

CD22 Associates with Protein Tyrosine Phosphatase 1C, Syk, and Phospholipase C- γ 1 upon B Cell Activation

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Summary

Cross-linking B cell antigen receptor (BCR) elicits early signal transduction events, including activation of protein tyrosine kinases, phosphorylation of receptor components, activation of phospholipase C- γ (PLC- γ), and increases in intracellular free Ca^{2+} . In this article, we report that cross-linking the BCR led to a rapid translocation of cytosolic protein tyrosine phosphatase (PTP) 1C to the particulate fraction, where it became associated with a 140–150-kD tyrosyl-phosphorylated protein. Western blotting analysis identified this 140–150-kD protein to be CD22. The association of PTP-1C with CD22 was mediated by the NH_2 -terminal Src-homology 2 (SH2) domain of PTP-1C. Complexes of either CD22/PTP-1C/Syk or CD22/PTP-1C/Syk/PLC- γ 1 could be isolated from B cells stimulated by BCR engagement or a mixture of hydrogen peroxide and sodium orthovanadate, respectively. The binding of PLC- γ 1 and Syk to tyrosyl-phosphorylated CD22 was mediated by the NH_2 -terminal SH2 domain of PLC- γ 1 and the COOH-terminal SH2 domain of Syk, respectively. These observations suggest that tyrosyl-phosphorylated CD22 may provide the scaffolding to ensure efficient interaction between Syk and PLC- γ 1 and the activation of PLC- γ 1 by Syk. The recruitment of PTP-1C to BCR-associated CD22 may downmodulate the activity of this complex by dephosphorylation of CD22, Syk, and/or PLC- γ 1. Transient expression of CD22 and a null mutant of PTP-1C (PTP-1C^M) in COS cells resulted in an increase in tyrosyl phosphorylation of CD22 and its interaction with PTP-1C^M. By contrast, CD22 was not tyrosyl phosphorylated or associated with PTP-1C^M in the presence of wild-type PTP-1C. These results suggest that tyrosyl-phosphorylated CD22 may be a substrate for PTP-1C or that PTP-1C regulates tyrosyl phosphorylation of CD22.

Ligation of the B cell antigen receptor (BCR)¹ induces activation of the BCR-associated protein tyrosine kinases (PTKs), tyrosyl phosphorylation of phospholipase C- γ (PLC- γ), and increases in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) (1, 2). Accessory molecules such as CD45, CD19, CD21, and CD22 have also been implicated to play essential regulatory functions in BCR-mediated signal transduction (3–5).

One BCR-associated PTK activated upon BCR engagement, Syk, has been linked to the activation of PLC- γ iso-

forms and increases in $[Ca^{2+}]_i$. First, cross-linking of a CD16-Syk chimera induces Ca^{2+} fluxes in cytotoxic T cells (6) and basophils (7). Second, deletion of the Syk gene in a chicken B cell line, DT40, eliminated the ability of these cells to phosphorylate PLC- γ 2 on tyrosine and to generate a Ca^{2+} signal upon BCR cross-linking (8). Third, PLC- γ 1 can form a complex with Syk and a 120-kD phosphoprotein in human B cells (9). The interaction between Syk and PLC- γ 1 is mediated by the interaction of the Src-homology 2 (SH2) domains of PLC- γ 1 with tyrosyl-phosphorylated Syk (10). The significance of this interaction is evident by our recent observation that Syk can phosphorylate PLC- γ 1 on a key regulatory tyrosyl residue in vitro and that a direct physical contact between the two is critical for this reaction to occur in vivo (11).

CD22, a 140–150-kD glycoprophosphoprotein restricted to the B cell lineage, regulates BCR-mediated signal trans-

¹Abbreviations used in this paper: BCR, B cell antigen receptor; $[Ca^{2+}]_i$, intracellular free calcium; GST, glutathione S-transferase; HA, hemagglutinin; IP, immunoprecipitates; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; MBP, maltose-binding protein; PI3K, phosphatidylinositol 3-kinase; PLC- γ , phospholipase C- γ ; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; PY, phosphotyrosine; SH2, Src-homology 2.

duction (5). It is a member of the Ig gene superfamily and is a sialic acid-binding animal lectin (12). A small portion of cell surface CD22 molecules is associated with the BCR, and cross-linking of the BCR induces rapid tyrosyl phosphorylation of CD22 at a rate similar to the activation of BCR-associated PTKs (13–15). Cross-linking CD22 on human B cells also potentiates BCR-induced increases in $[Ca^{2+}]_i$ and cell proliferation (16, 17). These studies suggest that a functional relationship exists between CD22 and the BCR. However, how CD22 regulates BCR-mediated signal transduction remains to be defined.

Recent studies have shown that protein tyrosine phosphatases (PTPs) can downmodulate receptor-mediated signal transduction (18, 19). PTP-1C contains two SH2 domains in the NH₂-terminal half of the molecule and is expressed in multiple hematopoietic lineages (20–23). This enzyme is essential for the development and normal function of the hematopoietic system. Mutations in the *Ptp-1c* gene lead to hematopoietic abnormalities in *motheaten* (*me/me*) and *motheaten viable* (*me^v/me^v*) mice (24, 25), including elevated granulocyte and macrophage activities, erythrocytosis, and decreased NK and T cell activity (26). PTP-1C has been described as interacting functionally with multiple receptors in different hematopoietic lineages, including those for erythropoietin-1 (EPO-1) (27, 28), IL-3 (29), stem cell factor (30), and CSF-1 (31). The defects in the B cell lineage of the *me/me* and *me^v/me^v* mice are characterized by a severe reduction in conventional B cells compounded with profound systemic autoimmunity (32). These defects in the B cell compartment may be related to the functions of PTP-1C in regulating B cell activation, as PTP-1C can interact with both the FcγIIb receptor (33) and components of the BCR complex (34, 35) and influences the threshold of antigen required for negative selection of B cells (36).

Herein, we report that CD22 plays a central role for the *in vivo* formation of a multimolecular complex with PTP-1C, Syk, and PLC-γ1. The binding of these enzymes to CD22 is mediated by their SH2 domains. Moreover, we also provide evidence suggesting that PTP-1C can dephosphorylate CD22. This would provide a potential mechanism to downmodulate the BCR-mediated Ca²⁺ signal.

Materials and Methods

Cells and Antibodies. The Burkitt's lymphoma cell line Daudi was maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, nonessential amino acids, sodium pyruvate, and penicillin-streptomycin. COS cells were maintained in DMEM supplemented with the same additives. Hybridomas secreting the CD22 mAb HD39 and the CD19 mAb HD37 were kindly provided by Dr. Anthony Pezzutto (Universität Heidelberg, Germany). Antiphosphotyrosine (PY) mAb PY-20, rabbit anti-Csk, rabbit anti-Lyn serum, and rabbit anti-Syk serum were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phosphatidylinositol 3-kinase (PI3K) serum, biotinylated anti-PY mAb, 4G10, and mouse anti-PLC-γ1 mAb were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Goat anti-mouse IgM F(ab')₂, goat anti-human IgM F(ab')₂, and normal goat IgG

F(ab')₂ were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Rat anti-mouse CD22 mAb, NIM-R6 (37), and rabbit anti-human CD22 serum raised against a peptide corresponding to the cytoplasmic tail of human CD22 (14) have been described. Human PTP-1C was overexpressed in 293 cells by using an adenovirus expression system, purified to homogeneity, and used as the immunogen for the generation of the rabbit anti-PTP-1C serum (38). Anti-HA1 mAb 12CA5 was obtained from Berkeley Antibody Co. (Richmond, CA).

Plasmid Constructs. The full-length cDNA encoding human PTP-1C (PTP-1C^{WT}) (20) was cloned into the expression vector pRc/CMV (Invitrogen, San Diego, CA). Site-directed mutagenesis of the full-length PTP-1C cDNA replacing Cys455 in the phosphatase domain by a Ser yielded the null mutant (PTP-1C^M). A maltose-binding protein (MBP) fusion protein plasmid containing the double SH2 domains of PTP-1C has been described (20). To express an MBP fusion protein containing the NH₂-terminal SH2 domain of PTP-1C, an EcoRI/SpeI fragment containing the first 109 amino acids of PTP-1C was used to replace the double SH2 domain of this plasmid. For the generation of the COOH-terminal SH2 domain of PTP-1C, a fragment of cDNA encoding the COOH-terminal SH2 domain of PTP-1C was amplified using a full-length cDNA clone of PTP-1C as the template and the following primers: 5' primer, 5' GCG GGA TCC GAT CCC ACT AGT GAG AGG 3' and 3' primer, 5' GCG AAG CTT CTA CGG CTG CCG CAG GTA GAC 3'. The underlined nucleotides represent BamHI and HindIII sites in the 5' and 3' primers, respectively. The PCR product was then cut with these enzymes and ligated into BamHI/HindIII-cut pMAL-c2 (New England Biolabs, Beverly, MA).

For the generation of glutathione S-transferase (GST) fusion proteins containing the SH2 domains of Syk, PCR was used to amplify cDNAs encoding either one or both SH2 domains of Syk using phSyk-1 (39) as template and the following primer pairs: GST-SykSH2(N): 5' primer, 5' GCG GGA TCC AGC GCC AAC CAC CRG CCC 3'; 3' primer, 5' GCG GAA TTC CTT GGG CCG GTT GAA GGG 3'; GST-SykSH2(C): 5' primer, 5' GCG GGA TCC GAT GAA AAA ATG CCT TGG 3'; 3' primer, 5' GCG GAA TTC TCA GCC GAT TTT TTG ACA TGG 3'; GST-SykSH2(NC): 5' primer, 5' GCG GGA TCC AGC GCC AAC CAC CRG CCC 3'; 3' primer, 5' GCG GAA TTC TCA GCC GAT TTT TTG ACA TGG 3'. Underlined nucleotides represent BamHI and EcoRI sites in the 5' and 3' primers, respectively. PCR products were cut with these enzymes and ligated into BamHI/EcoRI cut pGEX-2T (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ).

Plasmids encoding GST fusion proteins containing either one or both SH2 domains of PLC-γ1 have been described (11). All fusion protein constructs were transformed into *Escherichia coli* strain BL21(DE3) for protein production.

The full-length mCD22 cDNA clone, pmCD22-4, has been reported (35). To detect transiently expressed mCD22 by Western blot analysis, mCD22 was epitope tagged with the influenza hemagglutinin peptide HA1 (40). The complete coding region of mCD22, without the stop codon, was amplified by PCR using the template pmCD22-4, a 5' primer of 5' GAT CGG ATC CGC GGC CTG GGA GAG AAA TAG CAG 3', and a 3' primer of 5' GCG GAA TTC GTG CTT GAG GGT CAC ATA GTC 3'. The PCR product was cut with BamHI and EcoRI. The purified PCR product was then ligated in-frame with a double-stranded oligonucleotide containing the HA1 sequence with a 5' EcoRI overhang, a 3' stop codon, and a 3' XhoI overhang, and cloned into BamHI/XhoI-cut pcDNA3 (Invitrogen).

COS Cell Transfection. The DEAE-dextran method was used to transiently express cDNAs in COS cells. Briefly, COS cells were seeded at $\sim 3 \times 10^6$ cells per 150-mm plate 16 h before transfection. Cells were washed twice with serum-free DMEM before transfection medium containing 400 $\mu\text{g/ml}$ DEAE-dextran, 0.1 mM chloroquine, and 3 $\mu\text{g/ml}$ each of the cDNA constructs was added. After a 3–4-h incubation at 37°C, cells were pulsed with 10% DMSO in PBS at room temperature for 2 min and then returned to full medium. After 24 h, the medium was replaced with fresh DMEM containing 5% FCS. Cells were analyzed 72 h after transfection.

B Cell Stimulation. Daudi cells were pelleted and resuspended at $10 \times 10^6/\text{ml}$ in fully supplemented RPMI 1640. Cells were equilibrated at 37°C for 10–15 min and then stimulated by F(ab')_2 fragments of goat anti-human IgM (final concentration 10 $\mu\text{g/ml}$) or a mixture of H_2O_2 (2.5 mM) and sodium orthovanadate (100 μM) (referred to as peroxyvanadate). Controls for F(ab')_2 fragments of goat anti-human IgM were either F(ab')_2 fragments of normal goat IgG or F(ab')_2 fragments of goat anti-mouse IgM, and the control for peroxyvanadate was medium only. Stimulation was stopped at various time points by dilution of the cell suspensions into >10 vol of ice-cold PBS containing 0.02% NaN_3 . Cells were pelleted and washed again with ice-cold PBS before lysis in 0.5% NP-40 buffer (see below).

Subcellular Fractionation. Cells were resuspended at $20 \times 10^6/\text{ml}$ in PBS containing 1.5 mM MgCl_2 , 1 mM EGTA, protease inhibitors (2 mM PMSF, 10 $\mu\text{g/ml}$ aprotinin, 10 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ pepstatin, 100 $\mu\text{g/ml}$ soybean trypsin inhibitor), and phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4 , and 5 mM $\text{Na}_4\text{P}_2\text{O}_7$). Cells were disrupted by nitrogen cavitation, 350 psi for 20 min at 4°C. The nuclei were isolated by centrifugation at 1,000 g for 20 min. Postnuclear supernatants were then centrifuged at 100,000 g for 45 min. Supernatants after this spin (S100) were considered to represent the cytosolic fraction. The pellets (P100) containing cell organelles and plasma membrane were washed once in PBS containing protease inhibitors and then solubilized for 30 min at 4°C in 0.5% NP-40 lysis buffer containing

150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA plus protease and phosphatase inhibitors as described above. Debris was removed by centrifugation at 14,000 g for 30 min. The supernatants constituted the solubilized P100 fraction used for protein immunoprecipitation.

Immunoprecipitation and Western Blotting. Immunoprecipitation, SDS-PAGE, and Western blotting were conducted as described (41). Binding of primary antibodies to blots was detected with either horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) or horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) and an enhanced chemiluminescence kit (Amersham Corp., Arlington Heights, IL). In Western blotting using fusion proteins, blots were incubated with 200 nM of fusion proteins in Tris-buffered saline (10 mM Tris, pH 8.0, 150 mM NaCl) with 0.01% Tween 20 and 5% BSA for 2 h at room temperature. Binding of GST and MBP fusion protein was detected by a monoclonal anti-GST (Santa Cruz Biotechnology) or a rabbit anti-MBP serum (New England Biolabs) plus the appropriate horseradish-conjugated second-step goat antibody and enhanced chemiluminescence.

Results

Cross-Linking of the BCR Induces Recruitment of PTP-1C to the Particulate Fraction of B Cells. To understand the potential function of PTP-1C in regulating BCR-mediated signal transduction, we examined the effect of cross-linking the BCR on the subcellular localization of PTP-1C. Resting and activated B cells were fractionated into S100 (cytosolic) and detergent solubilized P100 (membrane) fractions. The presence of cytosol-associated Csk only in the cytosolic fraction and membrane-associated Lyn only in the membrane fraction confirmed the purity of the subcellular fractions obtained (Fig. 1A). The majority of PTP-1C re-

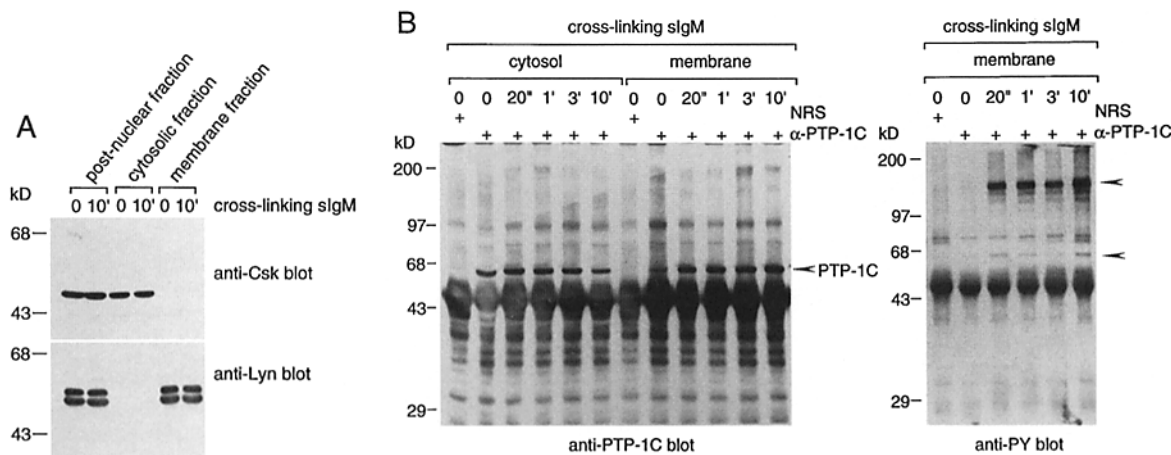


Figure 1. Anti-IgM-induced recruitment of PTP-1C to the detergent-solubilizable particulate fraction (P100). Daudi B cells were stimulated with goat anti-human IgM F(ab')_2 (10 $\mu\text{g/ml}$) for the indicated lengths of time. Stimulation was terminated by dilution of cell suspensions into >10 vol of ice-cold PBS. Particulate (P100) and cytosolic (S100) fractions were isolated as described in Materials and Methods. The P100 fraction was then solubilized with 0.5% NP-40 lysis buffer (*membrane*). The purity of these fractions was determined by immunoblotting for Csk, a cytosol-associated PTK, and Lyn, a membrane-associated PTK (A). PTP-1C was immunoprecipitated from the cytosolic and membrane fractions, resolved by reducing SDS-PAGE, and immunoblotted with anti-PTP-1C (B, left). The arrow indicates the mobility of PTP-1C. A duplicate blot of PTP-1C immunoprecipitates obtained from the membrane fraction was also probed with the anti-PY mAb 4G10 (B, anti-PY blot, right). Arrows indicate the mobilities of the 64- and 140–150-kD phosphoproteins. Negative control was immunoprecipitation using normal rabbit serum (NRS) and unstimulated B cell lysates.

sided in the cytosol of unstimulated Daudi B cells (Fig. 1 B, left). Detectable, albeit relatively limited, amounts of PTP-1C were also found associated with the membrane. Stimulation of the BCR with anti-human IgM induced a rapid recruitment of additional PTP-1C to the membrane detectable within 20 s; this increased level remained steady for at least 10 min of stimulation. Anti-PY blot of the PTP-1C immunoprecipitates (IP) from the membrane fractions revealed

the presence of two groups of proteins, ~140–150 and 64 kD, specifically phosphorylated on tyrosine (Fig. 1 B, right).

Cross-Linking the BCR and CD22 Induces a Specific Interaction between PTP-1C and CD22. Stimulation of the BCR induces a rapid tyrosyl phosphorylation of CD22 (140–150 kD) (12, 13). Therefore, we examined whether the 140–150-kD protein coimmunoprecipitating with PTP-1C was indeed CD22. Anti-PY blotting of CD22 IP showed a low

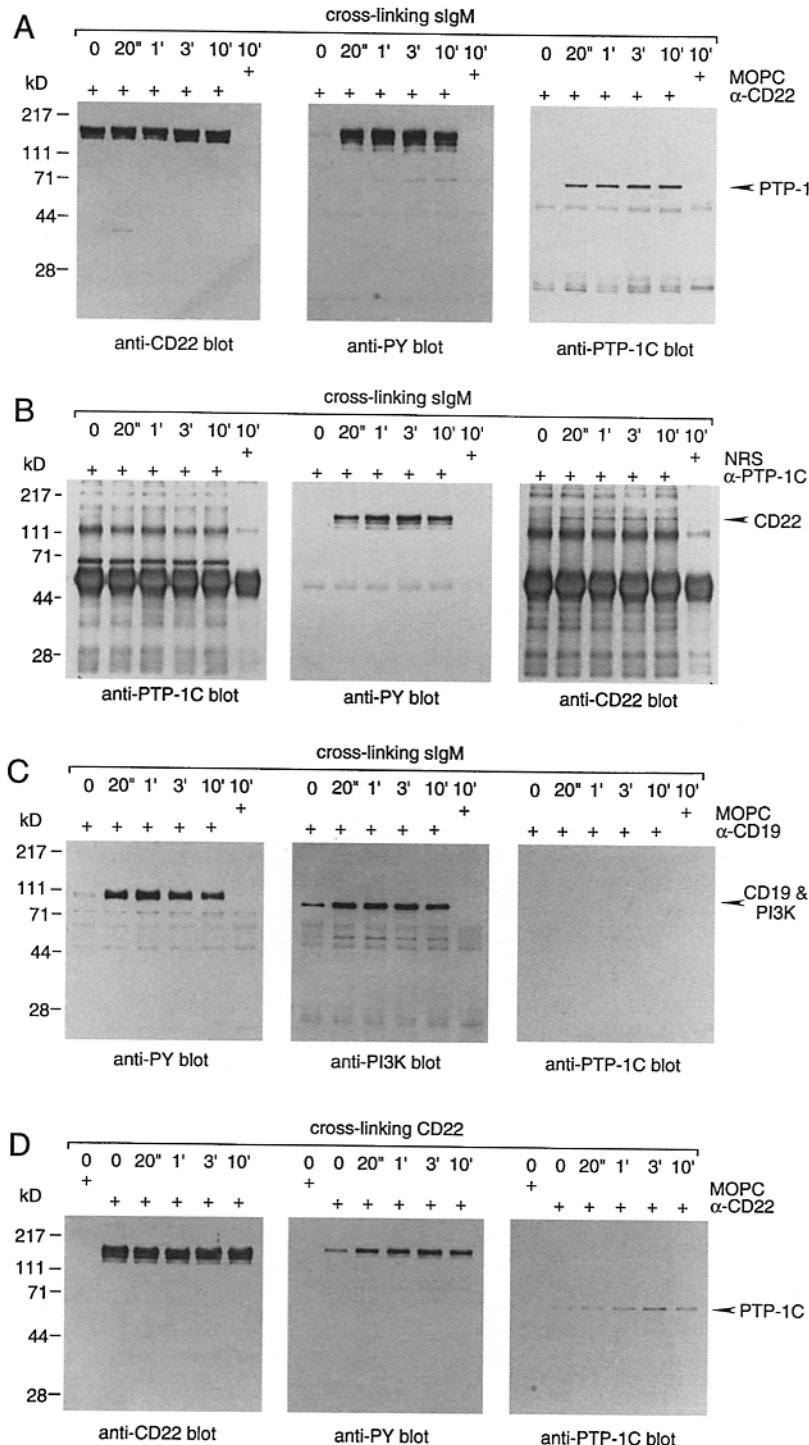


Figure 2. Anti-IgM- and anti-CD22-induced association between PTP-1C and CD22 in B cells. Daudi B cells were stimulated with goat anti-human IgM F(ab')₂ (10 μg/ml) for the indicated lengths of time, lysed in NP-40 lysis buffer, and immunoprecipitated with the CD22 mAb HD39 (A), an anti-PTP-1C serum (B), or the CD19 mAb HD37 (C). IgG1 control myeloma protein (MOPC) or normal rabbit serum (NRS) was used as control for HD39 and HD37 or anti-PTP-1C, respectively. IPs were resolved by SDS-PAGE on replicate gels and Western blotted onto membranes. Membranes containing CD22 and PTP-1C IPs were probed with an anti-CD22 serum, 4G10 (anti-PY), or an anti-PTP-1C serum. Membranes containing CD19 IP were probed with 4G10 (anti-PY), an anti-PI3K serum, or an anti-PTP-1C serum. Arrows show the mobility of the indicated proteins. (D) Daudi B cells were stimulated by 10 μg/ml of anti-CD22 HD39. CD22 was immunoprecipitated from NP-40 lysates using HD39, resolved by SDS-PAGE on replicate gels, and immunoblotted with either an anti-CD22 serum, 4G10 (anti-PY), or an anti-PTP-1C serum. Controls were unstimulated B cell lysates immunoprecipitated with MOPC.

basal level of tyrosyl phosphorylation before stimulation. Upon BCR stimulation, CD22 became heavily tyrosyl phosphorylated and associated with a 72-kD phosphoprotein (Fig. 2 A, middle). Probing of the replicate blots with a rabbit anti-PTP-1C serum showed that the enzyme was induced to interact with CD22 (Fig. 2 A, right). Conversely, when PTP-1C was immunoprecipitated from the same cells, anti-PY blot revealed the presence of a 140–150-kD phosphoprotein that could be recognized by anti-CD22, confirming the BCR-induced interaction between CD22 and PTP-1C (Fig. 2 B, middle and right). CD19 is another B cell accessory molecule that becomes tyrosyl phosphorylated and associates with PI3K upon BCR stimulation (42, 43, and Fig. 2 C, left and middle); no association between CD19 and PTP-1C could be detected after BCR ligation (Fig. 2 C, right), suggesting that the BCR-induced association between CD22 and PTP-1C was specific. Moreover, cross-linking of CD22 alone on Daudi cells also induced tyrosyl phosphorylation of CD22 and the recruitment of PTP-1C to CD22 (Fig. 2 D).

The NH₂-terminal SH2 Domain of PTP-1C Mediates Direct Interaction between PTP-1C and Tyrosyl-phosphorylated CD22. We next examined if the SH2 domains of PTP-1C

could bind to tyrosyl-phosphorylated CD22. Daudi cells were stimulated with either goat anti-human IgM or peroxyvanadate, which has been shown to elicit proximal signal transduction events in lymphocytes similar to those elicited by the Ag receptors (44, 45). The NH₂-terminal SH2 domain (MBP-1CSH2[N]) and the double SH2 domains (MBP-1CSH2[NC]), but not the COOH-terminal SH2 domain (MBP-1CSH2[C]), of PTP-1C specifically precipitated tyrosyl-phosphorylated 140–150-kD proteins from either anti-IgM- or peroxyvanadate-activated Daudi cells (Fig. 3 A, left). Western blotting with an anti-CD22 serum identified the 140–150-kD phosphoprotein precipitated by MBP-1CSH2(NC) as CD22 (Fig. 3 A, right). When these fusion proteins were used to probe Western blots containing CD22, both MBP-1CSH2(N) and MBP-1CSH2(NC) bound to tyrosyl-phosphorylated CD22 isolated from Daudi cells after anti-IgM stimulation (Fig. 3 B). By contrast, MBP-1CSH2(C) did not bind to CD22 isolated from either resting or anti-IgM-stimulated B cells. Hence, the NH₂-terminal SH2 domain of PTP-1C apparently is essential for CD22 binding. Although MBP-1CSH2(N) could detect tyrosyl-phosphorylated CD22 on Western blot, CD22 was not detected in the MBP-1CSH2(N) precipi-

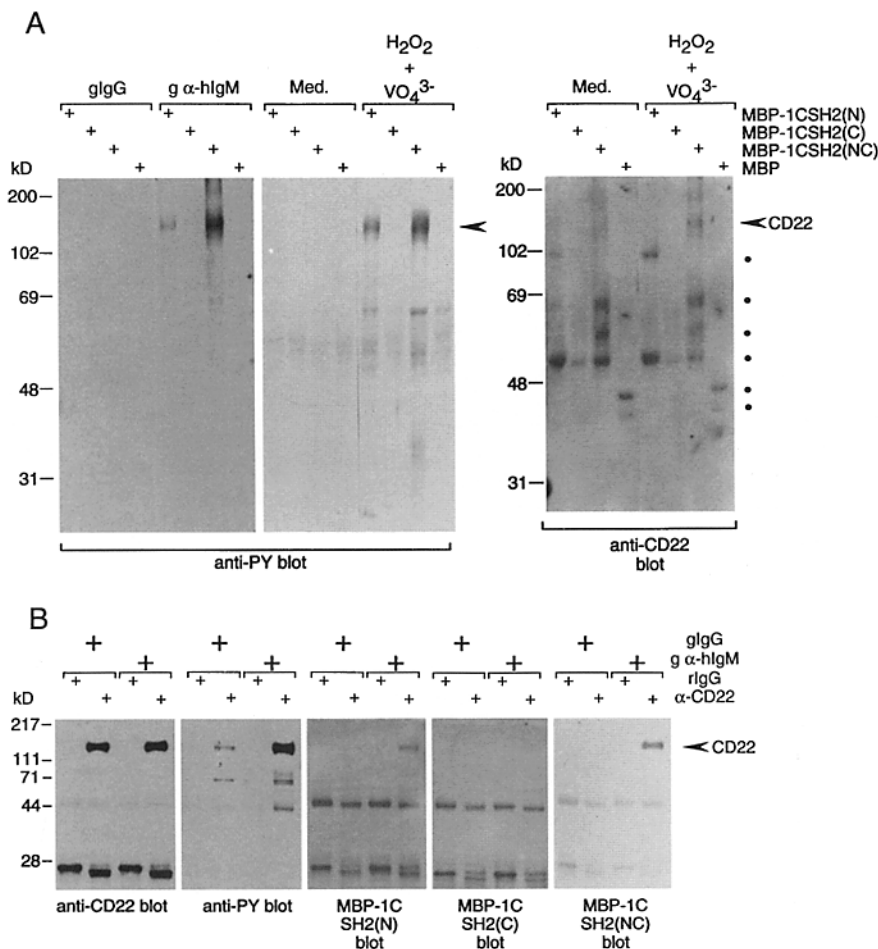


Figure 3. The NH₂-terminal SH2 domain of PTP-1C mediates direct interaction between PTP-1C and tyrosyl-phosphorylated CD22. (A) Daudi B cells were stimulated with goat anti-human IgM F(ab')₂ (g α-hIgM) (10 μg/ml) or hydrogen peroxide (2.5 mM) plus sodium orthovanadate (100 μM) (H₂O₂ + VO₄³⁻) for 5 min. Control cells were stimulated with normal goat IgG F(ab')₂ (gIgG) (10 μg/ml) or medium alone. NP-40 cell lysates were precipitated with either MBP-1CSH2(N), MBP-1CSH2(C), MBP-1CSH2(NC), or MBP plus amylose-agarose. Bound proteins were resolved by SDS-PAGE on duplicate gels and immunoblotted with either 4G10 (anti-PY) (left) or anti-CD22 serum (right). Arrows indicate either the mobility of a 140–150-kD phosphoprotein recognized by the SH2 domains of PTP-1C (left) or that of CD22 (right). Proteins of 40, 44, 50, 55, 69, and 102 kD are MBP, MBP fusion proteins, or their degradation products (dots). (B) CD22 IP obtained from unstimulated or anti-IgM-stimulated Daudi cells were resolved by SDS-PAGE on replicate gels and immunoblotted with either anti-CD22 serum, 4G10 (anti-PY), MBP-1CSH2(N), MBP-1CSH2(C), or MBP-1CSH2(NC). Blotting with MBP did not detect any specific protein (data not shown).

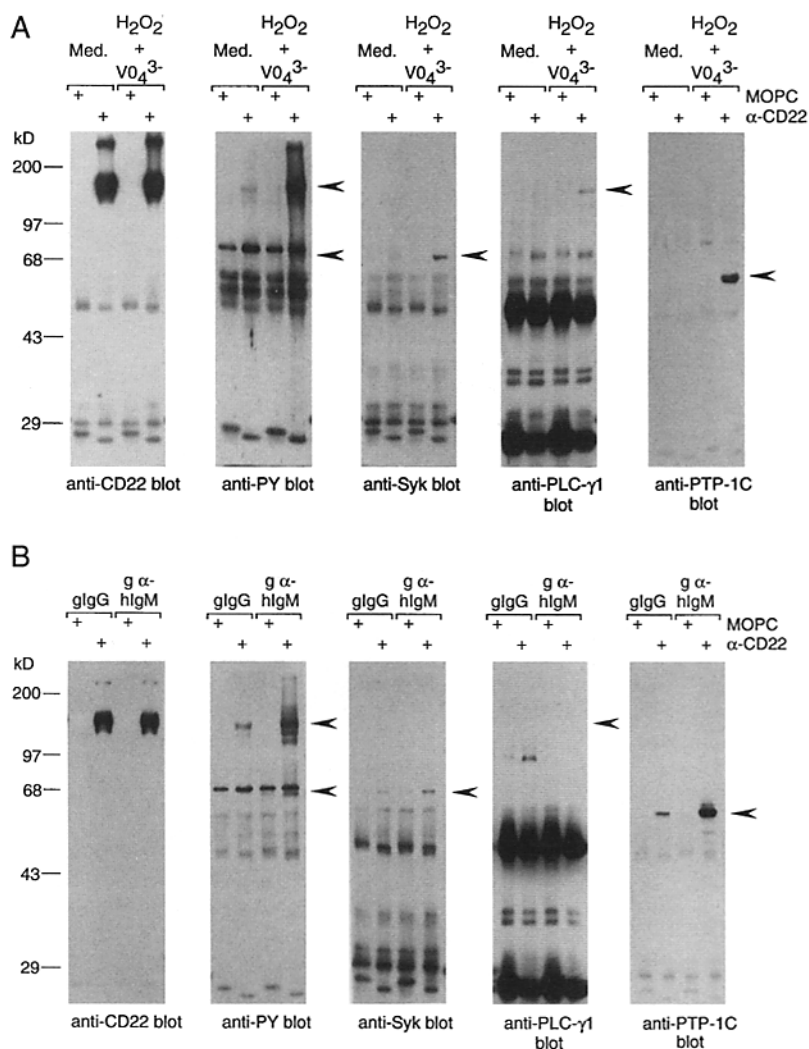


Figure 4. In vivo complexes of CD22/PTP-1C/Syk and CD22/PTP-1C/Syk/PLC- γ 1. Daudi B cells were stimulated with either goat anti-hlgM (A) or peroxyvanadate (B) as described in Fig. 3. CD22 was immunoprecipitated from NP-40 lysates, resolved by SDS-PAGE on replicate gels, and immunoblotted with the indicated antibodies. Arrows indicate the mobilities of proteins detected by the different antibodies.

tates. It is possible that MBP-1CSH2(N) precipitated a very small amount of highly tyrosyl-phosphorylated CD22, which the anti-CD22 serum used could not detect.

In Vivo Complex of CD22, PTP-1C, Syk, and PLC- γ 1. Since CD22 was the major membrane protein with which PTP-1C could interact upon BCR stimulation of Daudi B cells (Figs. 1–3), we hypothesized that this recruitment might be a way by which B cells could direct PTP-1C to its substrate(s), particularly phosphoproteins associating with CD22. Low basal levels of tyrosyl phosphorylation were detected on CD22 isolated from unstimulated Daudi cells, whereas heavily tyrosyl-phosphorylated proteins of 130–150 and 72 kD were detected after either anti-sIgM (Figs. 2 A and 4 B) or peroxyvanadate (Fig. 4 A) stimulation. Probing of replicate blots with anti-Syk serum revealed that the 72-kD protein was Syk; a small amount of unphosphorylated Syk was also associated with CD22 in unstimulated cells. PLC- γ 1 was detected in CD22 IP from peroxyvanadate-stimulated cells and comigrated with CD22 (Fig. 4 A), but not from anti-sIgM-stimulated cells (Fig. 4 B). PTP-1C was detected in the CD22 IP from stimulated

cells, as shown in Figs. 1–3. The absence of detectable PLC- γ 1 in anti-IgM-stimulated cells might be due to the stronger stimulation delivered by peroxyvanadate. Collectively, stimulation of B cells through their antigen receptors or using peroxyvanadate could indeed induce the in vivo formation of complexes consisting of either CD22/PTP-1C/Syk or CD22/PTP-1C/Syk/PLC- γ 1.

The COOH-terminal SH2 Domain of Syk Binds Directly to Tyrosyl-phosphorylated CD22. The molecular basis for the interaction between Syk and CD22 was examined using GST fusion proteins containing either one or both SH2 domains of Syk. A 140–150-kD tyrosyl-phosphorylated protein was the major protein that GST-SykSH2(C) or (NC) precipitated from either anti-sIgM- or peroxyvanadate-activated B cells (Fig. 5 A, left). The NH₂-terminal SH2 domain of Syk also weakly precipitated a 140–150-kD phosphoprotein from peroxyvanadate-stimulated Daudi cells (Fig. 5 A, left). Western blotting of the fusion protein precipitates obtained from lysates of peroxyvanadate- (Fig. 5 A, right) or anti-sIgM- (data not shown) activated B cells with an anti-CD22 serum revealed that GST-SykSH2(C) or GST-SykSH2(NC)

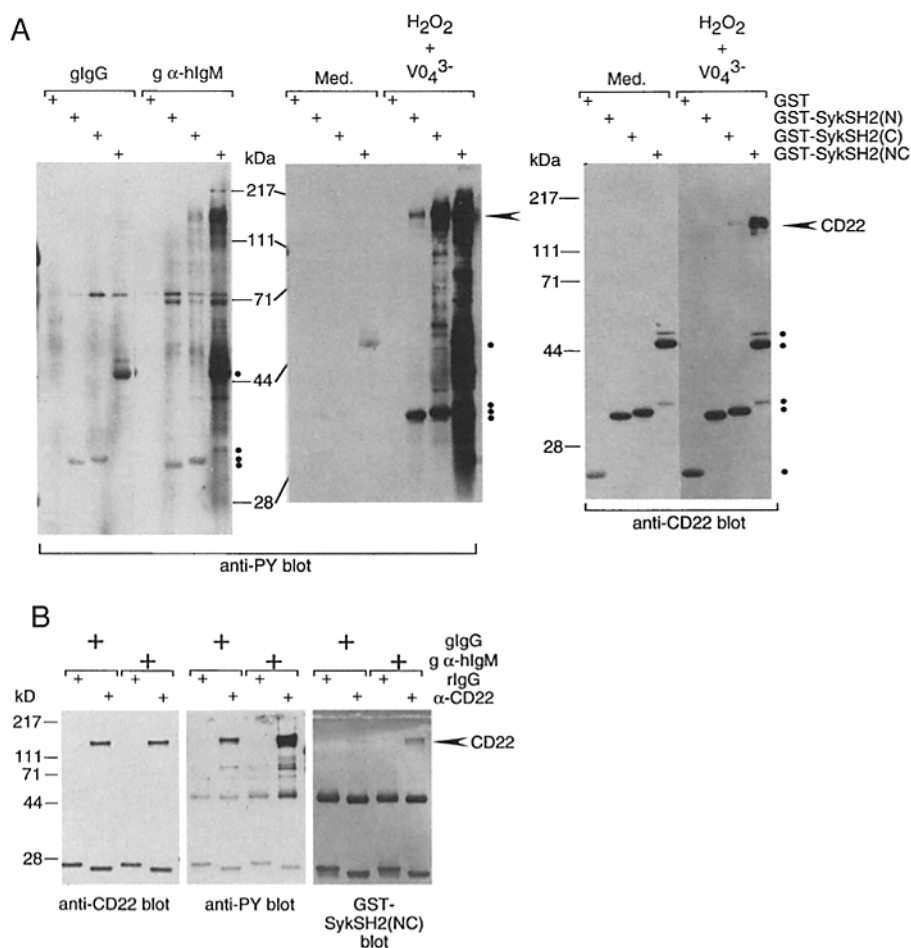


Figure 5. The COOH-terminal SH2 domains of Syk mediate a direct interaction between Syk and tyrosyl-phosphorylated CD22. Daudi B cells were stimulated as described for Fig. 3. NP-40 cell lysates were precipitated with either GST, GST-SykSH2(N), GST-SykSH2(C), or GST-SykSH2(NC) plus glutathione-agarose. Bound proteins were resolved by SDS-PAGE on duplicate gels and immunoblotted with either 4G10 (*anti-PY*) (*left*) or anti-CD22 serum (*right*). Arrows indicate either the mobility of a 140–150-kD phosphoprotein recognized by the SH2 domains of Syk (*left*) or that of CD22 (*right*). Low molecular mass proteins of 26–50 kD are GST, GST-SykSH2 fusion proteins, or their degradation products (*dots*). (*B*) CD22 IP obtained from unstimulated or anti-IgM-stimulated Daudi cells were resolved by SDS-PAGE on replicate gels and immunoblotted with either anti-CD22 serum, 4G10 (*anti-PY*), or GST-SykSH2(NC). Blotting with GST did not detect any specific protein (data not shown).

could precipitate CD22. Western blotting using the GST SH2 fusion proteins showed GST-SykSH2(NC) could bind directly to tyrosyl-phosphorylated CD22 isolated from anti-IgM-stimulated Daudi cells (Fig. 5 B). Taken together, these results suggest that Syk can directly interact with tyrosyl-phosphorylated CD22 via its COOH-terminal SH2 domain.

The NH₂-terminal SH2 Domain of PLC-γ1 Binds Directly to Tyrosyl-Phosphorylated CD22. The SH2 domains of PLC-γ1 can bind to Syk (10, 11). We have located the Syk-binding region of PLC-γ1 to its SH2(C) domain (11). In addition to Syk, the individual SH2 domains of PLC-γ1 also precipitated a number of tyrosyl-phosphorylated proteins from stimulated B cell lysates (Fig. 6 A, *left*). Thus, GST-γ1SH2(N) precipitated tyrosyl-phosphorylated proteins of 140, 59, and 52 kD, whereas GST-γ1SH2(C) precipitated tyrosyl-phosphorylated proteins of 84, 72, 59, and 52 kD. The double (NC) SH2 domains precipitated a combination of the proteins recognized by the individual SH2 domains. Sequential Western blotting of these precipitates with an anti-Syk followed by an anti-CD22 serum showed that, indeed, the 72-kD protein was Syk whereas the 140 kD protein was CD22 (Fig. 6 A, *right*). Although other undefined tyrosyl-phosphorylated proteins also coprecipitated, the interaction between the SH2(N) of PLC-γ1 was probably di-

rect, since GST-γ1SH2(NC) could bind directly to tyrosyl-phosphorylated CD22 isolated from anti-IgM-stimulated Daudi cells on Western blots (Fig. 6 B).

Cotransfection of CD22 with PTP-1C^M, but Not PTP-1C^{WT}, in COS Cells Results in the Formation of a Stable Complex between CD22 and PTP-1C^M. The complex consisting of tyrosyl-phosphorylated CD22, Syk, and PLC-γ1 with PTP-1C in stimulated B cells (Fig. 4) suggested that PTP-1C might use one or more of these phosphoproteins as substrates. In an attempt to study the interaction between CD22 and PTP-1C, we transfected COS cells with a cDNA encoding a null mutant PTP-1C (PTP-1C^M) that lacked phosphatase activity and a cDNA encoding mCD22. To facilitate the detection of mCD22 by Western blotting, an HA tag was added in-frame before the stop codon of mCD22 (mCD22HA). mCD22HA was not tyrosyl phosphorylated when transfected alone (Fig. 7 A). Cotransfection of mCD22HA with PTP-1C^M resulted in an association between the two, accompanied by a dramatic increase in tyrosyl phosphorylation of mCD22HA (Fig. 7 A, *middle and bottom*; Fig. 7 B, *top and middle*). On the other hand, PTP-1C associating with mCD22HA was not tyrosine phosphorylated, indicating that the enzyme need not be active or tyrosyl phosphorylated to bind to mCD22HA in

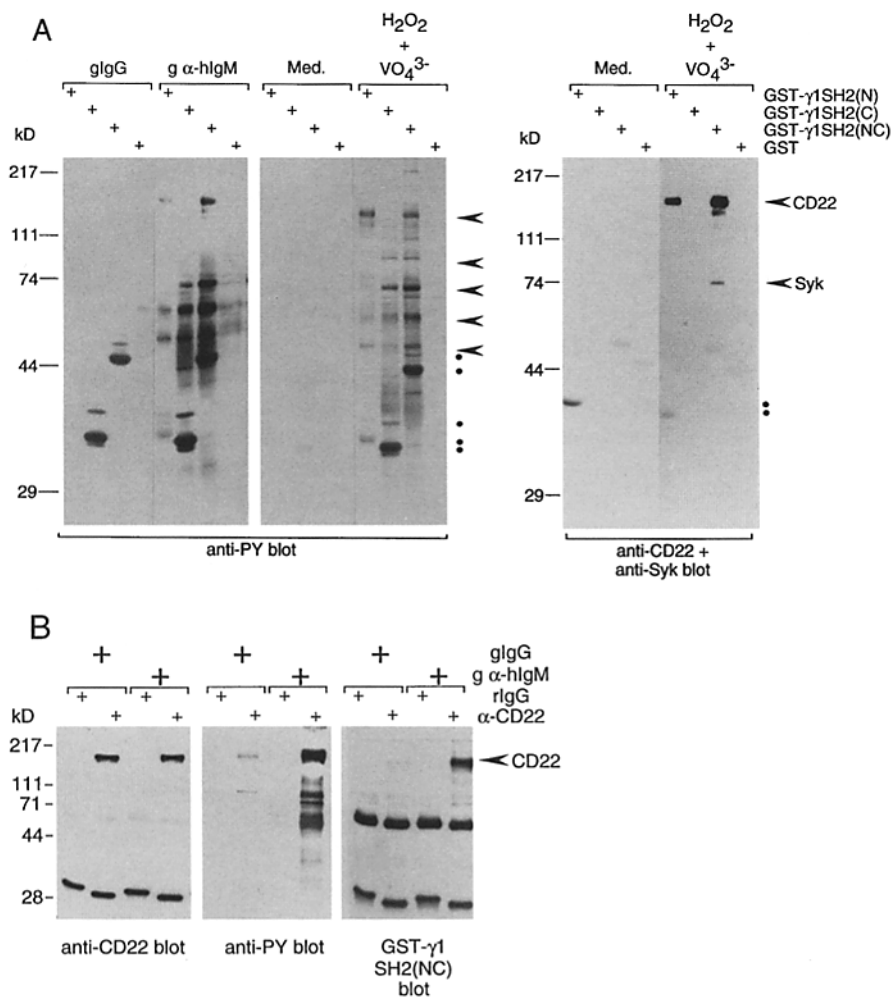


Figure 6. The NH₂-terminal SH2 domain of PLC-γ1 mediates a direct interaction between PLC-γ1 and tyrosyl-phosphorylated CD22. Daudi B cells were stimulated as described for Fig. 3. NP-40 cell lysates were precipitated with either GST-γ1SH2(N), GST-γ1SH2(C), GST-γ1SH2(NC), or GST plus glutathione-agarose. Bound proteins were resolved by SDS-PAGE on duplicate gels and immunoblotted with either 4G10 (*anti-PY*) (left) or anti-CD22 serum plus anti-Syk serum (*right*). Arrows indicate either the mobilities of phosphoproteins recognized by the SH2 domains of PLC-γ1 (*left*), CD22 (*right*), or Syk (*right*). Low molecular mass proteins of 35–48 kD are GST-PLC-γ1SH2 fusion proteins or their degradation products (*dots*). (B) CD22 IP obtained from unstimulated or anti-IgM-stimulated Daudi cells were resolved by SDS-PAGE on replicate gels and immunoblotted with either anti-CD22 serum, 4G10 (*anti-PY*), or GST-γ1SH2(NC). Blotting with GST did not detect any specific protein (data not shown).

vivo (Fig. 7, *A* and *B*, *middle*). When increasing quantities of wild-type PTP-1C (PTP-1C^{WT}) were mixed with decreasing quantities of PTP-1C^M and cotransfected with the mCD22HA, both the levels of tyrosyl phosphorylation in mCD22HA and the association between mCD22HA and PTP-1C decreased in a dose-dependent manner. The amount of immunoprecipitable mCD22HA and PTP-1C (mixture of PTP-1C^{WT} and PTP-1C^M) were comparable in the different transfectants (Fig. 7, *A*, *top*, and *B*, *bottom*). Therefore, the absence of the phosphatase activity in PTP-1C facilitated the association of PTP-1C with mCD22HA; expression of PTP-1C^{WT} alone with mCD22HA resulted in low levels of mCD22HA tyrosyl phosphorylation and some association with mCD22HA. Although the HA tag contained several tyrosyl residues, it was unlikely that this association was due to these tyrosyl residues, because identical results were observed using the native, nontagged, mCD22 cDNA (data not shown).

Overexpression of Either PTP-1C^M or PTP-1C^{WT} Does Not Alter the Overall Levels of Tyrosyl Phosphorylation of Cellular Proteins in COS Cells. The tyrosyl phosphorylation of mCD22HA and its association with PTP-1C^M may be occurring because (a) endogenous PTP activities, including

the low but detectable amount of PTP-1C (Fig. 7 *B*, *bottom*), negatively regulate the overall PTK activities in COS cells. In this case, transfection of PTP-1C^M would dominantly suppress the activity of endogenous PTP-1C or other PTP and release the PTKs from negative regulation, leading in turn to an elevated tyrosyl phosphorylation of cellular proteins including mCD22HA; or alternatively (b) certain PTKs (e.g., Src-family kinases) might phosphorylate mCD22 constitutively, whereas endogenous PTP-1C would specifically dephosphorylate mCD22 to reduce its level of tyrosyl phosphorylation. In this case, transfection of PTP-1C^M would dominantly suppress the activity of endogenous PTP-1C (or related enzymes), thereby allowing mCD22HA to become hyperphosphorylated to interact with PTP-1C^M. In the first instance, transfection of PTP-1C^M should increase the level of tyrosyl phosphorylation of other cellular proteins. In contrast, in the second case, transfection of PTP-1C^M would have no major effect on the level of tyrosyl phosphorylation of cellular proteins other than CD22. To distinguish between these two possibilities, we transfected COS cells with PTP-1C^{WT}/PTP-1C^M in different proportions. The level of tyrosyl phosphorylation of cellular proteins in general remained similar to the mock-trans-

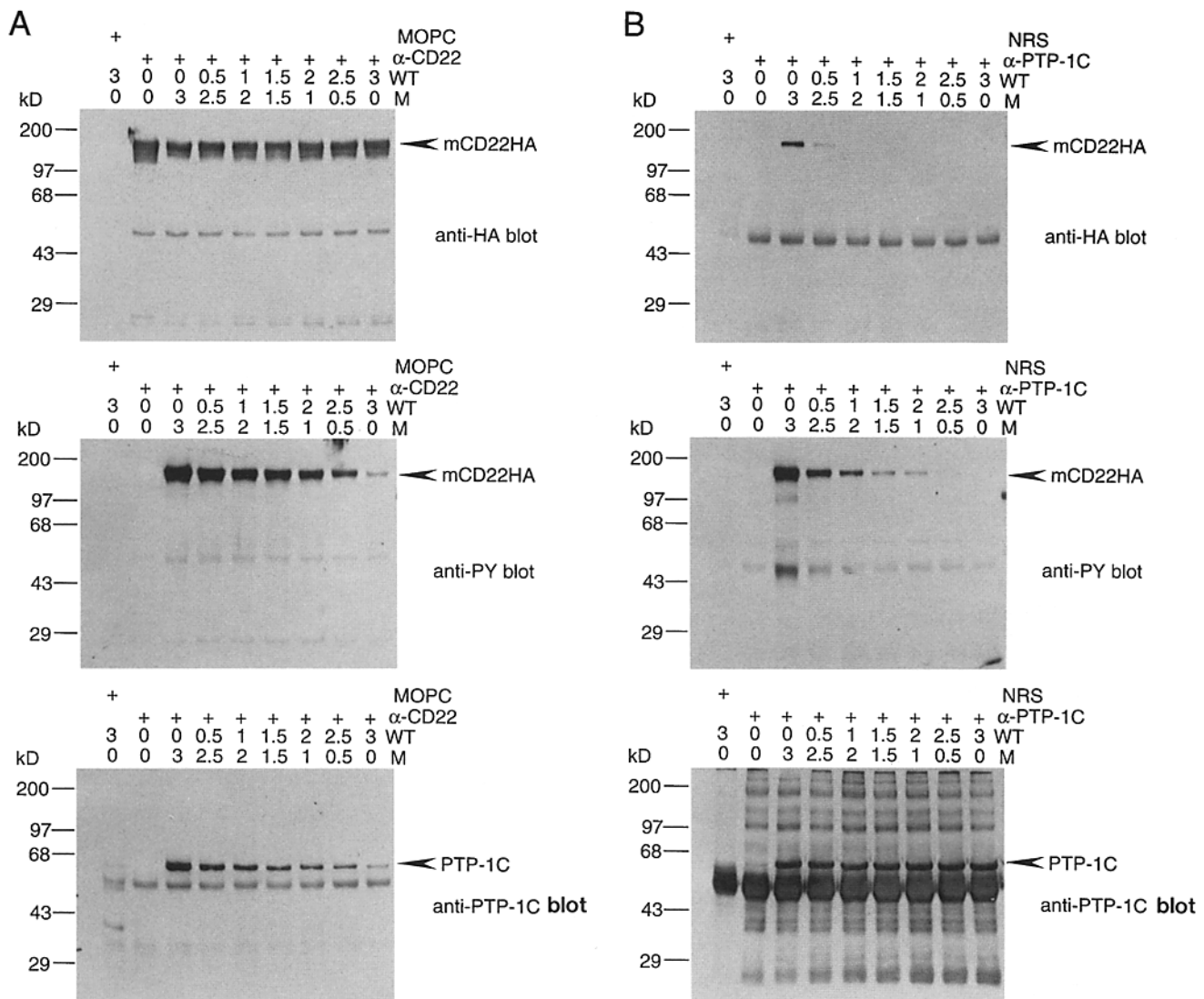


Figure 7. Cotransfection of CD22 with PTP-1C^M in COS cells results in the formation of a complex between CD22 and PTP-1C^M. COS cells were transiently transfected with mCD22HA cDNA (3 μ g/ml) and a mixture of cDNAs (total of 3 μ g/ml) encoding for both the wild-type PTP-1C (PTP-1C^{WT}) and a phosphatase-negative variant (PTP-1C^M) at the ratios indicated in the figure. Details of transfection and construction of cDNAs are described in Materials and Methods. 72 h after transfection, cells were lysed in 0.5% NP-40 lysis buffer. mCD22HA (A) and PTP-1C (B) were immunoprecipitated, resolved by reducing SDS-PAGE on replicate gels, and immunoblotted with an anti-HA tag mAb, 4G10 (*anti-PY*), or an anti-PTP-1C serum. Control precipitations were done using MOPC or normal rabbit serum (NRS) with lysates from COS cells transfected with 3 μ g/ml of mCD22HA and PTP-1C^{WT} cDNAs.

fected cells, regardless of the ratio of PTP-1C^{WT} to PTP-1C^M (Fig. 8). These results support the view that in COS cells, CD22 might serve as a substrate for PTP-1C.

Discussion

In this report, we describe the association of CD22 with key regulatory enzymes, including Syk, PLC- γ 1, and PTP-1C in B lymphocytes. The fact that tyrosyl phosphorylation of Syk and PLC- γ 1 correlates with their enzymatic activation suggests that PTP-1C may function to downregulate the BCR-mediated signal transduction cascade by catalyzing their dephosphorylation.

The NH₂-terminal SH2 domain of PTP-1C binds to ty-

rosyl-phosphorylated CD22 (Fig. 3). Since MBP-1CSH2(C) did not precipitate any detectable tyrosyl-phosphorylated proteins from B cell lysates, it is possible that the COOH-terminal SH2 domain of PTP-1C alone does not possess high binding affinity toward phosphorylated tyrosyl residues. Alternatively, SH2(C) of PTP-1C might not be folded to its optimal conformation when expressed as an MBP fusion protein by bacteria. However, the presence of SH2C clearly increases the overall affinity of interaction as revealed by the increased quantity of CD22 precipitated by MBP-1CSH2(NC) versus MBP-1CSH2(N), and also the elevated binding obtained when MBP-1CSH2(NC) was used for Western blotting of CD22 (Fig. 3 B). Taken together, our results confirm and extend recently published

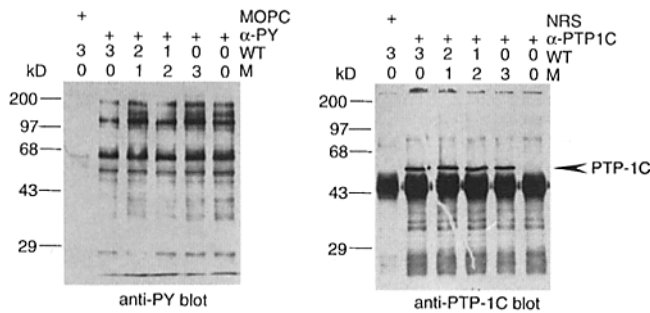


Figure 8. Expression of either PTP-1C^{WT} or PTP-1C^M in COS cells does not alter tyrosyl phosphorylation of cellular proteins. COS cells were transfected with a mixture of cDNAs (total of 3 μ g/ml) at the ratios indicated in the figure. 72 h after transfection, cells were lysed in 0.5% NP-40 lysis buffer. Cellular tyrosyl-phosphorylated proteins and PTP-1C were immunoprecipitated by PY20 (*anti-PY*) and an anti-PTP-1C serum, respectively, resolved by reducing SDS-PAGE, and Western blotted. The level of tyrosyl phosphorylation on cellular proteins was determined by blotting with 4G10, and the expression of PTP-1C was determined by blotting with an anti-PTP-1C serum.

findings using mouse B cells (34, 35). SH2(N) of PTP-1C also mediates the interactions between PTP-1C and c-kit (30) or the EPO-1 receptor (27). On the other hand, the specificity of the binding of PTP-1C to the Fc γ IIB receptor is determined by its SH2(C) domain (33). In all cases, the presence of the second SH2 domain considerably improves the overall ability of PTP-1C to interact with its ligands.

The interaction between CD22 and PTP-1C may serve multiple functions. First, the SH2 domains of PTP-1C have been shown to inhibit its phosphatase activity, presumably via an intramolecular interaction (46, 47); binding of the SH2(N) domain to CD22 may release this inhibition. In fact, synthetic phosphopeptides corresponding to the im-

munoreceptor tyrosine-based inhibitory motifs (ITIM) of both Fc γ IIB (33) and CD22 (35) increase the phosphatase activity of PTP-1C. Second, anionic phospholipids are potent activators of this enzyme (48); therefore, relocating PTP-1C to the plasma membrane may provide a rich source of anionic phospholipids to enhance its activity. Third, our data show that in addition to PTP-1C, both Syk and PLC- γ 1 can physically interact with CD22 (Figs. 4–6), and complexes of CD22/PTP-1C/Syk or CD22/PTP-1C/Syk/PLC- γ 1 assemble in stimulated B cells (Fig. 4). Hence, the interaction between CD22 and PTP-1C may juxtapose PTP-1C to its substrates, including CD22, Syk, and PLC- γ 1. Fourth, the anti-CD22-induced tyrosyl phosphorylation of CD22 and recruitment of PTP-1C (Fig. 2 D) may sequester the phosphatase from other signal transduction pathways. This is supported by the earlier findings that anti-CD22 can potentiate the increase in $[Ca^{2+}]_i$ and B cell proliferation induced by anti-IgM (16, 17).

Doody et al. (35) have identified pY783AIL, pY843SEL, and pY863VTL to be three PTP-1C-binding sites in the cytoplasmic tail of mouse CD22. Despite the relatively low overall sequence identity between the cytoplasmic tails of mouse and human CD22 (65%), all six tyrosyl residues are conserved between human and mouse (37) and the two known mCD22 alleles (49). Therefore, it is very likely that the corresponding tyrosyl residues in the cytoplasmic tail of human CD22 are the targets for human PTP-1C. Our observation that human PTP-1C^M can bind to mouse CD22 in COS cells supports this possibility (Fig. 7).

Syk associates with the BCR in both mouse (50) and human (14, 39) B cells. However, the exact nature of this interaction is not clear. In this study, we have provided evidence that the COOH-terminal SH2 domain of Syk can specifically precipitate and immunoblot tyrosyl-phosphory-

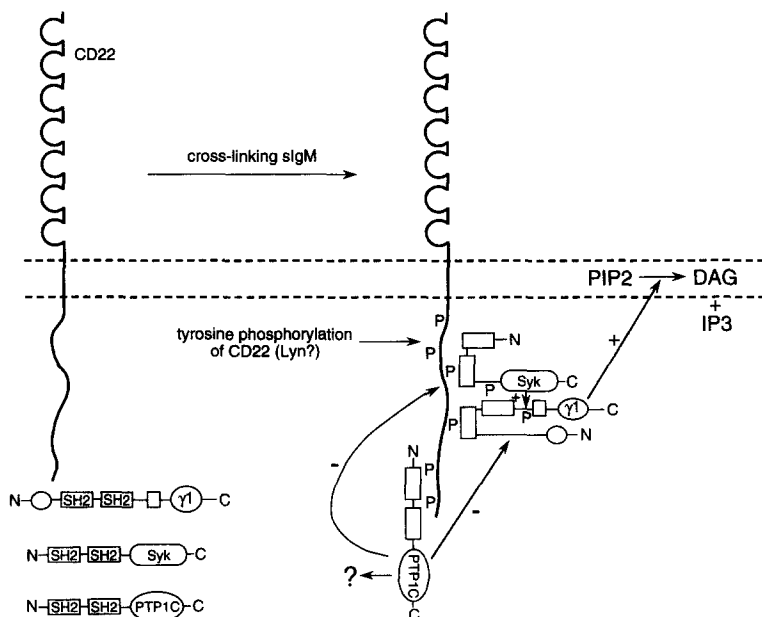


Figure 9. A model for the interaction between CD22, Syk, PLC- γ 1, and PTP-1C. After BCR ligation, CD22 is tyrosyl phosphorylated and thereby serves as a membrane anchor for Syk, PLC- γ 1, and PTP-1C.

lated CD22 obtained from either anti-sIgM- or peroxyvanadate-stimulated B cells (Fig. 5). Similar to the SH2(C) domain of PTP-1C, the SH2(N) domain of Syk may not possess high binding affinity toward phosphorylated tyrosyl residues, or it may not fold to its optimal conformation for ligand recognition when expressed as a bacterial GST fusion protein. Nevertheless, the association between the SH2(N) domain of Syk and CD22 may provide a physical link between Syk and the BCR. It is also possible that Syk may interact directly with the Ig α /Ig β heterodimer, since this association has been found to occur under detergent conditions in which CD22 is dissociated from the Ig α /Ig β complex (50; Law, C.-L., K.A. Chandran, S.P. Sidorenko, and E.A. Clark, manuscript in preparation).

The interaction between PLC- γ 1 and CD22 is mediated directly by the NH₂-terminal SH2 domain of PLC- γ 1 (Fig. 6). The COOH-terminal domain, on the other hand, mediates the binding of PLC- γ 1 to tyrosyl-phosphorylated Syk (Fig. 6 A) (11). The function of this latter interaction was revealed by the finding that Syk can phosphorylate a key regulatory tyrosyl residue, Y783, in PLC- γ 1 *in vitro* (11). However, when an enzymatically active mCD8-Syk chimera is expressed in COS cells, the basal [Ca²⁺]_i of these cells remains the same as mock transfectants (Law, C.-L., K.A. Chandran, and E.A. Clark, unpublished observations). This suggests that additional factors are needed to permit the efficient hydrolysis of phosphoinositides. Therefore, the presence of tyrosyl-phosphorylated cytoplasmic tails of CD22 to which both Syk and PLC- γ 1 can bind during B cell activation may provide the essential scaffolding needed for (a) efficient tyrosyl phosphorylation of PLC- γ 1 by Syk and (b) to localize and stabilize tyrosyl-phosphorylated PLC- γ 1 in the plasma membrane, where its phosphoinositide substrates are present.

It is still unknown which tyrosyl residues in the cytoplasmic tail of CD22 are recognized by the SH2 domains of Syk or PLC- γ 1. Since PTP-1C binds to pY783, 843, and 863 (35), Syk and PLC- γ 1 may bind to other tyrosyl residues by analogy to the EPO-1 receptor, which has distinct domains for JAK2 and PTP-1C binding (27). Based on the prediction by Songyang et al. (51, 52), Y817SVI and Y828ENV in CD22 are potential sites for Syk and PLC- γ 1 binding, respectively. It is also possible that Syk, PLC- γ 1, and PTP-1C may compete for the same site(s) in the cytoplasmic tail of CD22. Experiments are now in progress to assess these possibilities.

We (14) and others (15) have proposed that one or more of the tyrosyl residues of CD22 display the consensus motifs characteristic of immunoreceptor tyrosine-based activation motifs (ITAMs). The discovery that PTP-1C binds to phosphorylated CD22 suggests that ITIMs could also exist in the cytoplasmic tail of CD22 (35). Taking into consideration the binding of both Syk and PLC- γ 1 to phosphorylated CD22, we propose that the cytoplasmic tail of CD22 contains both ITAMs and ITIMs, which could play crucial roles in the regulation of PLC- γ 1 activation.

Two models can be proposed for the function of the interaction between CD22 with Syk, PLC- γ 1, and PTP-1C.

In the first one (Fig. 9), activation of B cells induces a rapid phosphorylation of CD22 (most likely the Src-family kinase Lyn); in turn, the phosphotyrosyl residues generated provide docking sites for the SH2 domains of Syk, PLC- γ 1, and PTP-1C. The interaction between these components may allow for the tyrosyl phosphorylation of PLC- γ 1 by Syk (11), thereby triggering phosphoinositide hydrolysis. On the other hand, the recruitment of PTP-1C may provide a means to terminate the signal by the dephosphorylation of either CD22, Syk, and/or PLC- γ 1, analogous to the function of PTP-1C in the inactivation of EPO-1 receptor-associated JAK2 (27). Alternatively, since the recruitment of PTP-1C to CD22 occurs within 20 s after BCR cross-linking (Figs. 1 and 2) at a rate similar to the activation of Ag receptor-associated PTKs, including Syk (39) and Lyn (53; Law, C.-L., K.A. Chandran, S.P. Sidorenko, and E.A. Clark, manuscript in preparation), it is also possible that individual cytosolic proteins may compete for binding to CD22. In this case, the on- and off-rates of the various SH2 domains involved will become the determining factors on how signal transduction by the BCR is regulated by these CD22-binding proteins.

To allow for the receptor-activated PTKs to propagate their signals during BCR cross-linking, PTP-1C may not be fully active initially. Although both the COOH-terminal segment (38) and the SH2 domains (46, 47) of PTP-1C can negatively regulate its activity, it is still not clear how PTP-1C activity is modulated in lymphocytes. The binding of PTP-1C to CD22 through its NH₂-terminal SH2 domain, however, is likely to increase, rather than repress, its phosphatase activity. PTP-1C can be tyrosyl phosphorylated by Src and Lck on Tyr538 (54, 55); tyrosyl phosphorylation of PTP-1C has been reported in CSF-1-stimulated macrophages (31), stem cell factor-stimulated Mo7e cells (30), and insulin-stimulated IM9 cells (56). Tyrosyl phosphorylation of PTP-1C (56) and its homologue PTP-2C (57) increases their phosphatase activities. Little, if any, tyrosyl phosphorylation can be detected in the CD22-associated PTP-1C after BCR ligation (Figs. 2 and 4). It is possible that at an early phase of B cell activation when PTKs are active, PTP-1C remains dormant because it is not tyrosyl phosphorylated. Experiments are now in progress to define the relationship between the activity of PTP-1C and its state of phosphorylation in B lymphocytes.

What are the substrates for PTP-1C? The data shown in Figs. 7 and 8 may shed some light on this question. Transfection of different ratios of wild-type to null mutated PTP-1C into COS cells did not affect the overall levels of tyrosyl phosphorylation of cellular proteins, whereas transfection of PTP-1C^M, but not PTP-1C^{WT}, brought about a high stoichiometry tyrosyl phosphorylation of mCD22HA and increased the association between the two proteins. Both responses could be abolished by increasing the proportion of PTP-1C^{WT} that was cotransfected with PTP-1C^M. These data suggest that phosphorylated CD22 is a substrate for PTP-1C. The ability for PTP-1C to dephosphorylate the same protein that it binds to is not unique to CD22, since PTP-1C has been shown to bind to and de-

phosphorylate the IL-3 receptor (29). Functionally, dephosphorylation of CD22 by PTP-1C may result in the dissociation of the CD22/Syk/PLC- γ 1 complex and the

termination of the activation of PLC- γ 1. This would be consistent with recent observations that Ag triggers a more elevated Ca²⁺ flux in PTP-1C-deficient cells (36).

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