

## **The Ligand-binding Domain of CD22 Is Needed for Inhibition of the B Cell Receptor Signal, as Demonstrated by a Novel Human CD22-specific Inhibitor Compound**

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### **Abstract**

CD22 is a B cell-specific transmembrane protein of the Siglec family. It binds specifically to  $\alpha$ 2,6-linked sialic acid (Sia) residues, which are also present on glycoproteins on the B cell surface. CD22 acts as a negative regulator in B cell receptor-mediated signaling by recruitment of Src homology 2 domain-containing tyrosine phosphatase (SHP)-1 to its intracellular tail. To analyze how ligand-binding of CD22 influences its intracellular signaling domain, we designed synthetic sialosides as inhibitors for the lectin domain of CD22. One of these compounds inhibited binding of human CD22-Fc to target cells over 200-fold better than Sia and was highly selective for human CD22. When Daudi cells or primary B cells were stimulated with anti-immunoglobulin (Ig)M in presence of this sialoside inhibitor, a higher  $\text{Ca}^{2+}$  response was observed, similar to CD22-deficient B cells. Accordingly, a lower tyrosine-phosphorylation of CD22 and SHP-1 recruitment was demonstrated in presence of the sialoside. Thus, by interfering with ligand binding of CD22 on the B cell surface, we have shown for the first time that the lectin domain of CD22 has a direct, positive influence on its intracellular inhibitory domain. Also, we have developed a novel low molecular weight compound which can enhance the response of human B cells.

Key words: B lymphocytes • CD22 • Siglecs • sialic acid •  $\text{Ca}^{2+}$  flux

### **Introduction**

CD22 is a B cell-specific transmembrane protein of the Ig superfamily with seven Ig-like domains. CD22 seems to have two distinct functions. First, it is associated with the B cell receptor (BCR) and inhibits the BCR signal, as has been demonstrated by characterization of CD22-deficient mice (1–4). B cells of these mice show an increased  $\text{Ca}^{2+}$  response when stimulated by anti-IgM. The inhibitory effect of CD22 is due to phosphorylation of three Ig-like tyrosine-based inhibitory motifs on its intracellular tail upon BCR stimulation. This results in recruitment and activation of Src homology 2 domain-containing tyrosine phosphatase (SHP)-1 (5), a tyrosine phosphatase which inhibits several signaling pathways, and binding of other intracellu-

lar proteins with unknown significance (for a review, references 6 and 7). Second, CD22 has properties of a lectin, belongs to a family of adhesion molecules, the Siglecs (sialic acid [Sia]-binding Ig-like lectins) and is also referred to as Siglec-2 (7, 8). CD22 has a high specificity for Sia in the  $\alpha$ 2,6-linkage (2,6Sia) (9, 10). When CD22 is transfected into heterologous cells, which do not display N-glycans with 2,6Sia, it can mediate Sia-dependent binding to various cell types (11, 12). In comparison, peripheral B cells usually display high levels of 2,6Sia on the cell surface, and CD22 is bound in cis on the majority of these cells (13, 14). Nevertheless, CD22 controls the homing of recirculating B cells back to the bone marrow by binding to ligands which are expressed on sinusoidal endothelium (15).

So far, it is not clear how these two apparently distinct functions of CD22 are linked. Binding to ligands on the B cell surface in cis may affect the subcellular localization and accessibility of the intracellular domain of CD22 and thereby control its inhibitory function. Solution of the 3-D

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structure of the first Ig domain of sialoadhesin (Sn, Siglec-1) in a cocrystal with 2,3 sialyllactose showed that all molecular contacts of conserved amino acids of the ligand-binding site are to the carbohydrate residues (16). Molecular modeling of a CD22 structure, as well as site-directed mutagenesis suggest a similar Sia binding site as in Sn (17). There is so far no evidence for protein epitopes contributing to CD22 ligand binding. Models trying to predict the influence of the ligand-binding domain on signaling have suggested that CD22 is sequestered away from the BCR by 2,6Sia carrying proteins, thereby releasing the BCR from the CD22 inhibition (18). Alternatively, Sia-binding could directly mediate CD22 interaction to the BCR (6) or the ligand-binding domain could have no influence on signaling at all.

We have addressed the role of the ligand-binding domain of CD22 by development of a new class of Sia analogs as artificial high affinity Siglec ligands. One of these sialosides could efficiently inhibit 2,6 Sia binding of human CD22 with a high specificity. The effect of this potent inhibitor sialoside on B cell signaling is described here.

## Materials and Methods

### *Fc-Chimaera Inhibition Assay with Sia Analogs*

**Fc-Chimeras.** Fc-chimeras containing the NH<sub>2</sub>-terminal three domains of human CD22 (hCD22<sub>d1-3</sub>-Fc) (19), murine CD22 (mCD22<sub>d1-3</sub>-Fc), or murine Sn (Sn<sub>d1-3</sub>-Fc) were produced in COS cells as described previously (9).

**Synthetic Sialosides.** The synthesis of Methyl- $\alpha$ -2-Neu5Ac (Me-Neu5Ac) has been described previously (20). Methyl- $\alpha$ -9-N-(biphenyl-4-carbonyl)-amino-9-deoxy-Neu5Ac (BPC-Neu5Ac) and Methyl- $\alpha$ -9-N-(biphenyl-4-acetyl)-amino-9-deoxy-Neu5Ac (BPAC-Neu5Ac) were prepared by acylation of methyl- $\alpha$ -9-amino-9-deoxy-Neu5Ac, obtained by a Mitsunobu reaction-based synthesis, using activated BPC and BPAC, as will be described in detail elsewhere. All these compounds were fully characterized by elemental analyses, MS and <sup>1</sup>H-NMR spectroscopy.

**Binding Assay.** Purified Fc-chimeras were labeled with carrier free Na<sup>125</sup>I (Amersham Pharmacia Biotech) and complexed with equimolar concentrations of anti-human IgG (Bioscience) to be used in binding assays with glutaraldehyde-fixed murine myeloma cells AG8 or human erythrocytes as described previously (9). For inhibition assays 10  $\mu$ l radio-iodinated Fc-chimeras were mixed with an equal volume of the sialoside, pH 7.5, and incubated for 1 h at 4°C before 10  $\mu$ l cells were added. After an overnight incubation at 4°C, the cells were washed and the bound radioactivity was quantified. The 50% inhibition concentrations (IC<sub>50</sub>) and the relative inhibitory potencies (rIP) were calculated as described previously (21).

### *Cellular Inhibition Assays with Sia Analogs*

Daudi cells (human B cell line) or mouse splenic B cells were treated with sialidase (from *A. ureafaciens*; Roche Laboratories) at 37°C. These cells or untreated controls were preincubated with sialosides on ice for 10 min. Then cells were stained with NeuGc2,6-PAA-bio (Neu5Gc $\alpha$ 2-6Gal $\beta$ 1-4GlcNAc-O(CH<sub>2</sub>)<sub>2</sub>-PAA) (unpublished results). This was followed by streptavidin-PE staining (plus B220-FITC for mouse cells). Cells were analyzed by flow cytometry.

### *Measurement of Intracellular Ca<sup>2+</sup> Mobilization*

Daudi cells were loaded with 4.5  $\mu$ M Indo-1 plus 0.003% pluronic F-127 (both Molecular Probes) in RPMI 1640 with 1% FCS for 45 min at 37°C. Primary human lymphocytes were prepared from blood by Ficoll-Paque (Amersham Pharmacia Biotech) purification of mononuclear cells. These were Indo-1-loaded similar to Daudi and then stained on ice with anti-CD20-FITC. Both types of cells were washed and preincubated for 5 min on ice with or without Sia analogs. The baseline Ca<sup>2+</sup> concentration (proportional to the FL5 to FL4 ratio) was recorded at 37°C and then 2  $\mu$ g/ml anti-IgM (clone BU.1; The Binding Site) was added. For primary lymphocytes, B cells were gated as CD20-positive and Ca<sup>2+</sup> responses were analyzed in this gate. The staining antibody had no effect on Ca<sup>2+</sup>, as controlled by unstained cells. Increases in intracellular free Ca<sup>2+</sup> were recorded in real time with the use of a FACSvantage™ (Becton Dickinson).

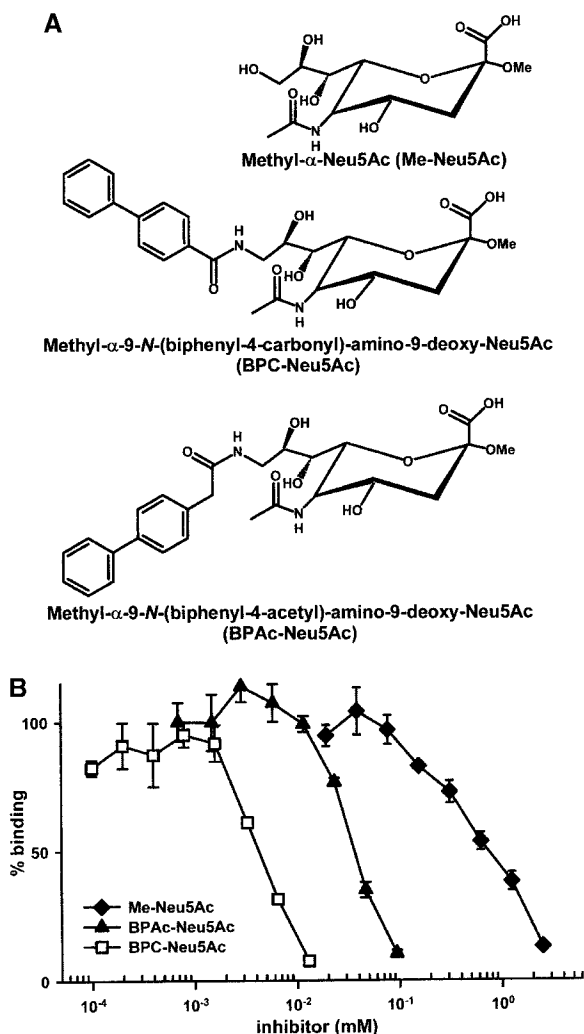
### *Immunoprecipitation of CD22*

6  $\times$  10<sup>6</sup> Daudi cells per immunoprecipitation were preincubated in 70  $\mu$ l on ice for 5 min with the indicated Sia analogue. Cells were stimulated after filling up with prewarmed RPMI 1640 medium to 1 ml at 37°C for the indicated time points by adding 2  $\mu$ g/ml anti-IgM antibody (BU.1). Cells were lysed in ice-cold NP-40 lysis-buffer containing protease inhibitors. Anti-CD22 (Dako) and anti-Vav (Santa Cruz Biotechnology, Inc.) were added together for immunoprecipitation. Precipitates were resolved by SDS-PAGE and analyzed by immunoblotting with anti-phospho-tyrosine (clone 4G10), anti-SHP-1, and anti-Vav (all UBI). Reblotting was done with anti-CD22 (Santa Cruz Biotechnology, Inc.).

## Results

Siglecs bind their ligand Sia with low affinity (8). Based on the crystal structure of Sn, complexed with 2,3 sialyllactose, synthetic sialosides were prepared with different substituents at the C9 of Me-Neu5Ac. These compounds were designed to allow additional interactions mainly with hydrophobic areas observed in the crystal structure of Sn to increase the affinity. About 50 such compounds were synthesized and used in hapten inhibition assays to determine their rIP (unpublished data). One of these compounds, BPC-Neu5Ac, had an >200-fold rIP for human CD22 when Me-Neu5Ac was used as a reference (Fig. 1), whereas several compounds with very similar structures, such as BPAC-Neu5Ac, bound much more weakly. Interestingly, this high rIP of BPC-Neu5Ac was not observed with murine CD22 (mCD22) or Sn. In contrast, BPAC-Neu5Ac was a more potent inhibitor for mCD22 than BPC-Neu5Ac (Table I).

To test the specificity of the synthetic sialosides also for cellular CD22, the human B cell line Daudi was stained with a biotinylated polyacrylamide-based synthetic ligand for mCD22 and hCD22, NeuGc2,6-PAA. Daudi cells could only be stained with this probe after sialidase treatment which led to unmasking of CD22. Pretreatment with BPC-Neu5Ac was most potent in inhibiting the binding of the synthetic CD22 ligand, confirming the selective high affinity of this compound for hCD22 (Fig. 2). When the same assay was performed on mouse B cells,



**Figure 1.** Inhibition of binding of CD22-Fc to target cells by synthetic sialosides. (A) Structures of sialosides and abbreviations used in this study. (B) Binding assays with human CD22. Binding of hCD22<sub>d1-3</sub>-Fc to murine AG8 cells was measured as described under Materials and Methods in the presence of sialosides at the concentrations indicated.

BPAC-Neu5Ac was the more potent inhibitor, confirming the results of Fig. 1 and Table I. Sialidase treated mouse CD22<sup>-/-</sup> B cells could not be stained with NeuGc2,6-PAA, showing the CD22 specificity of this synthetic ligand (Fig. 2).

The potent and selective sialoside inhibitor BPC-Neu5Ac allowed us to test directly in cellular signaling assays whether the Sia binding activity of CD22 is important for its biological function. Since CD22 inhibits the BCR-triggered Ca<sup>2+</sup> response, this response was analyzed in Daudi cells. While stimulating Daudi cells with anti-IgM in the presence of Me-Neu5Ac had no effect on the triggered Ca<sup>2+</sup> response, pretreatment with BPC-Neu5Ac led to an increase in the transient Ca<sup>2+</sup> peak (Fig. 3 A). Treatment with this sialoside led to a clearly increased Ca<sup>2+</sup> response also in anti-IgM stimulated primary human blood B lymphocytes (Fig. 3 B). The structurally highly similar compound, but weaker inhibitor BPAC-Neu5Ac had no effect

**Table I.** Comparison of Inhibitory Potential of Synthetic Sialosides

	hCD22		mCD22		mSn	
	IC <sub>50</sub> μM	rIP	IC <sub>50</sub> μM	rIP	IC <sub>50</sub> μM	rIP
Me-Neu5Ac	1,400	1	4,689	1	884	1
BPAC-Neu5Ac	35	29	123	48	3,000	0.3
BPC-Neu5Ac	4	224	1,220	5	52	13

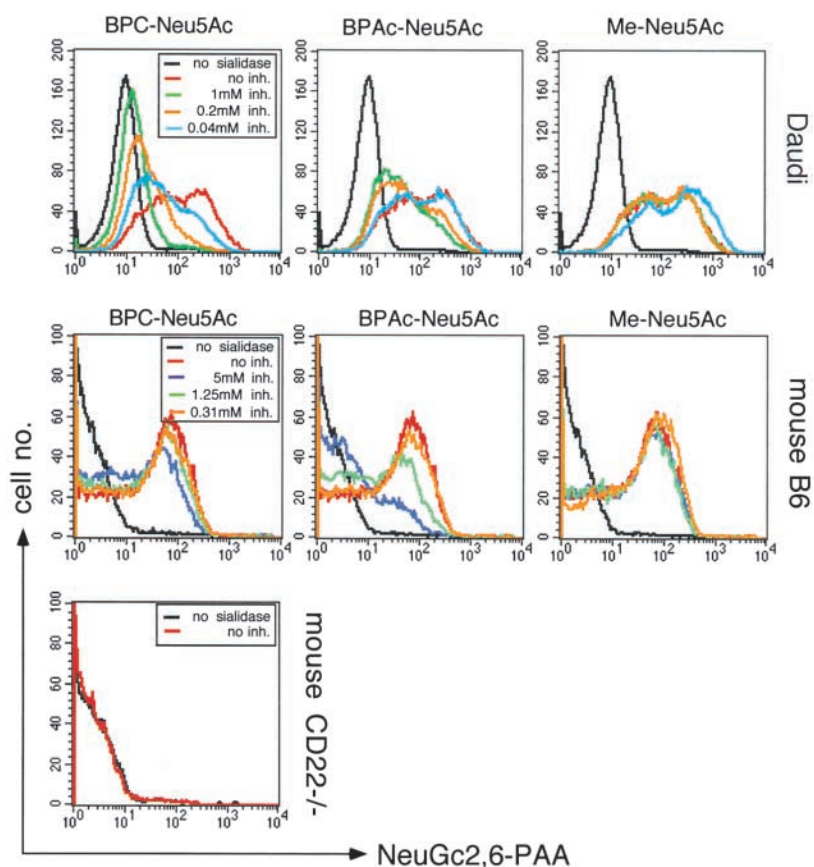
IC<sub>50</sub>- and rIP values of hCD22<sub>d1-3</sub>-Fc, mCD22<sub>d1-3</sub>-Fc, and mSn<sub>d1-3</sub>-Fc were determined from three to six independent experiments.

on the Ca<sup>2+</sup> response in primary cells (Fig. 3 B) or in the Daudi cell line (data not shown). This demonstrates that the increased Ca<sup>2+</sup> flux is specific for BPC-Neu5Ac and that a certain threshold affinity of the inhibitor for CD22 is needed to observe the effect.

The next question was whether BPC-Neu5Ac treatment interferes with CD22 phosphorylation. Fig. 4 shows that anti-IgM treatment of Daudi cells in presence of BPC-Neu5Ac led to a reduced tyrosine phosphorylation of CD22, when compared with treatment with Me-Neu5Ac. Me-Neu5Ac pretreatment served as a control, because it gave identical CD22 activation as no pretreatment in several experiments (data not shown). The presence of BPC-Neu5Ac also led to less SHP-1 coprecipitation with CD22 (Fig. 4). The weaker inhibitor BPAC-Neu5Ac had no influence on tyrosine phosphorylation of hCD22 (data not shown). These results show that by disturbing the ligand-binding domain the inhibitory function of CD22 can be reduced.

## Discussion

This work describes the newly developed sialoside BPC-Neu5Ac as a specific inhibitor for the ligand binding domain of hCD22. To unequivocally interpret our experiments on B cell signaling in presence of BPC-Neu5Ac, it was crucial to address the issue of specificity of this compound. The high specificity of BPC-Neu5Ac for hCD22 is supported by the following evidence: first, BPC-Neu5Ac inhibited binding of hCD22-Fc to Sia containing target cells very well, but not the binding of the closely related Siglec-Fc proteins, mCD22-Fc, Sn-Fc, and MAG-Fc (data not shown); second, BPC-Neu5Ac inhibited staining of sialidase-treated human B cells with the synthetic CD22 ligand NeuGc2,6-PAA, while it had no effect on staining of murine B cells. The specificity of the synthetic CD22 ligand for CD22 in this staining protocol was demonstrated with murine wild-type and CD22<sup>-/-</sup> B cells. A high specificity of the probe also for hCD22 is therefore very likely, although other probe-binding receptors on human B cells cannot be totally excluded; and third, a new crystal structure of BPC-Neu5Ac bound to Sn confirmed the predicted binding site to this Siglec (unpublished data). The

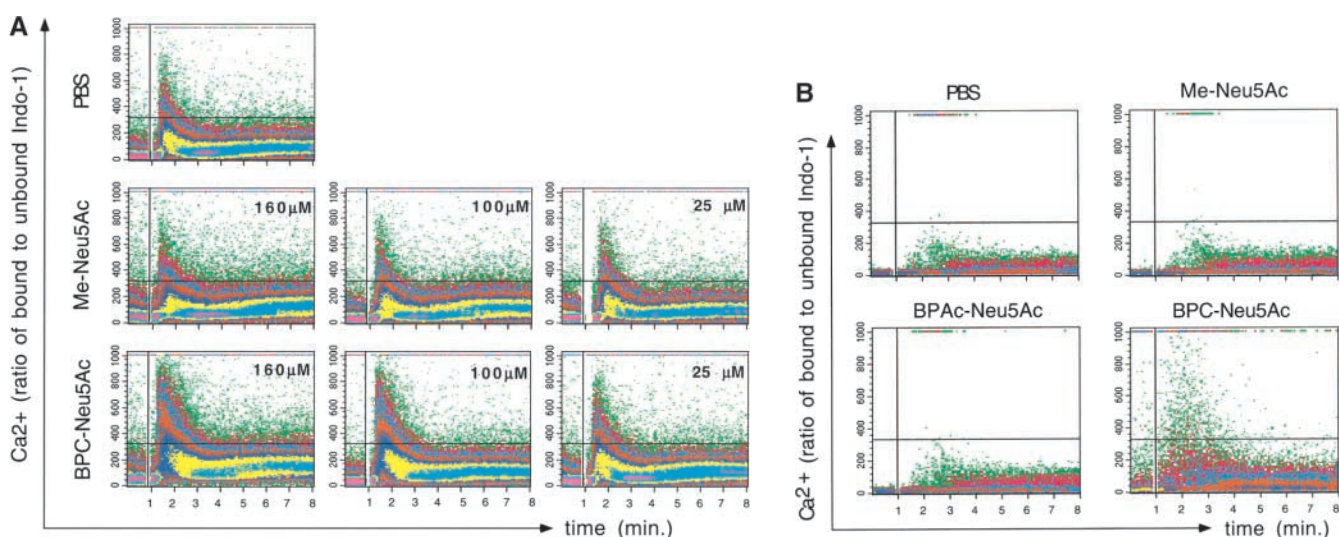


**Figure 2.** Specificity of the synthetic sialosides for cellular CD22. Human Daudi cells, mouse C57BL/6 (B6), or mouse CD22<sup>-/-</sup> B cells were not pretreated (black curves) or sialidase pretreated (all colored curves), then incubated with no inhibitor (no inh., in red) or the indicated inhibitors (same conc. used for one type of cells) and afterwards stained with NeuGc2,6-PAA. The assay shows inhibition of binding of NeuGc2,6-PAA (a synthetic CD22 ligand) to hCD22 and mCD22 by the indicated sialosides. Mouse cells are gated as B220<sup>+</sup>.

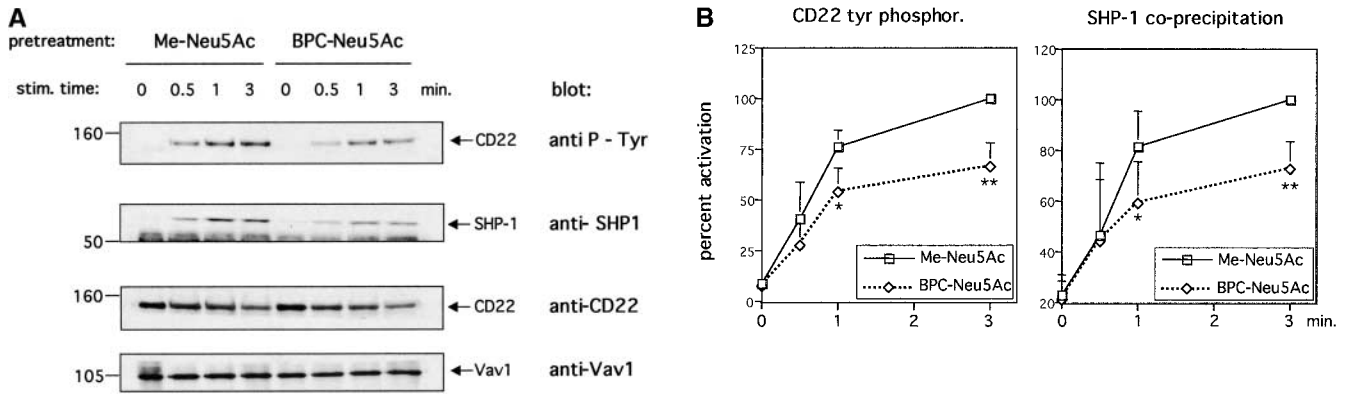
higher affinity of BPC-Neu5Ac for hCD22 than for mSn can be explained by molecular modeling of the CD22 binding site. The Val-109 and Leu-107 of Sn which make contact to the biphenyl group of the sialoside are substituted by Arg-111 and Met-109 in hCD22. The biphenyl

substituent could be sandwiched between these two side chains in hCD22 contributing a substantial binding affinity.

Together these data clearly suggest that the higher IgM triggered Ca<sup>2+</sup> signal of BPC-Neu5Ac treated B cells is due to a specific inhibition of the ligand-binding domain of



**Figure 3.** Presence of the sialoside BPC-Neu5Ac leads to increased BCR-triggered Ca<sup>2+</sup> responses. (A) Daudi B cells were stimulated with anti-IgM in presence of either PBS, Me-Neu5Ac, or BPC-Neu5Ac at the given concentrations. (B) Human blood lymphocytes were stimulated with anti-IgM in presence of either PBS, Me-Neu5Ac, BPAC-Neu5Ac, or BPC-Neu5Ac (250 μM each). CD20<sup>+</sup> gated cells are shown. Addition of antibody: at the vertical lines. Horizontal lines are drawn for quantitative comparison. One typical experiment, out of five experiments (for A) or two experiments (for B) is shown.



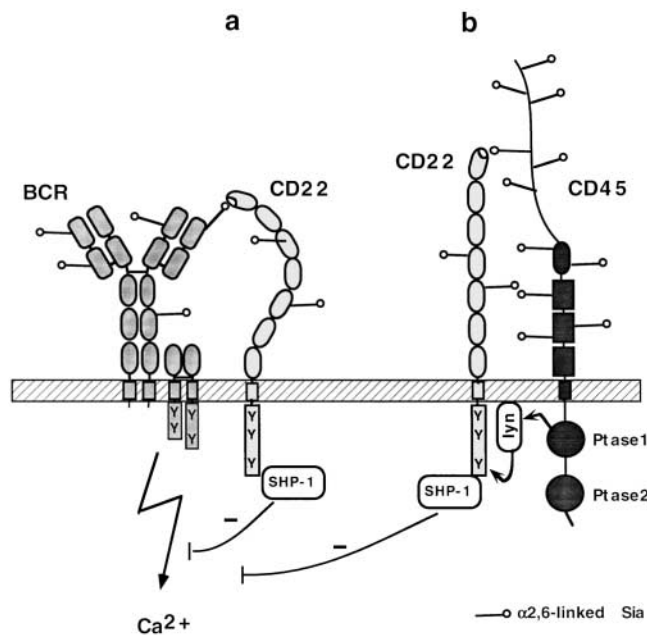
**Figure 4.** Lower tyrosine-phosphorylation of CD22 and less SHP-1 recruitment in presence of BPC-Neu5Ac. Daudi cells were stimulated with anti-IgM in presence of Me-Neu5Ac (control) or BPC-Neu5Ac (both 250  $\mu$ M). Cells were lysed and immunoprecipitated with anti-CD22 plus anti-Vav1. The anti-Vav1 I.P. served as loading control. (A) Immunoprecipitates were separated on a gel, blotted, and probed with the indicated blotting antibodies. The membrane containing the largest fragments was reprobed with anti-CD22. (B) Densitometric analysis of band intensities of the gel. Western blots were scanned and quantified. Intensities of P-tyr bands, or SHP-1 bands were divided by intensities of Vav1 bands. Vav1 was used as loading control, because the anti-CD22 antibody showed interference with the first antibody. The calculated ratio of the highest activation (3 min, Me-Neu5Ac) was set at 100%. Mean results from four experiments are shown ( $\pm$ SD). \* $P < 0.05$ ; \*\* $P < 0.01$  in Student's  $t$  test.

CD22. This interference with ligand-binding leads to an incomplete activation of the intracellular inhibitory domain of CD22. From the data presented it is obvious that the availability of 2,6Sia ligands on glycoproteins on the cellular surface is important for the function of this Siglec. B cells usually display high levels of 2,6Sia on the surface (13, 14). Upon in vitro activation, a subset of human peripheral B cells seems to downregulate surface expression of 2,6Sia (13). This could be due to downregulation of the  $\alpha$ 2,6 sialyltransferase ST6GalI which is highly regulated in several

cell types (22) or activation of a sialidase (13). Thus, the inhibitory activity of CD22 could be regulated by the differential expression of 2,6Sia on the B cell surface. The inhibitor BPC-Neu5Ac most likely also affects the cellular distribution of CD22 on the B cell membrane.

All available structural data show that the ligand-binding domains of Siglecs are specific for the sialylated carbohydrate moieties with no involvement of the core protein in binding (16). Also, recent surface plasmon resonance experiments have shown that the affinity of CD22 for 2,6Sia, coupled to different carriers, is very similar, irrespective of whether the sugar is attached to different protein backbones or even polyacrylamide (Bakker, T., and A. van der Merwe, personal communication). Thus, any glycoprotein on the B cell surface containing 2,6Sia as terminal sugars could be a potential ligand for CD22. Our  $Ca^{2+}$  data argue against the model that ligand binding of CD22 by other surface glycoproteins sequesters CD22 away from the BCR and thereby releases the BCR from CD22 inhibition (18). In this case, interference with the ligand binding by sialosides would release CD22 from this sequestering and lead to its availability for BCR inhibition, resulting in a lower  $Ca^{2+}$  signal.

In contrast, our results support the model that the lectin domain mediates CD22 interaction to specific transmembrane glycoprotein ligands which are positively involved in BCR signaling. Such a specificity would be in contradiction to the only carbohydrate-based binding specificity mentioned above. But Sia-binding of CD22 could support interaction to certain transmembrane proteins to which a specific interaction is accomplished by other mechanisms. Alternatively, CD22 binds to many glycoproteins, but the ones which are abundant, structurally well accessible and carry the highest level of appropriately linked Sia win. There are two likely candidates as ligands for CD22: one is membrane Ig. It has been shown that a low stoichiometric amount of CD22 can be coprecipitated with IgM from the



**Figure 5.** Model of possible ligands for CD22 on the B cell surface. (A) Interaction to surface Ig (BCR). (B) Interaction to CD45. Both CD22 interactions would be 2,6 Sia-dependent and could thus be inhibited by BPC-Neu5Ac, leading to impairment of CD22 activation, SHP-1 recruitment and a higher  $Ca^{2+}$  flux.

B cell surface (23, 24). CD22 lectin binding to sialylated membrane IgM may contribute to this constitutive association (Fig. 5 A). Proximity to the BCR is likely to be crucial for CD22 because the recruited SHP-1 needs to be near its tyrosine phosphorylated substrates of the antigen receptor signaling complex. A second likely candidate as CD22 ligand is CD45, since CD45 is an abundant protein on the B cell surface, is highly glycosylated and has been detected as a prominent ligand by immunoprecipitation with CD22-Fc (25). Also, CD45 is known to activate lyn, the src-like kinase responsible for CD22 phosphorylation (26) (Fig. 5 B). After initial binding to CD45, CD22 could subsequently interact with the BCR or even recruit CD45 to the BCR. These possibilities will be directly tested in future experiments. Additionally to the discussed cis-binding, trans interactions of CD22 to ligands on adjacent cells cannot be excluded. Such interactions which may relieve the B cell from CD22 suppression, could be relevant when B cells are closely packed together such as in primary follicles (5, 18). Recently, it was shown that trans interactions of CD22 with ligands on target cells can also influence Ag-specific B cell activation (27).

In summary, we have described a novel role for the CD22-lectin domain. Binding to ligands on the cell surface apparently supports the inhibitory function of CD22 on BCR-triggered  $Ca^{2+}$  flux. Our data are very well compatible with the study of Jin et al. (28) who have come to similar conclusions with a completely different experimental approach. In addition to addressing the biological role of the adhesion domain of CD22, the newly described sialoside could be potentially very important also therapeutically. Application of this structurally simple compound leads to an enhanced B cell response, which could be valuable for immunocompromised patients. Oligomerization of the sialoside can easily be used in the future to enhance affinity and avidity and improve the biological efficacy even further.

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