BD, binding domain; CD, β-methyl-cyclodextrin; PTyr, phosphotyrosine; SH2, Src homology 2; YPD, yeast extract-peptone-dextrose; PIR-B, paired Ig receptor B.

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plasma membrane, or by regulating the level of CD22 expression on the cell surface, or both. Studies have shown that CD22 is an effective target for treatment of B cell tumors because binding of mAbs leads to efficient internalization and intracellular routing of immunotoxins (25–27). CD22 undergoes constitutive internalization and is subsequently degraded in an acidic intracellular compartment without recycling back to the surface (28). In contrast, binding of mAb leads to a significant increase in the rate of CD22 internalization without a concomitant increase in intracellular degradation (28). These studies suggest that in a physiological setting, binding of ligand could promote a transient decrease in the expression of CD22, thereby attenuating its ability to modulate BCR-dependent signal transduction. Thus, it is of interest to delineate the specific mechanism by which ligand-mediated internalization of CD22 occurs.

Clathrin-mediated endocytosis is a well-characterized process in which transmembrane proteins are selectively internalized (29, 30). Clathrin-mediated internalization involves a process in which receptors are concentrated in clathrin-coated pits and are subsequently transported to endosomal compartments by coated vesicles that bud from the plasma membrane. It has been shown that specific internalization motifs within the cytoplasmic domain of receptors are required for recruitment of the receptor to clathrin-coated pits. These internalization motifs are tyrosine based, with the consensus sequence YXX∅ (∅ is a hydrophobic residue), and mediate an association with AP50, the medium subunit of the clathrin-associated AP-2 adapter complex (31–33). In the present study, we demonstrate that CD22 specifically associates with the AP-2 complex via binding to AP-50 and that this interaction is required for internalization.

Materials and Methods

Biological reagents

The mAbs used in these studies were: CY34 (mouse IgG1, anti-mouse CD22.2), NIM-R6 (rat IgG1, anti-mouse CD22), RG7/9.1 (mouse IgG2b, anti-rat κ L chain), and OKT3 (mouse IgG, anti-human CD3). The mAbs were purified from tissue culture supernatants using protein G-Sepharose 4B fast flow (Amersham Pharmacia Biotech, Piscataway, NJ) and were biotinylated, as previously described (34). Ab directed against α-adaptin (clone 100/2, mouse IgG2a, recognizes the AP-2 complex) was purchased from Sigma-Aldrich (St. Louis, MO). The anti-phosphotyrosine (PTyr) mAb 4G10 (mouse IgG2b) coupled to HRP was purchased from Upstate Biotechnology (Lake Placid, NY). The mouse anti-rat IgG1 mAb RG11/39.4 (IgG2b) coupled to FITC and CY34 conjugated to PE were purchased from BD PharMingen (San Diego, CA). Polyclonal goat anti-mouse IgG coupled to HRP was purchased from Biosource International (Camarillo, CA).

cDNA constructs

The cDNA encoding mouse CD22.2 was obtained from E. Clark (Primate Center, University of Washington, Seattle, WA). The cDNA encoding full-length mouse paired Ig receptor B (PIR-B) was obtained from H. Kubagawa (Department of Pathology, University of Alabama, Birmingham, AL). For transfection studies using Jurkat cells, the full-length cDNA encoding CD22 was subcloned into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) to generate p3CD22. To generate specific CD22 mutants, the QuikChange mutagenesis kit from Stratagene (La Jolla, CA) was used, as directed by the manufacturer, to introduce changes into the CD22 cDNA contained in pcDNA3.1. The carboxyl-terminal tyrosine motifs containing Y843 and Y863 were mutagenized either singly or in tandem to change the tyrosine residues to alanine (Fig. 1). An additional mutant was generated in which both tyrosines were mutated to phenylalanine. This mutant CD22 molecule was used only in the α-adaptin coimmunoprecipitation experiment. Microdeletion mutants were also made in which the Y843 and Y863 motifs were removed singly or in tandem. For these mutations, the tyrosine residue and three downstream, flanking amino acids were removed (Y843-L846 and Y863-L866). Additional mutations were introduced into the CD22 cDNA to create tandem premature stop codons in the cytoplasmic tail. Premature stop codons were introduced at aa positions 732 and 733 to truncate the CD22 cytoplasmic tail upstream of the membrane-proximal tyrosine residue (Trp^{733}), thereby eliminating all six tyrosine motifs. Similarly, premature stop codons were introduced at aa 843 and 844 to truncate the cytoplasmic domain just before Tyr^{843} (Fig. 1).

For yeast two-hybrid studies, PCR was used to amplify cDNA fragments that encode the complete cytoplasmic domain of CD22 and PIR-B. These cDNAs were then subcloned in-frame into the yeast two-hybrid GAL4 binding domain (BD) vector pGBT9 (Clontech Laboratories, Palo Alto, CA) to generate the BD-CD22.cyto and BD-PIR-B.cyto constructs for use in the yeast two-hybrid assay. The resultant vectors were subjected to DNA sequencing to ensure that there were no errors introduced into the CD22 or PIR-B coding sequences during PCR, and to ensure that the CD22 and PIR-B cDNAs had been ligated in-frame to the GAL4 BD coding sequence. The QuikChange mutagenesis kit from Stratagene was used to introduce premature stop codons as well as single amino acid changes into the cytoplasmic domain of CD22. Premature stop codons were engineered at aa positions 840 (BD-CD22:840Stop), 814 (BD-CD22:814Stop), and 770 (BD-CD22:770Stop), thereby removing two, four, or six of the tyrosine motifs in the cytoplasmic tail, respectively. Two site-specific mutations were introduced in which Tyr^{843} and Tyr^{863} of CD22 were changed to phenylalanine to generate BD-CD22:Y843F and BD-CD22:Y863F, respectively.

Cell lines

The human leukemic T cell line Jurkat was maintained in RPMI 1640 supplemented with 5% FBS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 50 μM 2-ME, 100 μg/ml streptomycin-penicillin, and 50 μg/ml gentamicin (Sigma-Aldrich). For CD22 internalization studies, Jurkat cells were transfected either with the empty pcDNA3.1 vector or with pcDNA3.1 containing the cDNAs encoding wild-type or mutated CD22. For transfection, 1×10^7 Jurkat cells were resuspended in 500 μl of IMDM and were electroporated with 10 μg of cDNA using a Bio-Rad Gene Pulser II electroporator (Hercules, CA) with settings of 960 mF and 0.25 KV. After 48 h, transfected cells were selected in medium containing 1 mg/ml G418 (Life Technologies, Grand Isle, NY). Drug-resistant transfectants were analyzed for expression of CD22 using immunofluorescence staining with biotinylated NIM-R6 anti-CD22 mAb and streptavidin-PE. Fluorescence-activated cell sorting was used to isolate bulk populations of transfected cells that expressed comparable levels of wild-type or mutant CD22 molecules on their surface. All transfectant populations were maintained in complete RPMI 1640 with 250–500 μg/ml G418. The B cell lines K46-17μmλ, A20.1, and IIA1.6, a FcγRIIb receptor-negative variant of A20.1, were maintained in IMDM, supplemented as described previously.

Mice and isolation of splenic B cells

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used at 9–12 wk of age. Splenic B cells were isolated, as previously described (35). Briefly, after preparing a single cell suspension of splenocytes, RBCs were lysed using Gey's solution. Next, T cells were depleted by complement-mediated lysis using a mixture of anti-Thy-1 mAbs HO13.4.9 and T24/4 plus low tox rabbit C′ (Life Technologies, Grand Island, NY), 10 μg/ml DNase, and 5 mM $MgCl_2$ at 37°C for 45 min. B cells were washed three times in complete IMDM

Yeast two-hybrid assay

The yeast strains HF7c and SFY526 (Clontech) were maintained on yeast extract-peptone-dextrose (YPD) agar plates. For transformation, yeast were grown on YPD medium before collection and resuspension in YPD with glucose (2% final concentration). The yeast were incubated at 30°C for 3–4 h with shaking. The cells were centrifuged and then resuspended in 1 ml of 100 mM LiAc and incubated for 5 min at 30°C. Next, the yeast were pelleted and the supernatant was removed. The following reagents were added sequentially to the yeast pellet: 240 μl of polyethylene glycol (50% w/v), 36 μl 1 M LiAc, 25 μl salmon sperm DNA (2 mg/ml), 5 ml of plasmid DNA (100 ng to 5 μg), and 45 ml of sterile H_2O. The yeast were then vortexed for 1 min and incubated at 42°C for 20 min. Afterward, the yeast were pelleted, resuspended in sterile water, and plated on synthetic dropout medium to select for transformants. The plates were incubated at 30°C for 2–4 days to allow for growth of yeast.

For the yeast two-hybrid assay, the BD-CD22.cyto or BD-PIR-B.cyto constructs were cotransformed into yeast with the pACT GAL4 activating domain (AD), which contained the cDNA encoding AP50 to produce an AD-AP50 fusion protein. Yeast that contained both GAL4 BD and AD constructs were selected on double-deficient medium lacking tryptophan and leucine. Potential interactions between CD22 or PIR-B and AP50 were assayed by plating yeast on selection medium lacking tryptophan, leucine, and histidine. An interaction between the GAL4 BD and AD fusion proteins results in the transcription of HIS3 and *lacZ* reporter genes conferring growth on defined medium lacking histidine. Yeast that exhibited growth

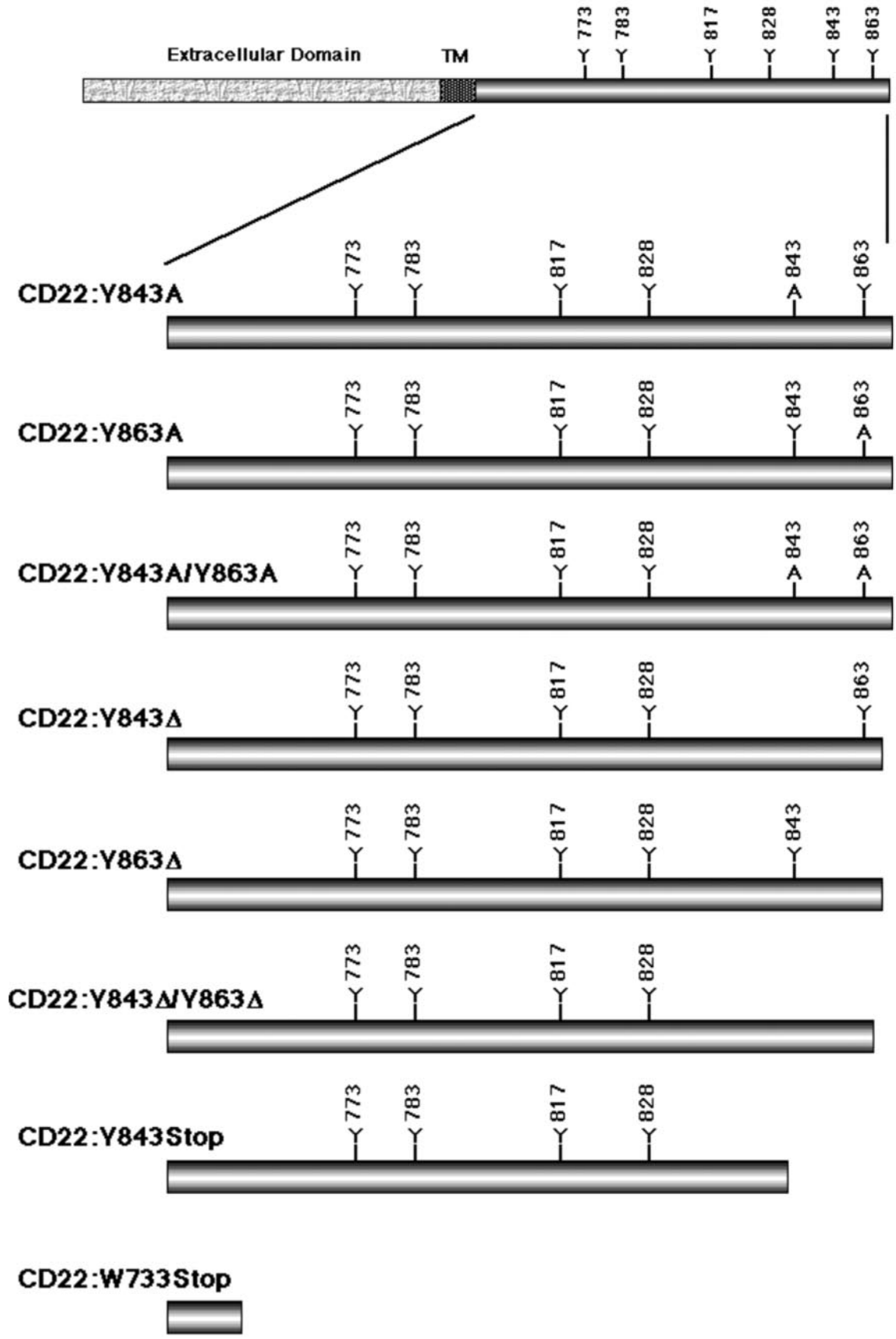

FIGURE 1. Schematic representation of CD22 mutants generated for use in internalization experiments. The full-length cDNA encoding CD22 was subcloned into the pcDNA3.1 expression vector and was subsequently mutagenized using the QuikChange mutagenesis kit from Stratagene. Tyr^{843} and Tyr^{863} were mutated to alanine either separately or together. Additional mutations were introduced in which the two carboxyl-terminal tyrosine motifs containing Tyr^{843} and Tyr^{863} were deleted singly or in combination. For microdeletions, the tyrosine residue and the three flanking amino acids on the carboxyl side were removed. Finally, the CD22 cytoplasmic domain was truncated at Tyr^{843} or Trp^{733} by the introduction of tandem stop codons.

on histidine-deficient medium were tested for β-galactosidase activity using either a colony lift filter-based assay, or a liquid-based assay to obtain semiquantitative results. The filter-based β-galactosidase assay was performed as described in the MATCHMAKER Two-Hybrid System manual (Clontech). In selected experiments, the SFY526 strain of yeast was cotransformed with GAL4 AD-AP50 and BD-CD22 constructs to measure activation of the *lacZ* gene using a liquid-based assay, as described in the MATCHMAKER Two-Hybrid System manual. For both assays, *o*-nitrophenyl β-D-galactopyranoside was used as a substrate. One β-galactosidase unit is defined as the amount of enzyme that hydrolyzes 1 μmol of ONPG to *o*-nitrophenol and D-galactose per minute.

Immunoprecipitation and immunoblotting

For experiments to monitor tyrosine phosphorylation of CD22 expressed in β cells, 2×10^7 β cells/sample were incubated in medium alone or in the presence of either anti-IgM mAb (B76 10 μg/ml) or pervanadate for varied periods of time at 37°C. Experiments to monitor coprecipitation of α-adaptin with CD22 also used 2×10^7 Jurkat cells/sample. The cells were washed in ice-cold PBS and lysed in 0.5 ml of lysis buffer (25 mM HEPES, pH 7.8, 150 mM NaCl, 10 mM EDTA, 0.1 mM Na_3VO_4) containing 1% Nonidet P-40. The cell lysates were incubated on ice for 1 h and then centrifuged at 13,000 × *g* for 15 min at 4°C. Detergent-soluble lysates were precleared by the addition of RG7 mAb conjugated to Sepharose 4B beads (Amersham Pharmacia Biotech) for 1 h at 4°C. CD22 was immunoprecipitated from precleared Jurkat lysates by the addition of CY34 conjugated to Sepharose 4B beads, after which the lysates were incubated at 4°C overnight. Immune complexes bound to beads were collected and washed five times with lysis buffer containing 0.2% Nonidet P-40. The beads were resuspended in 25 μl of SDS-PAGE sample reducing buffer, boiled for 4 min, and centrifuged at 13,000 × *g*. Immune complex proteins were separated by SDS-PAGE on 10% acrylamide gels, and the proteins were then transferred to Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech).

To detect tyrosine phosphorylation of CD22, the membranes were blocked by incubation in 3% blot-qualified BSA (Promega, Madison, WI) in TBST for 1 h at room temperature. The membranes were then washed extensively in TBST and incubated with the anti-PTyr mAb 4G10 coupled to HRP for 1 h at room temperature. The membranes were washed with TBST, and phosphorylation of CD22 was visualized using ECL with Supersignal reagent (Pierce, Rockford, IL). To detect α-adaptin in CD22 immune complexes, membranes were blocked in TBST with 10% milk for 1 h at room temperature. The membranes were then washed extensively in TBST and incubated with mAb against α-adaptin for 1 h at room temperature. Next, the membranes were washed in TBST and incubated with polyclonal goat anti-mouse IgG coupled to HRP. The presence of α-adaptin in CD22 immune complex material was visualized using ECL.

CD22 internalization assays

CD22 internalization assays were performed using Jurkat T cells (1×10^6/sample) that had been transfected with either wild-type or mutant forms of murine CD22 or the B cell lines K46-17μmλ, A20.1, and IIA1.6, all of which express endogenous CD22. The expression of CD22 on Jurkat cell lines was assessed using immunofluorescence staining, and the cells were sorted to select for bulk populations that expressed comparable levels of CD22. For these experiments, the level of CD22 expressed on transfected Jurkat cell lines was comparable to that expressed on the surface of the B cell lymphoma K46-17μmλ (data not shown). Experiments were performed to monitor ligand-induced internalization of CD22. To monitor ligand-mediated internalization, cells were incubated in medium alone at 37°C for 30 min. Different concentrations of anti-CD22 mAb (NIM-R6, 1–20 μg/ml) were then added, after which control and Ab-treated cells were incubated at 37°C for up to 60 min. At the appropriate time points, cells were removed from the incubator, washed in ice-cold staining buffer, and placed on ice. The cells were next stained with a distinct anti-CD22 mAb (CY34) coupled to PE. The cells were washed three times in staining buffer and were fixed in staining buffer containing 2% paraformaldehyde. It should be noted that the NIM-R6 and CY34 mAbs recognize distinct nonoverlapping epitopes on CD22 and therefore do not block binding of one another. Surface expression of CD22 was analyzed by flow cytometry. The relative level of CD22 expression was calculated based on the amount of CD22 expressed by cells that had been freshly harvested from tissue culture and stained immediately with CY34-PE on ice.

Results

CD22 interacts with AP50 via a tyrosine-based internalization motif

The yeast two-hybrid assay was used to identify proteins that interact with CD22 via phosphorylation-independent processes. These studies demonstrated that the cytoplasmic domain of CD22 is able to interact with AP50, the medium chain subunit of the AP-2 complex. The cytoplasmic tail of CD22 cloned into the GAL4 BD vector pGBT9 interacts with AP50 contained in the complementary GAL4 AD vector pACT, as determined by growth on histidine-deficient medium and the *lacZ* assay. In contrast, the cytoplasmic tail of the inhibitory protein PIR-B, which contains five tyrosine motifs and binds SHP-1 in a manner similar to CD22, did not interact with AP50 (data not shown). This result suggests that the interaction between CD22 and AP50 is specific.

Mutational analysis of the cytoplasmic domain of CD22 was performed to map the site of interaction with AP50. Initially, the cytoplasmic tail of CD22 was truncated by insertion of premature stop codons. Truncation of the cytoplasmic domain to generate BD-CD22Δ840 resulting in elimination of the fifth and sixth tyrosine motifs (Tyr^{843} and Tyr^{863}) abrogated the interaction with AP50 (Fig. 2). Subsequently, point mutations were introduced in which either Tyr^{843} or Tyr^{863} was changed to Phe. Mutation of Tyr^{863} to Phe had no effect on the interaction with AP50, whereas mutation of Tyr^{843} resulted in a significant decrease in the interaction between the BD-CD22Y843F and AD-AP50 fusion proteins

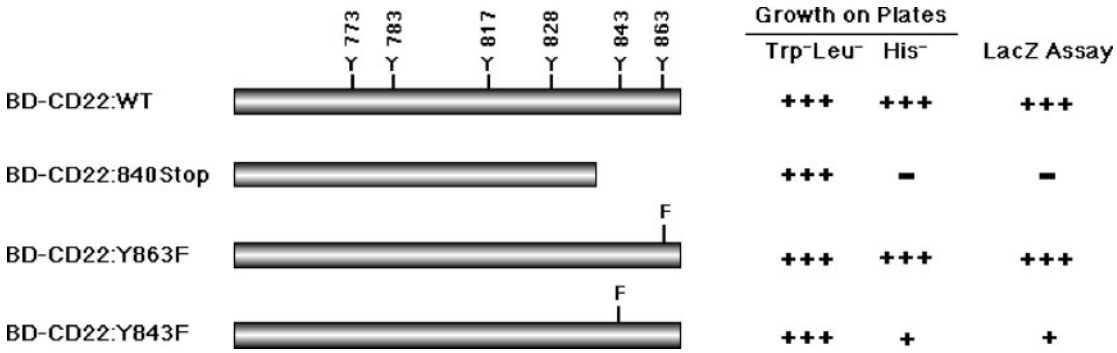

FIGURE 2. AP50 primarily interacts with Tyr^{843} in the cytoplasmic domain of CD22 based on yeast two-hybrid analysis. The full-length cDNA encoding the cytoplasmic domain of CD22 was subcloned into pGBT9. The QuikChange mutagenesis kit was used to introduce premature stop codons to truncate the cytoplasmic domain or to mutate selected Tyr residues to Phe. The cDNA encoding AP50 was subcloned into the pGAD424 vector. The HF7C strain of yeast was cotransformed with CD22 and AP50 vectors and was selected on double-deficient medium to ensure uptake of the respective plasmids. Colonies that grew on double-deficient medium were then transferred to histidine-deficient medium to monitor the interaction between AP50 and CD22. Colonies that grew on histidine-deficient medium were subjected to a *lacZ* assay to confirm the interaction between AP50 and CD22.

in the yeast two-hybrid assay. Subsequent experiments were performed in which a liquid β-galactosidase assay was performed to obtain a semiquantitative evaluation of the relative binding between BD-CD22:WT and AD-AP50 vs BD-CD22:Y843F and AD-AP50. The results shown in Fig. 3 are representative of three independent experiments and reveal that mutation of Tyr^{843} to Phe causes a 6-fold decrease in the β-galactosidase activity detected after cotransformation of yeast with the respective fusion protein constructs. These results indicate that AP50 primarily binds to CD22 via a tyrosine-dependent motif that encompasses Tyr^{843}. However, it is apparent that the interaction between CD22 and AP50 may involve additional flanking amino acids because mutation of Tyr^{843} alone is not sufficient to completely abrogate the association. It is equally possible that AP50 binds weakly to the carboxyl-terminal motif containing Tyr^{863}, and thus has the ability to interact with CD22 even though Tyr^{843} has been mutated. Finally, because it is unlikely that the CD22 cytoplasmic domain is phosphorylated in yeast, it is logical to conclude that the interaction between AP50 and CD22 is phosphorylation independent.

Internalization of CD22 is dependent on the interaction with the AP-2 complex

Because CD22 interacts with AP50, it is likely that the AP-2 complex is involved in recruitment of CD22 to clathrin-coated pits, thereby promoting constitutive endocytosis or ligand-induced internalization, or both. To explore this process further, experiments were performed using Jurkat cells transfected either with wild-type CD22 or specific CD22 mutants. All transfectant Jurkat cell lines were analyzed by immunofluorescence staining and flow cytometry to monitor the expression of CD22. Cell lines expressing wild-type CD22, and various truncation or point mutants of CD22 were subjected to fluorescence-activated cell sorting to isolate bulk populations of cells that expressed comparable levels of CD22 on their surface (data not shown). Previous studies have demonstrated that treatment of B cells with anti-CD22 mAb promotes internalization of CD22 (28). Based on this, experiments were performed to confirm that treatment with anti-CD22 mAb promotes internalization of CD22 on Jurkat cells in a manner similar to that observed for the K46-17μmλ B lymphoma cell line. As can be seen in Fig. 4*A*, CD22 expression on Jurkat cells incubated in medium alone remained stable over a period of 60 min. In contrast, treatment of Jurkat cells with anti-CD22 mAb led to internalization of CD22 in a time- and dose-dependent manner, in which optimal internalization of CD22 was induced by the addition of 20 μg/ml of anti-CD22 mAb. Similar results were obtained when the K46-17μmλ cell line was incubated with anti-CD22 mAb (Fig. 4*B*).

To confirm that the carboxyl-terminal tyrosines in the cytoplasmic tail of CD22 are indeed important for the association between CD22 and the AP-2 complex, experiments were performed to determine whether wild-type or mutant forms of CD22 coprecipitate with α-adaptin, another component of the AP-2 complex. Transfectant Jurkat cell lines expressing CD22:WT, CD22:733Stop, or CD22:Y843F/Y863F were lysed in buffer containing 1% Nonidet P-40, and CD22 was precipitated with anti-CD22 mAb (CY34) and protein G-Sepharose. As seen in Fig. 5, α-adaptin was observed to coprecipitate with wild-type CD22, but not with CD22 lacking the cytoplasmic domain (CD22:733Stop). Mutation of the carboxyl-terminal tyrosine residues 843 and 863 resulted in a significant decrease in the amount of α-adaptin that coprecipitated

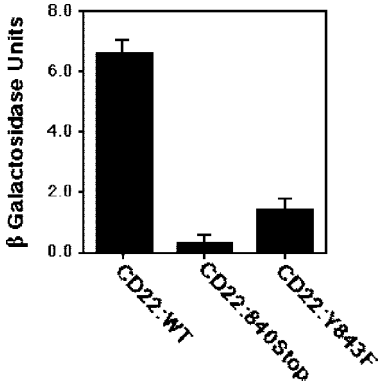

FIGURE 3. The tyrosine-based internalization motif containing Tyr^{843} is the primary binding site for AP50. To further quantitate the interaction between AP50 and specific CD22 mutants, CD22 and AP50 constructs were cotransformed into the SFY526 strain of yeast, after which the yeast were selected on double-deficient medium to ensure uptake of the two plasmids. Cotransformed yeast were then subjected to a liquid β-galactosidase assay, as described in *Materials and Methods*, to measure the interaction between CD22 and AP50. Color formation is presented as arbitrary β galactosidase units. The data represent the mean ± SEM for triplicate samples.

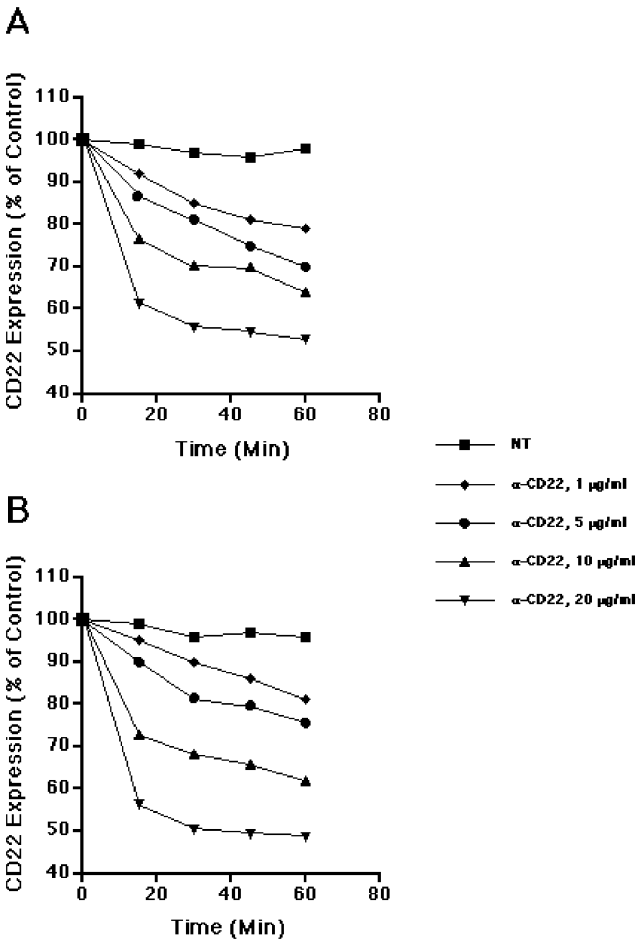

FIGURE 4. Treatment of cells with anti-CD22 mAb promotes internalization of CD22 in a time- and dose-dependent manner. Jurkat T cells (*A*) or K46-17μmλ B cells (*B*) were incubated in medium alone or in the presence of anti-CD22 mAb (NIMR-6, 1–20 μg/ml) for varied periods of time up to 1 h. The cells were harvested and washed in ice-cold staining buffer, after which they were stained with CY34 (anti-CD22 mAb) conjugated to PE. CD22 surface expression was assessed by flow cytometry. The data are depicted as the percentage of control CD22 expression. Control expression is defined as the amount of CD22 expressed on the surface of cells that were freshly harvested and stained immediately with CY34 conjugated to PE. The data are representative of three separate experiments.

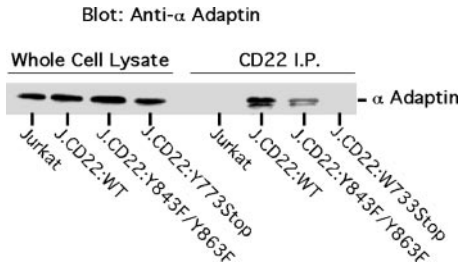

FIGURE 5. The interaction between CD22 and the AP-2 complex involves the carboxyl-terminal tyrosine motifs that encompass Tyr^{843} and Tyr^{863}. Transfectant Jurkat cells expressing wild-type or mutated forms of CD22 (1×10^7/sample) were lysed in 0.5 ml of buffer containing 1% Nonidet P-40 on ice for 1 h. Cell lysates were centrifuged at 13,000 × *g*, after which 20 μl of whole cell lysate was removed. The remaining lysate was precleared with RG7 mAb coupled to Sepharose 4B beads. Next, the lysates were incubated with anti-CD22 mAb (NIMR-6) coupled to Sepharose 4B beads to immunoprecipitate CD22. The immune complex material was boiled in SDS-PAGE sample buffer and separated on 10% acrylamide gels by SDS-PAGE. Whole cell lysate material was boiled with SDS-PAGE sample buffer and separated as well. The proteins were transferred to nitrocellulose, and the membrane was blocked in TBST with 10% milk. The membranes were probed with anti-α-adaptin mAb, washed, and then incubated with a secondary goat anti-mouse Ig Ab conjugated to HRP. Coprecipitation of α-adaptin was visualized using ECL.

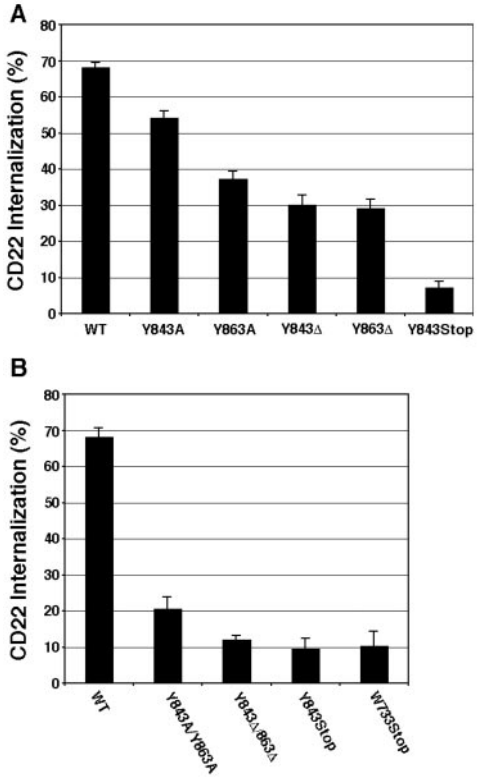

FIGURE 6. AP50 can functionally interact with either Tyr^{843} or Tyr^{863} and their respective flanking amino acids to mediate ligand-dependent internalization of CD22. Jurkat cells were transfected with cDNA encoding wild-type or mutant forms of CD22. *A*, Internalization analysis of CD22 molecules containing a single mutation in either tyrosine motif 5 or 6 compared with a truncation mutant that completely eliminates both the fifth and sixth tyrosine motifs. *B*, Internalization analysis of CD22 mutants that contain mutations in both the fifth and sixth tyrosine motifs compared with truncation mutants that eliminate either the two carboxyl-terminal tyrosines (Y843Stop) or all six cytoplasmic tyrosines (W733Stop). All transfectants were selected in medium with G418 and sorted using FACS to obtain populations of cells with comparable levels of CD22 expression. The transfectant cell lines (1×10^6/sample) were incubated in 1 ml of medium containing NIRM-6 (anti-CD22 mAb, 20 μg/ml) for 30 min at 37°C. The cells were harvested, washed in ice-cold staining buffer, and stained with CY34 conjugated to PE. CD22 expression is depicted as percentage of CD22 internalization and was calculated, as described previously. The data represent the mean ± SEM for triplicate samples. The results are representative of three independent experiments.

with CD22, but did not completely abrogate the association with the AP-2 complex. This finding suggests that, although Tyr^{843} and Tyr^{863} are important residues in the motifs recognized by AP50, other residues flanking Tyr^{843}, and possibly Tyr^{863}, are clearly involved in mediating binding. Moreover, previous studies raise the possibility that mutation of Tyr residues to Phe may not completely disrupt the AP50 binding motif as effectively as would have been the case if the Tyr residues were mutated to Ala. The specificity of the interaction between CD22 and α-adaptin was further demonstrated by the observation that α-adaptin could not be detected in immunoprecipitates from parental Jurkat cells lacking CD22. Thus, α-adaptin was not recovered due to nonspecific binding to either the anti-CD22 mAb or the bead matrix. In conclusion, these experiments confirm that CD22 associates with the AP-2 complex in vivo, presumably through a direct association with AP50. Furthermore, the data support the yeast two-hybrid studies, which indicate that the interaction involves one or both carboxyl-terminal tyrosine-based motifs of CD22.

Additional studies were performed to assess the effect of introducing mutations into the carboxyl terminus of CD22 on mAb-mediated internalization. A series of mutations were generated in the cytoplasmic domain of CD22, as depicted in Fig. 1, that resulted in alterations to either the fifth or sixth tyrosine-containing motif or both. Transfectant Jurkat cell lines that expressed comparable levels of wild-type or mutant forms of CD22 were incubated in the presence of anti-CD22 mAb for varied periods of time, as described in *Materials and Methods*. Surface expression of CD22 was assessed by flow cytometry, and the relative amount of internalization between control and mAb-treated samples was calculated. As can be seen in Fig. 6*A*, mutation of either Tyr^{843} or Tyr^{863} to alanine had a modest effect on internalization of CD22. This suggested that mutation of either tyrosine alone fails to block binding of AP50 to the other motif or to flanking amino acids within the same motif. To test this, additional mutants were generated in which the entire motif was deleted. Although deletion of either the fifth or sixth motif more effectively attenuated internalization of CD22 when compared with the Y843A and Y863A mutants, significant internalization of CD22 was still observed when cells were incubated in the presence of anti-CD22 mAb. In contrast, truncation of the cytoplasmic tail of CD22 just before Tyr^{843} inhibited internalization to a significant extent when compared with wild-type CD22 or any of the single motif mutants (Fig. 6*A*). These results support the hypothesis that mutation of either the fifth or sixth tyrosine motif in the cytoplasmic domain is not sufficient to prevent internalization, presumably due to the fact that AP50 has the ability to interact with either motif alone. The generation of constructs encoding CD22 that contains dual mutations in both the fifth and sixth tyrosine motifs was performed to further test this hypothesis. As can be seen in Fig. 6*B*, mutation of Tyr^{843} and Tyr^{863} to Ala inhibits CD22 internalization to a greater extent than mutation of either Tyr residue alone, although not to the extent seen with the CD22:Tyr^{843}Stop mutant. Deletion of the fifth and sixth motifs results in inhibition of CD22 internalization to a degree that is comparable to that observed for the CD22:

Tyr^{843}Stop mutant. This confirms that the carboxyl-terminal tyrosine residues that include Tyr^{843} and Tyr^{863}, as well as the respective flanking amino acids, are critical for AP50 binding and internalization in response to ligand binding.

Although the CD22:Tyr^{843}Stop mutation significantly decreased internalization of CD22 in response to anti-CD22 mAb, it did not completely block internalization. Previous studies have shown that clathrin-coated pit-dependent internalization can be blocked by cholesterol depletion using β-methyl-cyclodextrin (CD) (36, 37). CD was used to treat Jurkat cells expressing both wild-type CD22 and the CD22:Y843Stop mutant to determine whether the residual internalization observed with the mutant was affected by cholesterol depletion. As can be seen, ligand-mediated internalization of wild-type CD22 was inhibited by CD to a level comparable to that seen for the CD22:Y843Stop mutant in the presence of anti-CD22 mAb alone (Fig. 7). Treatment of cells expressing the CD22:Y843Stop mutant did not significantly decrease the residual level of CD22 internalization. These data indicate that the residual CD22 internalization observed for the CD22:Y843Stop mutant is insensitive to cholesterol depletion and therefore may be due to a clathrin-coated pit-independent mechanism. This conclusion was further supported by internalization experiments with cells expressing the CD22:W733Stop mutant. Mutant CD22 molecules that are truncated just before the first Tyr motif in the cytoplasmic tail exhibit internalization that is comparable to that observed for the CD22:Tyr^{843}Stop mutant (Fig. 6*B*). Because the CD22:W733Stop mutant does not contain any tyrosines in its cytoplasmic domain and clearly does not interact with the AP-2 complex (Fig. 5), the residual internalization is probably caused by a clathrin-coated pit-independent mechanism.

Internalization of CD22 is inhibited by cross-linking of the BCR

Previous studies have shown that binding of AP50 to tyrosine-based internalization motifs is blocked in response to phosphorylation of the tyrosine residue within the internalization motif (38). It has also been demonstrated that cross-linking of the BCR promotes tyrosine phosphorylation of CD22 and that the carboxyl-terminal motifs are involved. Therefore, studies were performed to determine whether cross-linking of the BCR blocks internalization in response to anti-CD22 mAb treatment. Normal splenic B cells were incubated in the presence of anti-CD22 mAb (20 μg/ml) with

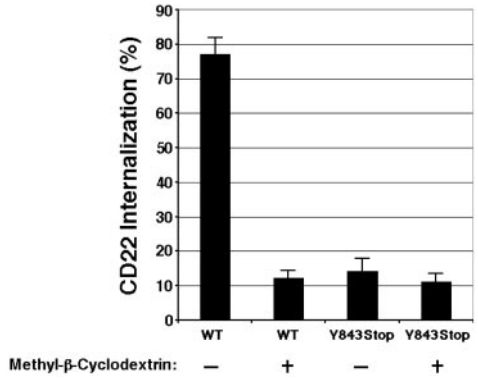

FIGURE 7. mAb-mediated internalization of wild-type, but not Y843Stop CD22 is blocked by pretreatment of cells with CD. Transfectant Jurkat cell lines were incubated with CD (10 mM) for 15 min before the addition of anti-CD22 mAb (NIMR-6, 20 μg/ml). The cells were incubated at 37°C for 30 min, after which they were washed and stained with CY34 conjugated to PE. Incubation of cells with CD alone had no effect on CD22 expression over the 30-min time period (data not shown). The data represent the mean ± SEM and are representative of two experiments.

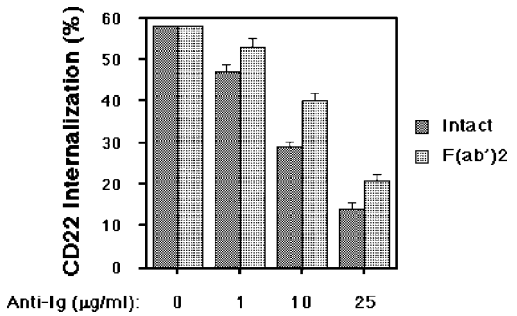

FIGURE 8. Cross-linking of the BCR blocks mAb-mediated internalization of CD22. Normal splenic B cells (1×10^6/sample) were isolated and incubated in the presence of anti-CD22 mAb alone, or with increasing concentrations of intact or $F(ab')_2$ of polyclonal anti-Ig Ab (1–25 μg/ml) for 15 min at 37°C. The cells were then harvested, washed in ice-cold staining buffer, and stained with CY34 conjugated to PE. The data are presented as the mean ± SEM of the percentage of CD22 internalized.

or without polyclonal anti-murine Ig Ab, after which CD22 expression was assessed by flow cytometric analysis. Whereas treatment of B cells with anti-CD22 alone promoted active internalization of CD22, addition of either intact anti-Ig or $F(ab')_2$ significantly inhibited internalization in a dose-dependent manner (Fig. 8). Inhibition of CD22 internalization correlated with tyrosine phosphorylation of CD22, as determined by Western blotting (data not shown). The observation that both intact and $F(ab')_2$ inhibit CD22 internalization supports the conclusion that FcγRIIb receptor engagement does not play a major role in inhibiting CD22 internalization. However, it should be noted that intact anti-Ig Ab blocked internalization more effectively than $F(ab')_2$, suggesting that FcγRIIb receptor signaling may play a minor role in inhibition of CD22 internalization. To further corroborate the hypothesis that phosphorylation of CD22 blocks internalization, experiments were performed in which CD22 internalization was assessed using the A20.1 B lymphoma cell line and the FcγRIIb-deficient IIA1.6 variant. Anti-CD22 mAb-mediated internalization was monitored for both cell lines in the presence or absence of intact polyclonal anti-Ig Ab. Simultaneous incubation of the A20.1 and IIA1.6 variant cells with anti-CD22 mAb and intact anti-Ig polyclonal Ab blocked internalization to an equal extent in both cell lines (data not shown), suggesting that inhibition may be mediated predominantly by phosphorylation of CD22 as opposed to FcγRIIb-dependent signaling.

The inhibitory effect of anti-Ig Ab was observed to be transient in nature. Incubation of B cells in the presence of anti-CD22 mAb for 30 min or longer resulted in progressive internalization of CD22 even in the continued presence of anti-Ig Ab (Fig. 9*A*). This finding suggested that tyrosine phosphorylation of CD22 and the associated inhibition of AP50 binding may occur only transiently due to the activation of one or more feedback regulatory mechanisms. Western blot analysis to monitor tyrosine phosphorylation of CD22 confirmed that this was indeed true (Fig. 9*B*). Tyrosine phosphorylation of CD22 was significantly decreased by 30 min and was undetectable at 60 min. Thus, there was a direct correlation between tyrosine phosphorylation of CD22 and inhibition of its internalization in response to anti-Ig treatment.

Discussion

Previous studies have demonstrated that anti-CD22 mAbs effectively induce endocytosis of CD22 (28). These results suggest that ligand binding to CD22 may play an important role in modulating its expression on the B cell surface, thereby affecting its ability to

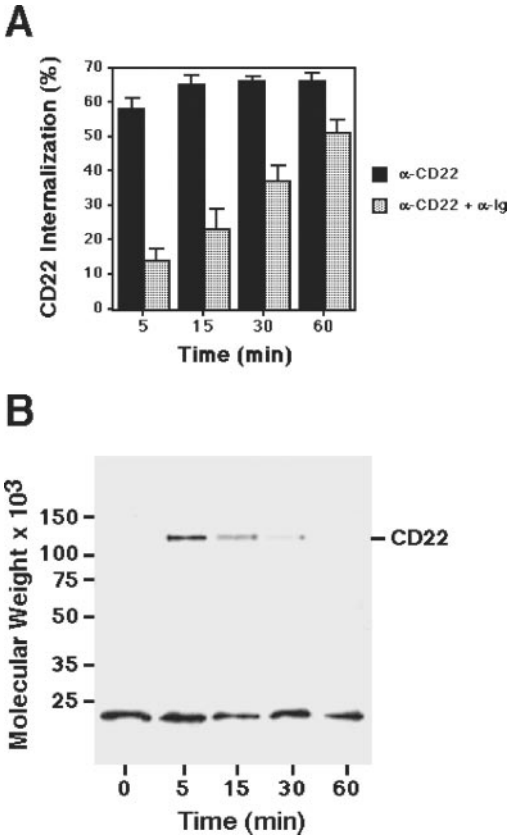

FIGURE 9. Inhibition of CD22 internalization in response to BCR cross-linking is transient. *A*, Analysis of CD22 internalization. Normal splenic B cells (1×10^6/sample) were isolated and incubated at 37°C in the presence of anti-CD22 mAb (20 μg/ml) with or without polyclonal anti-Ig Ab (10 μg/ml) for the time points indicated. The cells were harvested, washed in ice-cold staining buffer, and stained with CY34 conjugated to PE. The level of CD22 expressed on the surface of the cells was measured by flow cytometric analysis, and the data were presented as the mean ± SEM of the percentage of CD22 internalized. *B*, Analysis of CD22 tyrosine phosphorylation. Splenic B cells (1×10^6/sample) were incubated in the presence of anti-Ig Ab (10 μg/ml) for the periods of time indicated. The cells were washed in ice-cold PBS and lysed in buffer containing 1% Nonidet P-40. CD22 was immunoprecipitated from detergent-soluble lysates, after which SDS-PAGE was performed. CD22 was transferred to nitrocellulose and blotted with the anti-PTyr mAb 4G10 coupled to HRP. Tyrosine phosphorylation of CD22 was visualized using ECL.

regulate signal transduction via the BCR. In the present study, a yeast two-hybrid assay was used to demonstrate that AP50, the medium subunit of the AP-2 complex, interacts with the cytoplasmic domain of CD22. Mutational analysis revealed that the carboxyl-terminal portion of the CD22 cytoplasmic tail, encompassing tyrosine residues 843 and 863, is critical for binding of AP50 to CD22 in the yeast two-hybrid system. Additionally, this region was found to be essential for the interaction between CD22 and the AP-2 complex in Jurkat cells and for mAb-mediated internalization of CD22. Mutational analysis revealed that changing either Tyr^{843} or Tyr^{863} to Ala, or deletion with their respective flanking amino acids, significantly inhibited ligand-dependent internalization of CD22. However, these mutations did not completely abrogate mAb-mediated internalization of CD22 expressed on Jurkat cells. In contrast, mutation of the carboxyl terminus to change both Tyr^{843} and Tyr^{863} to Ala, or to delete the respective tyrosine-containing motifs, resulted in inhibition of internalization that was comparable to that observed for the CD22:Y843Stop mutant. These data indicate that AP50 can bind to either of the tyrosine-based motifs in the cytoplasmic domain of CD22 that encompass Tyr^{843} or Tyr^{863}, resulting in efficient targeting to clathrin-coated pits and endocytosis.

Although mutagenesis of the carboxyl-terminal tyrosine motifs significantly decreased CD22 endocytosis, residual internalization of CD22 was consistently observed with these mutants. However, this internalization was not sensitive to treatment of cells with CD, which has previously been shown to block clathrin-coated pit-dependent internalization (36). It is possible that the residual internalization observed is due to mAb-mediated capping of CD22, which could bypass AP-2-dependent targeting to clathrin-coated pits. Alternatively, a previous report studying internalization of human CD22α in response to anti-CD22 mAb has shown that internalization can be mediated by a membrane-proximal 11-aa motif (QRRWKRTQSQQ) (39). It is interesting to note that the cytoplasmic domain of CD22α lacks the 23 carboxyl-terminal aa found in human CD22β, which is analogous to the murine CD22 analyzed in these studies. This truncation, in addition to other amino acid changes, eliminates the three carboxyl-terminal tyrosine motifs found in CD22β that include Tyr^{828}, Tyr^{843}, and Tyr^{863} (40, 41). Thus, residual endocytosis of the CD22 mutants examined in this study may be mediated by a process that involves a membrane-proximal motif, which functions in a manner that is independent of AP-2-mediated targeting to clathrin-coated pits.

It has been demonstrated that sorting of receptors into the endocytic pathway via clathrin-coated pits involves the recognition of a tetrapeptide sequence by AP50 that characteristically contains a tyrosine flanked by two polar and one hydrophobic amino acid residue (YppØ) (31–33). The optimal sequence for AP50 binding contains a Tyr at the anchor position, an arginine at position Y + 2, and a leucine at position Y + 3; however, functional binding of AP50 to variations of this optimal motif has been observed (33). Although all six of the tyrosine motifs in the cytoplasmic tail of CD22 contain a hydrophobic amino acid in the +3 position, only two of the six motifs (i.e., those encompassing Tyr^{828} and Tyr^{843}) contain polar residues in both the +1 and +2 positions. Despite the fact that Tyr^{828} and its flanking amino acids exhibit characteristics of a tyrosine-based internalization motif, neither yeast two-hybrid analysis nor expression of CD22 mutants in Jurkat cells supports a role for Tyr^{828} in mediating binding of AP50. In contrast, the same analyses suggest that Tyr^{863} and its flanking amino acids constitute a functional internalization motif that interacts with AP50 even though it does not contain polar residues in both the +1 and +2 positions. Thus, it is not clear why AP50 selectively interacts with the two carboxyl-terminal motifs of CD22 and not other similar motifs that are more membrane proximal. However, it is theoretically possible that factors distinct from the amino acid sequence of specific motifs, including the proximity of Tyr^{843} and Tyr^{863} to the carboxyl terminus or the three-dimensional structure of the cytoplasmic tail, affect the ability of AP50 to engage specific tyrosine-based motifs in the CD22 cytoplasmic domain.

It has been documented that CD22 is involved in regulating signal transduction via the BCR as well as B cell trafficking (3, 4, 42). Therefore, it is logical to propose that modulation of CD22 expression in response to ligand binding could affect one or both of these biological functions in the B cell, although it remains to be determined whether ligand binding plays an active role in modulating CD22 expression in vivo. Additionally, it is likely that ligand-dependent modulation of CD22 may be regulated by the microenvironment that the B cell is in and the relative expression of cell-associated ligands vs soluble ligands in the plasma or interstitial fluid, as well as the developmental/differentiation state of the B cell. First, modulation of CD22 would presumably be associated

with binding to soluble ligands in the plasma or interstitial fluids. However, it is likely that soluble sialylglycoconjugates bind to CD22 with low avidity and would have to compete with highly clustered cell-associated sialic acid-containing ligands. Thus, the specific microenvironment in which the B cell is localized and the relative level of CD22 ligands expressed on adjacent cells may determine whether CD22 can bind to soluble vs cell-associated ligands. Studies have also shown that B cells express high levels of ST6GalI, the sialyltransferase that generates ligands (i.e., α2,6-sialylglycoconjugates) that are recognized by CD22 (2, 4, 5, 43, 44). Therefore, CD22 expressed on the surface of a majority of B cells is masked by *cis* interactions with α2,6-sialylated proteins, which prevents binding to ligands in the plasma or on other cells. It has been shown, however, that B cell activation is associated with unmasking of CD22 either due to decreased expression of ST6GalI or increased sialidase activity (45, 46). Thus, it is possible that B cell activation promotes binding of CD22 to ligands in trans, which could in turn lead to modulation of its expression. Studies suggest that the level of CD22 expression does indeed vary in selected B cell populations in response to activation. Immunization of mice with sheep RBCs leads to the generation of a $CD38^{dim/-}$ population of B cells that exhibit characteristics similar to germinal center cells, including high levels of PNA, CD95, and CD24 (47). This population of responding B cells is also characterized by decreased CD22 expression. In another study, a major subpopulation of memory B cells has been characterized that lack B220 and CD138 as well as CD22 (48). Whether these $CD22^{dim/-}$ B cell populations arise due to activation-dependent down-modulation of CD22 in response to ligand binding, or as a result of transcriptional or posttranslational regulation of CD22 expression is not known.

Previous studies have shown that Tyr^{843} and Tyr^{863} are involved in the recruitment of SHP-1 and Syk via their respective SH2 domains after tyrosine phosphorylation of CD22 (7–10). In contrast, studies using the yeast two-hybrid assay suggest that binding of AP50 to Tyr^{843} and Tyr^{863} occurs in the absence of tyrosine phosphorylation. Indeed, inducible phosphorylation of CD22 appears to correlate with inhibition of AP50 binding based on experiments demonstrating that stimulation of splenic B cells through the BCR results in tyrosine phosphorylation of CD22, the kinetics of which correlate with inhibition of CD22 internalization. It is formally possible that another mechanism could be involved, although the presumption that tyrosine phosphorylation of CD22 abrogates binding of AP50 is supported by previous studies demonstrating that binding of AP50 to the YVKM motif in the cytoplasmic tail of CTLA-4 is inhibited by tyrosine phosphorylation (49–51). It has been proposed that up-regulation of CTLA-4 expression in response to T cell stimulation is due to its phosphorylation, which precludes binding of AP50 while at the same time promoting recruitment of SH2 domain-containing effector proteins. Additionally, it has been shown using the Ramos and Daudi B cell lines that signaling through the BCR can lead to rapid, transient up-regulation of CD22 expression in a tyrosine phosphorylation-dependent manner (52). Inhibition of tyrosine kinase activation blocked up-regulation of CD22 expression, raising the possibility that phosphorylation of CD22 and inhibition of AP50 binding are partially responsible for the transient increase in expression. Alternatively, it is possible that phosphorylation of CD22 in response to BCR cross-linking maintains expression of CD22 on the surface of the cell, even in the presence of ligand(s) that might otherwise induce its internalization. This would effectively enhance the ability of CD22 to regulate BCR signaling by recruiting intracellular SH2 domain-containing effector proteins (e.g., SHP-1 and/or Syk) via phosphorylated Tyr^{843} and Tyr^{863}, as well as other phosphotyrosine motifs in the cytoplasmic domain. In either case, it is likely that phosphorylation of the CD22 cytoplasmic domain would only transiently affect CD22 internalization due to regulatory feedback mechanisms that limit the duration and/or magnitude of CD22 phosphorylation. This conclusion is supported by findings in the current study demonstrating that inhibition of CD22 internalization in response to anti-IgM treatment of splenic B cells is transient, even in the continued presence of anti-IgM Ab.

In conclusion, these studies demonstrate that active internalization of CD22 is predominantly mediated through its interaction with the AP50 subunit of the AP-2 complex. This interaction represents a key point at which numerous processes may converge to regulate the overall level of CD22 expression on the surface of the B cell and thereby regulate the ability of CD22 to modulate signaling through the BCR.

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