Analysis of Tyrosine Phosphorylation-dependent Interactions between Stimulatory Effector Proteins and the B Cell Co-receptor CD22*

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The B cell-restricted transmembrane glycoprotein CD22 is rapidly phosphorylated on tyrosine in response to cross-linking of the B cell antigen receptor, thereby generating phosphotyrosine motifs in the cytoplasmic domain which recruit intracellular effector proteins that contain Src homology 2 domains. By virtue of its interaction with these effector proteins CD22 modulates signal transduction through the B cell antigen receptor. To define further the molecular mechanism by which CD22 mediates its co-receptor function, phosphopeptide mapping experiments were conducted to determine which of the six tyrosine residues in the cytoplasmic domain are involved in recruitment of the stimulatory effector proteins phospholipase Cγ (PLCγ), phosphoinositide 3-kinase (PI3K), Grb2, and Syk. The results obtained indicate that the protein tyrosine kinase Syk interacts with multiple CD22-derived phosphopeptides in both immunoprecipitation and reverse Far Western assays. In contrast, the Grb2-Sos complex was observed to bind exclusively to the fourth phosphotyrosine motif (Y863ENV) from CD22 and does so via a direct interaction based on Far Western blotting. Although both PLCγ and PI3K were observed to bind to multiple phosphopeptides in precipitation experiments, subsequent studies using reverse Far Western blot analysis demonstrated that only the carboxy-terminal phosphopeptide of CD22 (Y663VTL) binds directly to either one. This finding suggests that PLCγ and PI3K may be recruited to CD22 either through a direct interaction with Tyr663 or indirectly through an association with one or more intermediate proteins.

CD22 is a B cell-restricted transmembrane glycoprotein that is also an I-type lectin, which specifically recognizes α2–6-linked sialic acid residues (1). CD22 expression is tightly linked with that of membrane IgM and IgD on mature B cells (2, 3), and studies have demonstrated that it physically associates with the B cell antigen receptor (BCR) complex, albeit at a low stoichiometry (4, 5). Moreover, CD22 is phosphorylated rapidly on tyrosine residues in response to BCR cross-linking, and this promotes the recruitment of several effector proteins that contain Src homology 2 (SH2) domains (1). By virtue of its ability to recruit intracellular effector proteins, CD22 functions as a co-receptor that is able to modulate B cell activation in response to BCR cross-linking (6–9).

CD22 contains three immunoreceptor tyrosine-based inhibitory motifs in its cytoplasmic domain, similar to other inhibitory co-receptors (10). Studies have shown that tyrosine phosphorylation of CD22 promotes the recruitment of the protein tyrosine phosphatase SHP-1, which has been shown to regulate signal transduction negatively via growth factor and cytokine receptors (6, 11, 12). The SH2 domain-mediated binding of SHP-1 to CD22 results in potentiation of its catalytic activity (6). Thus, it was hypothesized that recruitment of SHP-1 to the CD22-BCR complex is responsible for attenuation of signal transduction. Independent ligation of CD22 using immobilized anti-CD22 mAb was observed to potentiate B cell proliferation in response to anti-Ig and interleukin-4 and actually decreased the threshold of stimulus required by more than 10-fold (6). This finding was interpreted as providing evidence that sequestration of CD22 away from the BCR leads to enhanced signal transduction. Furthermore, co-cross-linking experiments in which CD22 is co-localized in the membrane with the BCR complex leads to suppression of mitogen-activated protein kinase activation (13). Additional proof that CD22 negatively regulates signal transduction via the BCR has been provided by a series of studies examining the B cell compartment in CD22-deficient mice, revealing that the loss of CD22 expression causes B cells to become hyperresponsive to acute stimulation through the BCR (14–17).

Although the studies described above indicate that CD22 functions as an inhibitory co-receptor, existing evidence indicates that CD22 may transduce stimulatory signals under certain circumstances. It has been shown that BCR cross-linking promotes the recruitment of multiple stimulatory effector proteins to CD22 including Syk, phospholipase Cγ (PLCγ), phosphoinositide 3-kinase (PI3K), and Lyn (18–21). Additionally, it has been shown that human tonsil B cells lacking CD22 are unresponsive to stimulation through the BCR based on their inability to mobilize Ca2+ or to proliferate (22). These findings suggest that expression of CD22 and its association with stimulatory effector proteins could be involved in potentiating signal transduction through the BCR (18–20). Experiments have

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† The abbreviations used are: BCR, B cell antigen receptor; SH2, Src homology 2; SH3, Src homology 3; mAb, monoclonal antibody; PLC-γ, phospholipase Cγ; PI3K, phosphoinositide 3-kinase; Tyr(P), phosphotyrosine; HRP, horseradish peroxidase; GST, glutathione S-transferase; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ECL, enhanced chemiluminescence; TBST, TBS with Tween; HBB, HEPES balanced buffer.
also demonstrated that ligation of CD22 alone can indeed deliver stimulatory signals to the B cell independent of BCR cross-linking. Incubation of tonsilar B cells in the presence of an anti-CD22 mAb that blocks binding of CD22 to ligand induces proliferation and antibody production in the presence of interleukin-2 (21). Moreover, the anti-CD22-blocking mAb induced tyrosine phosphorylation of CD22 and recruitment of stimulatory effector proteins (21). These data suggest that engagement of CD22 by CD22 ligand(s) may induce a stimulatory signal independent of BCR cross-linking.

Delineation of the specific molecular mechanisms underlying the function of co-receptors like CD22 will yield important insight regarding the factors that regulate the balance between tolerance and immunity. Because CD22 exhibits the ability to recruit both inhibitory (i.e., SHP-1) and stimulatory effector (i.e., Syk, PLCγ, and PI3K) proteins, it is essential to define further the nature of these interactions to develop an understanding of the molecular mechanism(s) underlying the function of CD22. Toward this goal, phosphopeptides were synthesized based on the cytoplasmic domain of murine CD22 and were used to identify the binding sites for several stimulatory effector proteins. The results obtained indicate that the three distal tyrosine residues in the cytoplasmic domain of CD22 are involved in the recruitment of stimulatory effector proteins and that all of the effector proteins studied exhibit direct binding either to native phosphorylated CD22 or CD22-derived phosphopeptides. Finally, the current study has identified a novel interaction between CD22 and the Grb2-Sos complex.

**EXPERIMENTAL PROCEDURES**

**Biological Reagents**—Biotinylated phosphopeptides derived from CD22 (see Fig. 1) were purchased from Quality Controlled Biochemicals (Hopkinton, MA). Additionally, six control peptides were also purchased from Quality Controlled Biochemicals in which the tyrosine residues were changed to phenylalanine.

Antibodies used in these studies included mouse anti-bovine PLC-γ1 mixed monoclonal antibody (IgG isotype, Upstate Biotechnology, Lake Placid, NY), rabbit anti-rat PI3K whole antiseraum (Upstate Biotechnology), polyclonal rabbit anti-mouse Syk antibody (Upstate Biotechnology), polyclonal rabbit anti-Grb2 antibody (Santa Cruz, CA), and mouse anti-Sos1 monoclonal antibody (IgG3 isotype, Transduction Laboratories, Lexington, KY). The anti-phosphotyrosine (Tyr(P)) mouse monoclonal antibody 4G10 conjugated to horseradish peroxidase (HRP) was purchased from Upstate Biotechnology. Additional antibody reagents used in Western blotting included goat anti-mouse IgG conjugated to HRP and goat anti-rabbit IgG conjugated to HRP (Santa Cruz Biotechnology, Santa Cruz, CA) and streptavidin conjugated to HRP (BioRad, Hercules, CA). Antibody reagents used in antigen-antibody interactions (Far Western) included goat anti-peptide antibody (IgG3 isotype, Transduction Laboratories, Lexington, KY) and streptavidin conjugated to HRP (BioRad, Hercules, CA). Antibody reagents used in Immunoprecipitation and Immunoblotting included mouse anti-bovine PLC-γ1 mixed monoclonal antibody (Upstate Biotechnology), polyclonal rabbit anti-mouse Syk antibody (Upstate Biotechnology), polyclonal rabbit anti-Grb2 antibody (Santa Cruz, CA), and mouse anti-Sos1 monoclonal antibody (IgG3 isotype, Transduction Laboratories, Lexington, KY). The anti-phosphotyrosine (Tyr(P)) mouse monoclonal antibody 4G10 conjugated to horseradish peroxidase (HRP) was purchased from Upstate Biotechnology. Additional antibody reagents used in Western blotting included goat anti-mouse IgG conjugated to HRP and goat anti-rabbit IgG conjugated to HRP (Santa Cruz Biotechnology, Santa Cruz, CA) and streptavidin conjugated to HRP (BioRad, Hercules, CA). Antibody reagents used in antigen-antibody interactions (Far Western) included goat anti-peptide antibody (IgG3 isotype, Transduction Laboratories, Lexington, KY) and streptavidin conjugated to HRP (BioRad, Hercules, CA).

**Cell Line**—The B lymphoma cell line K46-17-muλ (K46) was generously provided by Dr. Michael Reth (Max Planck Institute for Immunobiology, Freiburg, Germany). K46 cells were cultured in Iscove's modified Dulbecco's medium (Life Technologies, Inc.) supplemented with 5% fetal calf serum (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 μg/ml streptomycin-penicillin, and 50 μg/ml gentamycin (Sigma Chemical Co.) at 37 °C under 5% CO2.

**GST Fusion Proteins**—GST fusion proteins were produced as described below. The plasmids encoding GST-Grb2-SH2 and GST-Grb2-SH2/3 fusion proteins as well as the GST-Syk-SH2 fusion protein have been described previously (19). The plasmid encoding the GST-PLC-γ fusion protein containing the NH2- and COOH-terminal SH2 domains of PLC-γ was provided by Dr. K. Mark Coggeshall (Ohio State University, Columbus) and the polyclonal rabbit anti-glutathione S-transferase (GST) antibody used in these studies.

**Analysis of Tyr(P)-dependent Recruitment of Effector Proteins to CD22**—The cytoplasmic domain of CD22 contains six sites that have been described previously (19). The plasmid encoding the GST-PLC-γ fusion protein containing the NH2- and COOH-terminal SH2 domains of PLC-γ was provided by Dr. K. Mark Coggeshall (Ohio State University, Columbus) and the polyclonal rabbit anti-glutathione S-transferase (GST) antibody used in these studies.

**RESULTS**

**Analysis of Tyr(P)-dependent Recruitment of Effector Proteins to CD22**—The cytoplasmic domain of CD22 contains six...
tyrosine residues that are conserved between mouse and man (1). Based on previous studies, it is apparent that one or more tyrosine residues are phosphorylated in response to BCR cross-linking, thereby generating Tyr(P) motifs that are able to recruit SH2 domain-containing effector proteins. To explore the potential role of CD22 Tyr(P) motifs in the recruitment of effector proteins, we synthesized 12 peptides (10 amino acids each) based on the sequence of murine CD22 (Fig. 1). Six peptides were synthesized in which the tyrosine residue of each was phosphorylated (phosphopeptides 1–6). Six complementary peptides were generated in which the tyrosine residues were replaced with phenylalanine (Y → F mutants). Therefore, additional studies were performed to determine if Grb2 co-precipitates with native CD22 from activated B cells. K46 cells were incubated in medium alone or were stimulated either with F(ab’2) fragments of polyclonal anti-Ig or pervanadate to stimulate tyrosine phosphorylation of CD22 after which the cells were lysed, and CD22 was immunoprecipitated from cell lysates, resolved by SDS-PAGE, and then transferred to nitrocellulose membranes that were subsequently blocked in 10% milk before incubation with antibodies specific for PLCγ, PI3K, Syk, or Grb2. Precipitation of specific effector proteins was visualized by the addition of secondary antibody coupled to HRP, after which the blots were developed using ECL detection reagents.

With the exception of Grb2, which binds specifically to phosphopeptide motif 4 containing Tyr(P)2, the other effector proteins were observed to interact with multiple phosphopeptides, although with varying affinities. Syk bound to phosphopeptide motifs 2, 3, 5, and 6, exhibiting the greatest affinity for the motifs containing Tyr773 and Tyr863. PLCγ and PI3K exhibited a binding preference for CD22 phosphopeptide motif 6 containing Tyr863, although both PI3K and PLCγ also associated to a lesser extent with motif 4. Finally, PLCγ was observed to interact weakly with motif 2. Subsequent experiments revealed that in all cases, the interaction of PLCγ, PI3K, Syk, and Grb2 with peptide was dependent on the presence of Tyr(P) (Fig. 3; Syk data not shown). Although these studies indicate that multiplex effector proteins can bind to CD22 in a Tyr(P)-dependent manner, it was not possible to determine whether the interactions resulted from the direct binding of effector proteins to CD22-derived phosphopeptides. Additionally, it was not possible to determine if the interactions between effector proteins and specific motifs derived from CD22 are physiologically relevant because studies have not been performed to identify the specific tyrosines that are phosphorylated in the cytoplasmic domain of CD22.

Grb2 Binds Directly to CD22 via Tyr(P) Motif 4—The association of Grb2 with CD22 phosphopeptide motif 4 represents a novel interaction between CD22 and a stimulatory effector protein. Therefore, additional studies were performed to determine whether Grb2 co-precipitates with native CD22 from activated B cells. K46 cells were incubated in medium alone or in the presence of anti-Ig antibody or pervanadate to stimulate tyrosine phosphorylation of CD22 after which the cells were lysed, and CD22 was immunoprecipitated. Experiments demonstrated that Grb2 is inducibly recruited to CD22 in response to stimulation of B cells with F(ab’)2 fragments of polyclonal anti-Ig or pervanadate (Fig. 4). As expected, binding of Grb2 correlates with tyrosine phosphorylation of CD22 as demonstrated by stripping and reprobing the membrane with anti-Tyr(P) mAb. These results clearly demonstrate the recruitment of Grb2 to CD22 in response to stimulation of the B cell through the BCR complex.

Next, Far Western and reverse Far Western assays were performed to determine if Grb2 binds directly to CD22 or whether it is recruited via an intermediate linker protein. For the Far Western assay, K46 cells were incubated in medium alone or were stimulated either with F(ab’)2 fragments of polyclonal anti-Ig or pervanadate. CD22 was immunoprecipitated from cell lysates, resolved by SDS-PAGE, and then transferred to nitrocellulose. CD22 derived from untreated or activated B cell was probed with GST fusion proteins containing the SH2 domain of Grb2. Binding of fusion proteins to CD22 was determined by stripping and reprobing the membrane with anti-GST mAb. These results clearly demonstrate the recruitment of Grb2 to CD22 in response to stimulation of the B cell through the BCR complex.
Recruitment of Stimulatory Effector Proteins by CD22

Fig. 3. Effector proteins bind specifically to tyrosine-phosphorylated but not Tyr→Phe mutant peptides from CD22. K46 cells (2 × 10^7/sample) were washed, pelleted, and lysed in buffer containing 1% Nonidet P-40 for 1 h on ice. Lysates were centrifuged and precleared after which either biotinylated phosphopeptides or Tyr→Phe mutant peptides were added at a final concentration of 10 μM. Peptide-effector protein complexes were recovered using streptavidin-conjugated beads. The beads were washed in lysis buffer containing 0.2% Nonidet P-40, SDS-PAGE reducing sample buffer was added, and the proteins were resolved by SDS-PAGE on 10% gels. Proteins were transferred to nitrocellulose membranes, and the membranes were blocked in 10% milk. After probing the membranes with antibodies specific for PLC-γ, PI3K, or Grb2, the presence of specific effector proteins was visualized using secondary antibody coupled to HPR and ECL.

Fig. 4. Grb2 is recruited to native CD22 in an activation-dependent manner. K46 cells (2 × 10^7/sample) were stimulated with F(ab')2 fragments of polyclonal goat anti-mouse Ig (αlg, 3 μg/ml) or with pervanadate (PV) for the length of time indicated. Stimulation of cells was terminated by the addition of ice-cold PBS (>10 volumes). The cells were washed two times in PBS, pelleted, and lysed in buffer containing 1% Nonidet P-40. Subsequently, the lysates were centrifuged at 13,000 × g, precleared with Sepharose 4B beads coupled to RG7 mAb, and then incubated with anti-CD22 mAb (CY34) coupled to Sepharose 4B beads. The beads were washed and boiled with SDS-PAGE sample buffer after which the immunocomplex proteins were resolved by SDS-PAGE on 12% acrylamide gels. The proteins were transferred to nitrocellulose, and the membranes were probed with polyclonal rabbit anti-Grb2 antibody. The association between CD22 and Grb2 was visualized using secondary goat anti-rabbit Ig coupled to HRP and ECL (upper panels). Subsequently, a Far Western assay was performed. CD22 on the membranes was denatured and then renatured as described under "Experimental Procedures." The membranes were blocked by incubating them for 1 h in buffer containing 5% milk. The membranes were washed, and then probed with polyclonal rabbit anti-GST antibody. Binding of GST fusion proteins to CD22 was detected by the addition of a secondary goat anti-rabbit Ig antibody coupled to HRP followed by development of the blots with ECL. The interaction was only observed under conditions that led to tyrosine phosphorylation of CD22, as detected by anti-Tyr(P) immunoblotting of the membranes after they had been stripped. This observation is consistent with the hypothesis that Grb2 binds directly via its SH2 domain to phosphorylated Tyr^{828} in the cytoplasmic tail of CD22. To examine further this possibility reverse Far Western blotting was used to determine whether the CD22 phosphopeptide corresponding to motif 4 (Tyr^{828}) was able to interact with the recombinant SH2 or SH2/SH3 domain of Grb2. GST alone, GST-Grb2-SH2, or GST-Grb2-SH2/3 fusion proteins were resolved by SDS-PAGE and were transferred to nitrocellulose after which the membranes were probed with biotinylated phosphopeptides representing the six motifs from CD22. Peptide binding was detected using streptavidin coupled to HRP and chemiluminescence. Only phosphopeptide motif 4 bound to the Grb2 fusion protein (data not shown). The specificity of peptide binding in the reverse Far Western assay is demonstrated in Fig. 6. As depicted, phosphopeptide 4 bound to the Grb2 SH2 domain but not to GST alone. In contrast, the Tyr→Phe mutant peptide did not bind to Grb2 even though the GST-Grb2 fusion proteins were present at equal levels on the membrane (Fig. 6). Together, these data show that the SH2 domain of Grb2 is capable of specific and exclusive binding to CD22 at Tyr^{828}.

Grb2 is an adapter protein that stimulates Ras activation by engaging and promoting membrane translocation of Sos, a Ras-specific guanine nucleotide exchange enzyme, to the membrane (28–30). To test the possibility that Sos is co-localized with Grb2 in CD22 phosphopeptide precipitates, lysates from unstimulated K46 cells were incubated with phosphorylated or Tyr→Phe peptides corresponding to the motif containing...
Proteins were visualized by ECL. Secondary goat anti-rabbit Ig antibody coupled to HRP. GST fusion and probing with rabbit anti-GST polyclonal antibody followed by a alent loading of fusion proteins was confirmed by stripping the blots CD22 peptides to GST fusion proteins was detected using ECL. Equiv-
etic precipitation controls with anti-Sos and anti-Grb2 polyclonal antibodies were included. The resolved proteins were transferred to nitrocellulose, and the membrane was probed with rabbit anti-Grb2 polyclonal antibody followed by rabbit anti-mouse polyclonal antibody coupled to HRP. Sos was visualized using ECL.

Because murine CD22 does not contain the YXXM motif,
subsequent experiments were performed to determine if PI3K binds directly to CD22. A Far Western assay was performed in which K46 cells were incubated in medium alone or were stimulated with anti-CD22 mAb or pervanadate. CD22 was isolated from cell lysates, resolved by SDS-PAGE, and transferred to nitrocellulose. Duplicate membranes were probed with anti-tyrosine-phosphorylated Ig or anti-phosphotyrosine Ig. The membranes were incubated overnight in hybridization buffer containing GST-PI3K fusion protein. The fusion proteins were added at a final concentration of 4 μg/ml. The membranes were washed and probed with polyclonal rabbit anti-GST antibody. Binding of the GST-PI3K fusion protein to CD22 was detected by the addition of a secondary goat anti-rabbit Ig antibody coupled to HRP followed by development of the blots with ECL.

The results from several studies indicate that CD22 negatively regulates signaling through the BCR via recruitment and activation of the protein tyrosine phosphatase SHP-1 (6, 13–17). Although there has been a great deal of interest in the finding that CD22 recruits SHP-1, it is evident that tyrosine phosphorylation of CD22 also mediates the recruitment of several stimulatory effector proteins. The current study, and work from other laboratories, clearly establishes that tyrosine phosphorylation of CD22 leads to the recruitment of PLCγ, PI3K, Syk, and Grb2 (18–20). The functional significance underlying the ability of CD22 to recruit both inhibitory as well as stimulatory effector proteins is not well understood. However, it is possible that recruitment of stimulatory effector proteins may facilitate their dephosphorylation by SHP-1. This would presumably involve multiple CD22 molecules because motif 6 (Tyr863), for example, appears to be involved in mediating binding of SHP-1 as well as Syk, PLCγ, and PI3K, effectively precluding recruitment of multiple SH2-containing proteins to a single CD22 molecule. Thus, it is possible that SHP-1 bound to one CD22 molecule could act on PLCγ, Syk, or PI3K bound to another CD22 molecule that is localized in the same region of the plasma membrane.

Alternatively, it is interesting to note that CD22 has the ability to recruit a wide range of stimulatory effector proteins that, in aggregate, would theoretically be sufficient to promote signal transduction via both Ca2+- and mitogen-activated protein kinase-dependent pathways. Recent studies have demonstrated that treatment of B cells with anti-CD22 mAbs promotes tyrosine phosphorylation of CD22 and the recruitment of effector proteins (21). Moreover, it has been shown that anti-CD22 mAbs, which recognize the ligand binding site on CD22, have the ability to induce B cell proliferation and antibody secretion (21). These findings indicate that under certain circumstances CD22 may function as a stimulatory receptor independent of the BCR.

The novel finding that Grb2 interacts with CD22 was characterized further in the present study by demonstrating that GST-Grb2 binds directly to the fourth phosphopeptide motif of five distinct phosphopeptide motifs. Therefore, experiments were performed to determine which phosphopeptides are able to bind directly to a GST-Syk dual SH2 domain fusion protein using the reverse Far Western assay. The results depicted in Fig. 9 demonstrate that GST-Syk dual SH2 domain fusion protein using the reverse Far Western assay. The results depicted in Fig. 9 demonstrate that GST-Syk dual SH2 domain fusion protein using the reverse Far Western assay.
CD22 (Tyr^{828}) based on the reverse Far Western assay. This finding is in agreement with previous predictions that the SH2 domain of Grb2 selectively binds to Tyr(P) motifs exhibiting the sequence YXX(N/P) (31). Additional experiments indicate that binding of Grb2 to Tyr^{828} of CD22 mediates Sos recruitment to the complex, presumably through the association of Grb2 SH3 domains with proline-rich sequences in Sos. The recruitment of Sos to CD22 suggests that CD22 may be able to affect Ras activation positively or negatively. If CD22 is physically localized in the membrane with the BCR complex, then recruitment of Sos could potentiate BCR-dependent Ras activation. In contrast, if CD22 is physically excluded from the BCR activation complex, then it could actively compete with the BCR for recruitment of the Grb2-Sos complex, thereby attenuating Ras activation.

Experiments also demonstrated that the sixth phosphopeptide motif from CD22 (Tyr^{863}) is able to interact directly with a GST-PLCγ fusion protein based on both Far Western (20) and reverse Far Western assays. The observation that the amino- and carboxyl-terminal SH2 domains of PLCγ bind preferentially to peptides containing Y(V/L)X(P/V) and Y(V/L)X(V/L), respectively (31), supports the conclusion that the sixth tyrosine motif in the cytoplasmic domain of CD22 does indeed play a role in the recruitment of PLCγ. Although it is formally possible that motif 2 (Y^{783}AIL) or 4 (Y^{828}ENV) could play a role, as well, because they were observed to precipitate PLCγ from cell lysates, it is likely that these motifs would recruit PLCγ through an indirect interaction involving one or more intermediate proteins. Whether PLCγ is indeed recruited to CD22 primarily via Tyr^{863} as opposed to Tyr^{783} or Tyr^{828}, remains to be established definitively as it is not known which of the six tyrosine residues in the cytoplasmic domain of CD22 are phosphorylated in response to cross-linking of the BCR.

Results from the current study demonstrate that Tyr(P) motifs 4 and 6 are able to precipitate PI3K from B cell lysates. This finding is in agreement with a previous report demonstrating that phosphopeptides containing the human equivalent of either motif 4 or 6 were able to bind to PI3K when added to human B cell lysates (18). The results of these analyses reveal that both the amino- and carboxyl-terminal SH2 domains of PI3K exhibit specificity for the sequence YXXM (31). Whereas human CD22 does indeed contain such a tyrosine motif (YNPM), the analogous tyrosine motif in mouse CD22 is Y^{773}NPAM (1). Thus there is no consensus motif in murine CD22 to which PI3K would be predicted to bind based on previous analyses using degenerate phosphopeptide libraries (31). It is interesting to note that precipitation experiments using a phosphopeptide based on the human sequence for CD22 containing the YNPM motif fail to precipitate PI3K from cell lysates (18). Similarly, an association between phosphopeptide motif 1 from mouse CD22 (Y^{773}NPAM) and PI3K was not observed in the current studies. Nevertheless, the sixth phosphopeptide motif from CD22 (Y^{865}VTL) was observed to bind directly to a GST fusion protein of PI3K. Although previous studies have indicated that the SH2 domains of the p85 subunit of PI3K exhibit selectivity for the sequence YXXM, the amino-terminal SH2 domain exhibits additional specificity for a hydrophobic residue at the +1 position in relationship to tyrosine (31). Motif 6 in the cytoplasmic domain of CD22 (Y^{865}VTL) does have a hydrophobic residue in the +1 position, which may be important for binding of PI3K. As is the case with PLCγ, it is not possible to establish definitively if PI3K is primarily recruited to CD22 via a direct interaction with motif 6 or indirectly through binding to motif 6 via an intermediate protein(s).

Like the stimulatory effector proteins discussed previously, it is clear that CD22 and Syk can interact directly with one another based on the results from Far Western blotting experiments (20). However, in contrast to Grb2, PLCγ, or PI3K, Syk interacts with multiple CD22 phosphopeptides. Indeed, both peptide precipitation experiments and reverse Far Western blotting indicate that Syk can bind to multiple Tyr(P) motifs in the cytoplasmic tail of CD22, including motifs 2, 5, and 6. Binding of Syk to the immunoreceptor tyrosine-based activation motifs of CD79a and CD79b involves both of its SH2 domains and requires that both tyrosine residues within the immunoreceptor tyrosine-based activation motif be phosphorylated (32, 33). Thus, optimal recruitment of Syk to CD22 may occur only when both of its SH2 domains are bound to phosphorylated tyrosine residues in the cytoplasmic domain.

It is interesting to note that binding inhibition and phosphopeptide precipitation experiments indicate that the inhibitory type protein tyrosine phosphatase SHP-1 can associate with motifs 2, 5, and 6 from CD22 (6). Additional studies using reverse Far Western blotting suggest that phosphopeptides 5 and 6 can interact directly with a GST.SHP-1 fusion protein. These results have been corroborated by studies in which each of the tyrosine residues in the cytoplasmic tail of CD22 has been mutated to phenylalanine. Mutation of Tyr^{865} or Tyr^{863} dramatically decreases the binding of SHP-1 to CD22, suggesting that motifs 5 and 6 are important for recruitment of this protein tyrosine phosphatase (34). Findings from the current study suggest that PLCγ, PI3K, and perhaps Syk may bind directly to the same motif (i.e. motif 6) in the cytoplasmic tail of CD22. If this is indeed true, then it is likely that these effector proteins would compete for available binding sites on CD22. Furthermore, it is possible that binding of positive effector proteins to CD22 would decrease binding of SHP-1 and vice versa. It follows then that the extent to which a specific effector protein or class of effector proteins actually interacts with CD22 could have a significant effect on the functional role that

### TABLE I

**Analysis of CD22 phosphopeptide binding to stimulatory effector proteins using the reverse Far Western assay**

Blots were probed with CD22 phosphopeptides at a final concentration of 100 nM as described under “Experimental Procedures.” Control peptides containing Tyr → Phe mutations were also used to probe duplicate membranes at a final concentration of 100 nM. Control peptides were not observed to bind to any of the GST fusion proteins tested (data not shown).

| GST fusion protein | Phosphopeptide binding |
|-------------------|------------------------|
|                   | Tyr^{773}  | Tyr^{783}  | Tyr^{817}  | Tyr^{826}  | Tyr^{843}  | Tyr^{863}  |
| GST alone         | −         | −          | −          | −          | −          | −          |
| GST-PI3-K         | −         | −          | −          | −          | −          | −          |
| GST-PLC-γ         | −         | −          | −          | −          | −          | −          |
| GST-Syk           | +         | +          | +          | −          | −          | −          |

_Equivalent loading and transfer of GST fusion proteins were confirmed based on Western blotting with anti-GST antibody. 1_
CD22 plays in regulating B cell activation. The association between CD22 and specific effector proteins could be determined by several factors including the relative level of effector protein expression in the cell, the subcellular localization of effector proteins relative to CD22, the relative affinity of effector protein SH2 domains for specific tyrosines in the cytoplasmic tail of CD22, and whether other proteins in the cell compete with CD22 for binding to a particular effector protein. To determine the precise mechanism(s) responsible for recruitment of the individual effector proteins described herein, additional studies will be required using cells that express CD22 in which each of the tyrosine motifs in the cytoplasmic domain has been selectively altered to affect the binding of specific effector proteins. By selectively inhibiting the binding of individual effector proteins, it should be possible to determine whether the inhibitory function of CD22 is mediated solely by the interaction with SHP-1. Conversely, it should be possible to determine if recruitment of stimulatory effector proteins but not SHP-1 enables CD22 to function primarily as a stimulatory co-receptor.

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