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Cell surface sialic acids do not affect primary CD22 interactions with CD45 and surface IgM nor the rate of constitutive CD22 endocytosis

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CD22/Siglec-2 is a B cell–specific molecule modulating surface IgM (sIgM) signaling via cytosolic tyrosine-based motifs. CD22 recognizes α2-6-linked sialic acids (Sias) via an amino-terminal Ig-like domain. This Sia-binding site is typically masked by unknown sialylated ligands on the same cell surface, an interaction required for optimal signaling function. We studied the effect of cell surface Sias on specific interactions of CD22 with other molecules and on its turnover via endocytosis. A novel approach for simultaneous biotinylation and cross-linking showed that CD22 associates with CD45 and sIgM at much higher levels than reported in prior studies, possibly involving cell surface multimers of CD22. Sia removal or mutation of a CD22 arginine residue required for Sia recognition did not affect these associations even in human:mouse heterologous systems, indicating that they are primarily determined by evolutionarily conserved protein–protein interactions. Thus masking of the Sia-binding site of CD22 involves many cell surface sialoglycoproteins, without requiring specific ligand(s) and/or is mediated by secondary interactions with Sias on CD45 and sIgM. Abrogating Sia interactions also does not affect constitutive CD22 endocytosis. Sia removal does enhance the much faster rate of anti-CD22 antibody-triggered endocytosis, as well as killing by an anti-CD22 immunotoxin. In contrast to the unstimulated state, sIgM cross-linking inhibits both antibody-induced endocytosis and immunotoxin killing. Thus the signal-modulating activity of CD22 Sia recognition cannot be explained by mediation of primary interactions with specific molecules, nor by effects on constitutive endocytosis. The effects on antibody-mediated endocytosis could be of relevance to immunotoxin treatment of lymphomas.

Key words: B lymphocytes/endocytosis/sialic acids/Siglecs/toxin conjugates

Introduction

CD22 is a B cell–specific glycoprotein expressed in the cytosol of pre- and pro-B cells, and on the plasma membrane of mature B cells (Cyster and Goodnow, 1997; Law *et al*., 1994; Tedder *et al*., 1997; Torres *et al*., 1992; Wilson *et al*., 1991). The predominant form of cell surface CD22 (CD22β) is a 140-kDa type I transmembrane protein. CD22 associates with the B cell receptor (BCR) both physically and functionally (Leprince *et al*., 1993; Peaker and Neuberger, 1993) and negatively regulates BCR signaling (Doody *et al*., 1995). Six tyrosine residues are located in the cytosolic portion of CD22, and BCR stimulation can induce phosphorylation on some of them (Schulte *et al*., 1992; Torres *et al*., 1992; Wilson *et al*., 1991). CD22-deficient mice generally show a hyperreactive B cell phenotype (Nitschke *et al*., 1997; O'Keefe *et al*., 1996; Otipoby *et al*., 1996; Sato *et al*., 1996), affirming that the primary function of CD22 is to dampen BCR signaling.

CD22/Siglec-2 is also an I-type lectin belonging to the Siglec (sialic acid binding Ig-like lectin) family (Crocker and Varki, 2001). It binds to glycans containing sialic acid (Sia) in a highly linkage specific manner. Human and mouse CD22 bind selectively to α2-6-linked Sias (Engel *et al*., 1993; Kelm *et al*., 1994; Powell *et al*., 1993, 1995; Powell and Varki, 1994; Sgroi *et al*., 1993). The amino-terminal Ig-like V-set domain is critical in this binding (Engel *et al*., 1995), requiring a conserved arginine residue that likely forms a salt bridge with the carboxylate groups of the Sia ligand (Van der Merwe *et al*., 1996). Like most of the other Siglecs, CD22 is natively bound to sialylated *cis* ligands on the same cell surface (Razi and Varki, 1998, 1999). This "masking" effect is abolished by sialidase treatment, and a small amount of "unmasking" has been found on activated human B cells (Razi and Varki, 1998). Using different approaches, two groups showed that this *cis* binding of Sia by CD22 is required for its optimal function as an inhibitory regulator of the BCR (Jin *et al*., 2002; Kelm *et al*., 2002). These data confirmed the biological importance of Sia recognition in CD22 function. However the mechanism of how *cis* Sia binding affects CD22 biological functions is not clear. In this article we test two hypotheses regarding to this matter: (1) that *cis* Sia binding helps keep specific partner proteins close to or away from CD22; and (2) that *cis* Sia binding restricts CD22 turnover from the cell surface by endocytosis, thus modulating CD22 cell surface levels.

The physiological *cis* ligands for CD22 are not well defined (Tedder *et al*., 1997). Many prior studies explored the nature of the sialylated ligands responsible for this *cis* masking of CD22 Sia binding activity. Several molecules that carry α2-6-linked Sias, for example, cell surface IgM (sIgM) or CD45, as well as circulating glycoproteins such as IgM and haptoglobin have been suggested as candidate ligands (Hanasaki *et al*., 1995a; Sgroi *et al*., 1993; Stamenkovic *et al*., 1991). Currently published models

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hypothesize that associations of CD22 with sIgM and CD45 are mediated by CD22 recognition of their Sia residues (Collins *et al.*, 2002; Cyster and Goodnow, 1997). Indirect support for this hypothesis comes from the finding that B cells from mice deficient in sialylated CD22 ligands show constitutive unmasking of CD22 and altered sIgM signaling responses (Hennet *et al.*, 1998). However, there is as yet no direct proof for a role of Sias in forming or maintaining specific interactions of other proteins with CD22. In addition, these candidate ligands have been suggested mostly based on the ability of recombinant soluble CD22 to bind them in a Sia-dependent manner *in vitro*. However, the dimeric CD22-Fc chimeras used for such studies can interact with any molecule with a high density of α2-6-linked Sias, especially when the chimera is immobilized on beads. Indeed, we have previously shown that although a low-density CD22-Fc column selectively interacted with sIgM and haptoglobin from blood plasma, a high-density column could bind most of the α2-6 sialylated glycoproteins in the sample (Hanasaki *et al.*, 1995a). Also, CD22 expressed on Chinese hamster ovary or COS cells (which are physiologically irrelevant to CD22 biology) can be masked if these cells are also induced to express α2-6-linked Sias by transfection with ST6Gal-I (Braesch-Andersen and Stamenkovic, 1994; Hanasaki *et al.*, 1995b).

On the other hand, attempts to study the native interactions in CD22-expressing B cells by coimmunoprecipitation from lysates have been fraught with difficulties. Mild detergent approaches have only detected very limited (~1–2%) interactions of CD22 with sIgM (Law *et al.*, 1994; Leprince *et al.*, 1993; Peaker and Neuberger, 1993). Also, because the single-site binding affinity of CD22 to α2-6-linked Sias is relatively poor (Powell *et al.*, 1995), it is unlikely to survive the repeated washing steps involved in standard immunoprecipitation protocols. Surface labeling followed by cross-linking thus provides the best hope of quantitatively detecting native interactions (Powell *et al.*, 1995). However, labeling and cross-linking are typically carried out in a stepwise fashion, and there is a serious risk of perturbing critical interactions during the first labeling step, prior to the cross-linking. We have therefore developed a novel approach for simultaneous biotinylation and cross-linking of cell surface molecules on rapidly chilled cells, which can circumvent many of the problems. We apply this approach to study the nature of CD22-associated cell surface molecules and further examine if these associations are Sia-dependent to test the first hypothesis.

Prior studies also have suggested that cell surface CD22 undergoes constitutive endocytosis and degradation, with a $t_{1/2}$ of ~8 h and that certain residues in the cytosolic tail are involved in mediating this turnover (Chan *et al.*, 1998; Shan and Press, 1995). Because CD22 expression level on B cells is significantly reduced in the ST6Gal-I knockout mouse, where CD22 ligand formation is abolished (Hennet *et al.*, 1998), we considered the possibility that *cis* binding Sia ligands might help maintain optimal CD22 levels on the cell surface by restricting its rate of endocytosis. To test this hypothesis, we examined not only the constitutive but also antibody-mediated endocytosis of CD22, an issue that is of importance to the anti-CD22-based immunotherapy of B cell leukemias and lymphomas (Herrera *et al.*, 2000, 2003; Mansfield *et al.*, 1997; Messmann *et al.*, 2000; Pagel *et al.*, 2002; Shen *et al.*, 1988; Tuscano *et al.*, 2003) and how Sia-binding as well as sIgM ligation affect these processes.

Results

Simultaneous cross-linking and biotinylation of cell surface molecules demonstrates major interactions of CD22 with CD45 and sIgM

Prior studies reported that a very small percentage (1–2%) of total cell surface CD22 is noncovalently associated with sIgM (Law *et al.*, 1994; Leprince *et al.*, 1993). Also, only 0.2–2% of sIgM was shown to associate with CD22 (Peaker and Neuberger, 1993). It is difficult to reconcile these very low level associations with the robust effects of CD22 on signaling via the BCR. For example, sequestration of CD22 away from sIgM via immobilized antibodies resulted in a >100-fold lowering of the threshold for BCR activation (Doody *et al.*, 1995). As discussed in the *Introduction*, the protocol of immunoprecipitation following mild detergent lysis used to reach these conclusions could have disrupted weak but significant interactions. To circumvent these problems, we developed a novel approach for simultaneous biotinylation and cross-linking of cell surface molecules in the cold.

The two primary amine-reactive reagents EZ-link Sulfo-NHS-Biotin and 3,3′-dithiobis (sulfosuccinimidylpropionate) (DTSSP) (for biotinylation and cross-linking, respectively) were mixed in various ratios and applied to cells that had just been rapidly chilled to prevent any lateral movement of molecules in the membrane. The reactive group of these two reagents is identical (a primary amine-reactive NHS ester), and they therefore compete for the same sites on cell surface proteins. We found that a ratio of 1:1 of the reagents allowed both biotinylation and cross-linking of cell surface molecules to proceed efficiently on ice in a short time. The labeled membrane proteins could then be solubilized and immunoprecipitated with anti-CD22 antibody, allowing recovery of biotinylated CD22 along with other associated biotinylated proteins, which could then be detected by streptavidin-based reagents. Because the DTSSP cross-linker is cleavable by reducing reagents, a nonreducing gel can indicate if CD22 forms any complexes, and a reducing gel can show what the individual components are. Surface biotinylation and streptavidin staining also ensures detection of only cell surface molecules that interact in the native situation, either through Sia binding or via other mechanisms. To our knowledge, this is the first attempt to define noncovalent cell surface associations using this approach.

We first examined Daudi cells, a human B cell line that constitutively expresses CD22. A nonreducing gel showed that a CD22-antibody-specific immunoprecipitate comprised a high-molecular-weight smear so large that it hardly entered into the 7.5% separating gel (Figure 1A). A reducing gel indicated that in keeping with previous reports (Engel *et al.*, 1995), CD22 in Daudi cells appears as two bands (which cannot be explained by differential sialylation or N-glycosylation, data not shown, and

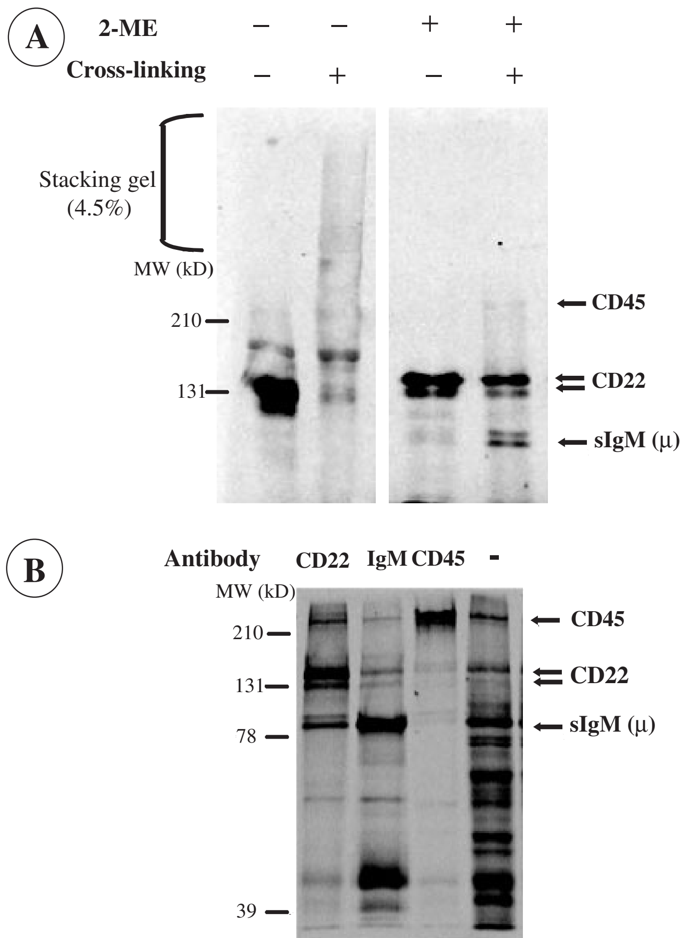

Fig. 1. Immunoprecipitation of CD22 following simultaneous cell surface biotinylation and cross-linking on Daudi cells. Daudi cells were subjected to simultaneous biotinylation (by Sulfo-NHS-Biotin) cross-linking (by DTSSP), and immunoprecipitation (by antibodies to CD22, clone To15; IgM, clone G20-127; and CD45, clone HI30), as indicated in *Materials and methods*. Samples were separated on 7.5% SDS–PAGE gels, following prior boiling with or without the presence of 2-ME. Gels were then subjected to transfer and detection of biotin-labeled proteins. (**A**) Nonreducing gel (left): a high-molecular weight smear is detected in the sample subjected to simultaneous biotinylation and cross-linking, but not in the sample without cross-linking. The stacking gel is also included to show the very high-molecular-weight smear. Reducing gel (right): CD22, CD45, and sIgM (μ) bands are indicated by arrows, showing that the high-molecular-weight protein complex in the second lane of left panel contains CD22. (**B**) CD45 and sIgM association with CD22 is shown by parallel immunoprecipitation. Equivalent amounts of pre-cleared cell lysate were subjected to immunoprecipitation by anti-CD22, anti-sIgM, or anti-CD45 antibodies. Presence of CD22, IgM (μ) and CD45 are indicated by arrows. An aliquot of the total lysate without immunoprecipitation (3 μg) was loaded on the same gel. The amount of protein was determined by the fluorescence intensity as quantified by ImageQuant software. The calculated association levels amongst these three molecules are shown in Table I. This is one representative experiment out of four.

neither of which is CD22α, based on antibody specificity). The reducing gel also showed that several other proteins are involved in forming this high-molecular weight complex with CD22 (Figure 1A). Some of these could be identified by their typical molecular weights and by parallel immunoprecipitation of the B cell surface proteins CD45 and sIgM (Figure 1B). Sequential immunoprecipitation and control antibody immunoprecipitation were done to confirm the identities of these proteins (data not shown). Comparisons with a small amount of total lysate (without immunoprecipitation) loaded on the same gel indicated that these findings are not simply explained by the fact that sIgM and CD45 are major components of the cell surface (Figure 1B). Because several other major bands in the total lysate were not represented in the immunoprecipitated complexes, the observed associations with CD22 must be specific. Other proteins also coimmunoprecipitated with CD22, and potential candidates are other components of BCR, such as Igα and Igβ (Leprince *et al*., 1993). However, in this article we focus only on the higher-molecular-weight proteins sIgM and CD45. A quantitative analysis of the results for these three proteins is presented in Table I.

Table I. Cell surface associations of CD22, CD45, and sIgM on Daudi cells

Antibody used	Associated molecule	% Association (mean ± SD)	Previously reported % association
Anti-CD22	sIgM (μ)	8.0 ± 1.3	0.2–2 (Peaker and emsp;Neuberger, 1993)
Anti-CD22	CD45	30.1 ± 5.1	
Anti-sIgM	CD22	15.6 ± 3.1	1–2 (Leprince *et al*., 1993; Law *et al*., 1994)
Anti-sIgM	CD45	5.4 ± 0.8	
Anti-CD45	CD22	5.6 ± 2.0	
Anti-CD45	sIgM (μ)	0.6 ± 0.2	

The proteins bands shown in Figure 1B were visualized and quantified using Storm 860 scanner and ImageQuant software. Percent of association of a given molecule A with B is determined by dividing the fluorescence intensity of A that coprecipitated with the nominally immunoprecipitated protein B by the fluorescence intensity of A being immunoprecipitated by the antibody specific to A. The results presented are mean ± SD from four experiments.

Interactions of CD22 with CD45 and sIgM are not primarily Sia-dependent

We did several experiments to test the hypothesis proposed in published models that the associations of CD22 with *cis* ligands, that is, sIgM and CD45, are mediated by CD22 recognition of their Sia residues (Collins *et al*., 2002; Cyster and Goodnow, 1997). First we compared the profile of proteins that coimmunoprecipitated with CD22 with and without cell surface sialidase treatment, followed by simultaneous biotinylation and cross-linking (Figure 3A). No difference was detected in any of the major protein components, and the small molecular weight shifts in some proteins confirmed the action of the sialidase. The data suggest that Sias are not involved in maintaining the associations of CD22 with sIgM or CD45. However, sialidase treatment of intact cells can also nonspecifically alter cell surface

negative charge. Furthermore, this approach does not rule out the possibility that Sias are involved in initiating the interactions but are then not required for their maintenance.

Thus we approached the same issue via another method: evaluating proteins coimmunoprecipitated with human CD22 that was stably transfected into a CD22-deficient mouse B cell line J2-44 (kindly provided by Dr. Henry Wortis) (Jin *et al.*, 2002). We transfected these cells with either wild-type human CD22 or CD22 with a point mutation that changes a specific arginine residue (Van der Merwe *et al.*, 1996) required for Sia recognition to an inactive alanine. As shown in Figure 2A, clones of transfected cells were selected that expressed similar levels of cell surface CD22 (detected by flow cytometry using anti-human CD22 antibodies, levels similar to those seen on Daudi cells). Unmasked Sia binding sites were also detected after sialidase treatment of these cells (Figure 2B) using exogenous α2-6 sialylated probes as previously reported (Razi and Varki, 1998). As expected, such binding sites were not detected on cells transfected with the arginine-mutated CD22. These cell lines provide a model situation wherein one form of CD22 is incapable of Sia-dependent interactions, even though cell surface Sias themselves are unperturbed. Both cell types were then rapidly chilled and subjected to simultaneous biotinylation and cross-linking of cell surface molecules, followed by immunoprecipitation of CD22.

As shown in Figure 3B, a pattern of cell surface associations with multiple proteins (including the endogenous mouse CD45 and sIgM, separately proven by specific immunoprecipitation, data not shown) was observed with the wild-type CD22 in a similar manner to those observed in the human cell system. As discussed for Figure 1, there are other proteins also coimmunoprecipitated with CD22 whose identities are not defined in this study. However, none of the major protein bands, including CD45 and sIgM, observed to be associated with wild-type CD22 were significantly reduced in the similar experiment involving the arginine-mutated CD22 (Figure 3B). Thus neither of these major associated molecules depends primarily on Sia recognition for their interaction with CD22. If there are any molecules that do interact with CD22 only via Sia binding, they must be present in small amounts and thus are not detectable by our method.

Interactions of CD22 with CD45 and sIgM are not affected with BCR activation

Because the importance of Sia binding in CD22 function has been clearly demonstrated by others with BCR-activated cells, we also examined the associations between CD22, CD45, and sIgM on Daudi cells following BCR activation by a F(ab′)$_2$ goat anti-human IgM Fc$_{5u}$ specific antibody (cellular activation was confirmed by enhanced calcium influx, data not shown). The BCR-stimulated Daudi cells were simultaneously biotinylated and cross-linked, and immunoprecipitation was done with anti-CD22, anti-IgM, and anti-CD45. As shown in Figure 3C, no major change in the interactions between the three molecules was detected after BCR activation. Similar results were found with CD22-transfected J2-44 cells following BCR stimulation (with or without arginine mutation of CD22, data not shown). Varying the concentrations of anti-IgM for activation or allowing activation for different periods of time (1–30 min) also did not show any changes in CD22 interactions with sIgM or CD45.

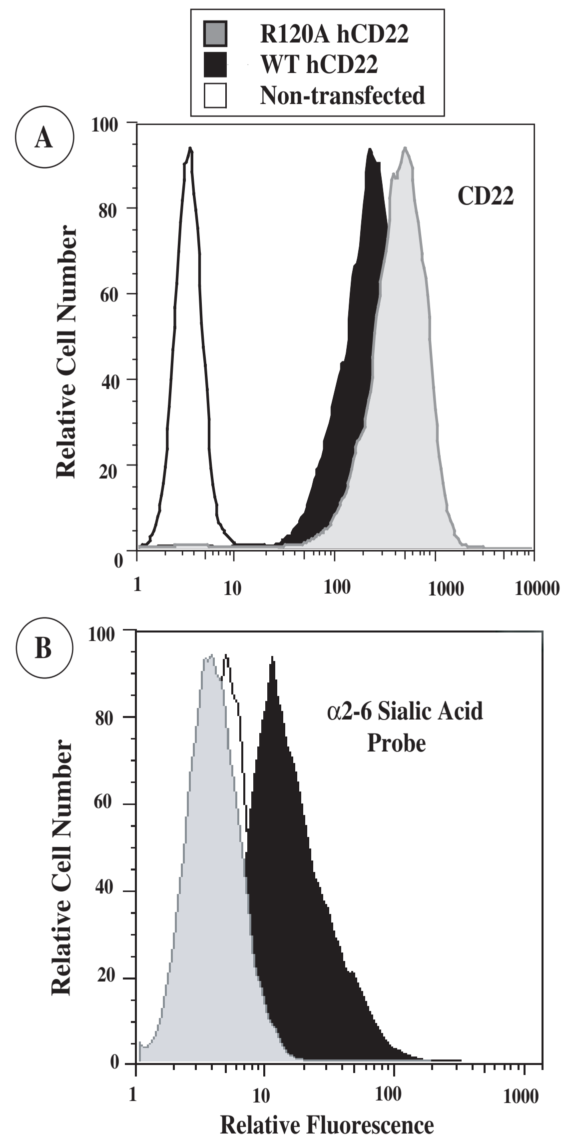

Fig. 2. Sia binding and masking on J2-44 cells transfected with human CD22 constructs. J2–44 cells stably transfected with human CD22 were tested for (**A**) CD22 expression and (**B**) binding of α2-6-sialyllactose-PAA-Biotin after sialidase treatment. (**A**) Both the wild-type and arginine-mutated human CD22 (R120A) transfected J2-44 cells have CD22 expression, detected by flow cytometry using anti-human CD22 antibody. Nontransfected J2-44 cells do not have CD22 expression. (**B**) J2-44 cells were pretreated by *A. ureafaciens* sialidase for 15 min at room temperature and subjected to flow cytometry, testing for binding to α2-6-sialyllactose-PAA-Biotin probe. Transfected wild-type CD22 is able to bind to α2-6-sialyllactose-PAA-Biotin probe after pretreatment by sialidase to unmask CD22–Sia binding site. No binding was detected on cells transfected with arginine mutated CD22. No binding to α2-6-sialyllactose-PAA-Biotin was detected in any of these cells without prior sialidase treatment (data not shown), indicating that the transfected wild-type CD22 is completely masked.

Constitutive endocytosis and turnover of CD22 is not affected by cell surface Sia

Taken together, the data indicate that the well-documented importance of Sia recognition in CD22 function (Jin *et al.*, 2002; Kelm *et al.*, 2002) cannot be explained by a primary role in forming associations with sIgM and CD45. We next tested another hypothesis, that *cis* binding Sia ligands might help maintain optimal CD22 levels on the cell surface by restricting its rate of endocytosis. This hypothesis was suggested by a previous finding that in the ST6Gal-I knockout mouse, where CD22 ligand formation is eliminated, B cell CD22 expression level is significantly reduced (Hennet *et al.*, 1998). The hypothesis was tested by comparing CD22 endocytosis rates in the presence or absence of Sia-based interactions. In the first approach, sialidase treatment was used to induce a sudden unmasking. Daudi cells were subjected to cell surface biotinylation in the cold, and the constitutive turnover of CD22 at 37°C with the presence or absence of sialidase was then studied by immunoprecipitation at various time points in a pulse-chase format. However, no difference in the rate of turnover was observed (data not shown). Of course, sialidase treatment could also cause multiple functional changes on the cell surface related to a general loss of net negative charge. Thus we also studied the rate of turnover of human CD22 and its arginine-mutated variant expressed by transfection into CD22-negative mouse B cells. Again, no difference in the rate of turnover was seen (data not shown).

In contrast to our surface biotinylation/chase approach, some previous studies describing CD22 endocytosis actually followed the internalization of labeled anti-CD22 antibodies (Chan *et al.*, 1998; Press *et al.*, 1989). In comparing these reports with those involving other means, such as surface labeling (Shan and Press, 1995), we noted that the reported rate of internalization for antibody-induced CD22 turnover was much faster than the constitutive one.

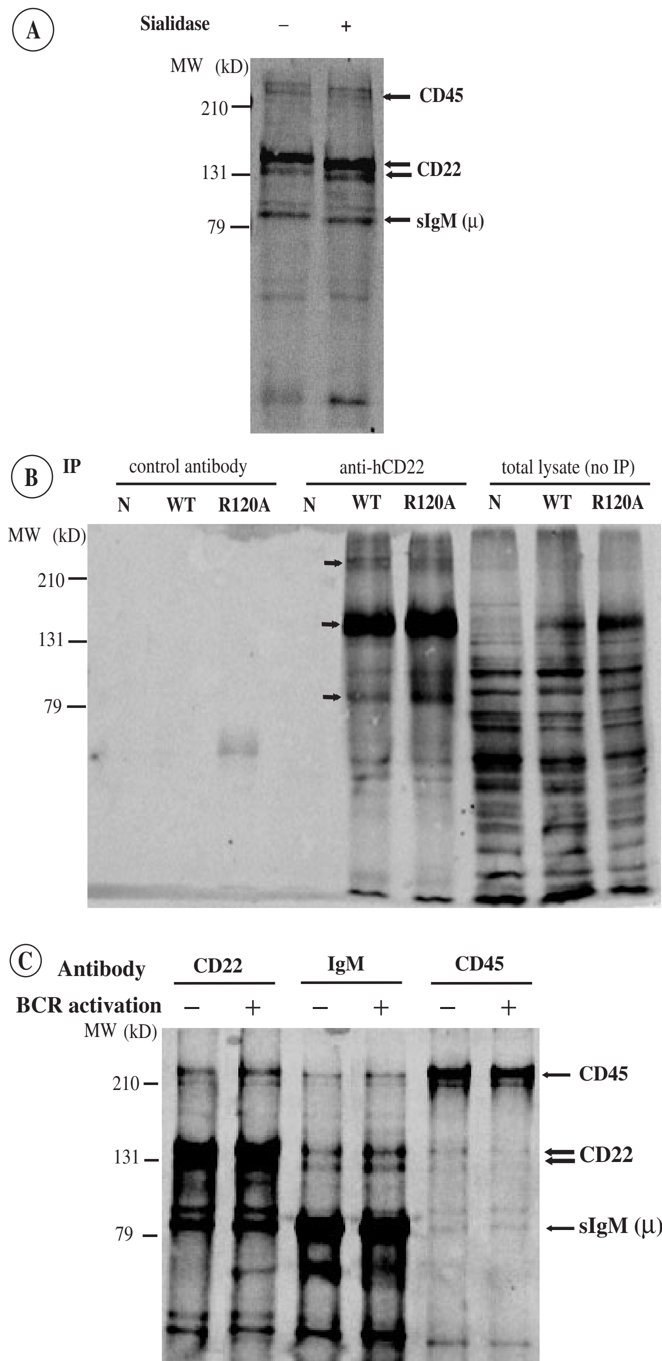

Fig. 3. None of the major proteins associated with CD22 do so in a Sia-binding-dependent manner. (**A**) Daudi cells with or without previous sialidase treatment were subjected to simultaneous biotinylation (by Sulfo-NHS-Biotin), cross-linking (by DTSSP), and immunoprecipitation (by antibody to human CD22, clone To15). Daudi cells were quickly chilled and treated with *A. ureafaciens* sialidase for 1 h at 4°C with gentle shaking. The sialidase was used at 10 mU/1 $\times 10^6$ cells in sialidase treatment buffer (20 mM HEPES, 140 mM NaCl). The cells were then subjected to simultaneous biotinylation, cross-linking, and immunoprecipitation. Only the results with the 2-ME-reduced samples are shown. CD22, IgM (μ) and CD45 are indicated by arrows. Neither CD45 nor sIgM (μ) nor the other unknown proteins associated with CD22 are significantly reduced in amount with prior sialidase treatment, and the small molecular weight shifts in some proteins confirmed the action of the sialidase. (**B**) Nontransfected parental J2-44 (N), wild-type (WT) hCD22, or arginine-mutated hCD22 (R120A) transfected J2-44 cells were subjected to simultaneous biotinylation (by Sulfo-NHS-Biotin), cross-linking (by DTSSP), and immunoprecipitation (IP, by antibody to human CD22, clone To15; or isotype matched control antibody). Samples were separated on 7.5% SDS–PAGE gels and subjected to transfer and detection of biotin-labeled proteins. Only the results with the 2-ME reduced samples are shown. Aliquots of total lysate without immunoprecipitation were run on the same gel. Migration positions of mouse CD45, human CD22 and mouse IgM (μ) are indicated by three arrows from top to bottom. The control antibody immunoprecipitation, anti-CD22 immunoprecipitation on nontransfected cells, and total lysate serve as controls for the specificity of CD22 association with the proteins seen in transfected cells immunoprecipitated by anti-CD22. Neither CD45 nor sIgM (μ) nor the other unknown proteins that are observed to be associated with wild-type CD22 are significantly reduced in associating with the arginine-mutated CD22. Thus none of these major associated molecules depends primarily on Sia recognition for their interaction with CD22. This is one representative experiment out of four that gave similar results. (**C**) BCR activation of Daudi cells was achieved by treatment with 1 μg/ml $F(ab')_2$ fragments of goat anti-human IgM Fc_{5u} specific antibody) for 10 min at 37°C. The activation was stopped by addition of 10 volumes of ice-cold media. Cells were then washed and subjected to simultaneous biotinylation and cross-linking, followed by immunoprecipitation with anti-CD22, anti-sIgM, or anti-CD45 antibodies. None of the major associations was changed following BCR activation.

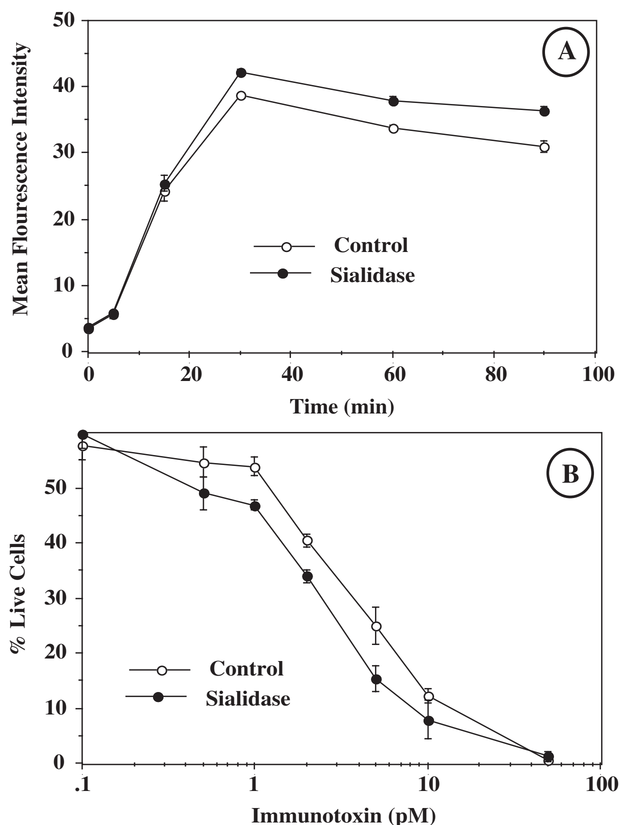

Fig. 4. Antibody-induced CD22 turnover and immunotoxin-mediated killing efficiency are slightly enhanced on sialidase-treated cells. Daudi cells with or without sialidase treatment were studied for (**A**) antibody-induced CD22 turnover and (**B**) CD22 immunotoxin killing. (**A**) Daudi cells were surface labeled with FITC-conjugated anti-human CD22 in cold and incubated in AIM-V media at 37°C for 0–90 min to allow CD22 endocytosis. At each time point, 10 volumes of ice-cold media were used to stop the process, and 0.2 M glycine (pH 2.5) was then applied to remove remaining surface bound antibody. Cells were then subjected to flow cytometric analysis for endocytosed CD22. 10 mU *A. ureafaciens* sialidase/10^6 cells was added right before incubation at 37°C. Error bars are SD values of triplicates. Student's *t*-test is used for statistical analysis. The differences at 30, 60, and 90 min are statistically significant ($p < 0.001$). This is one representative experiment out of three. (**B**) Daudi cells were incubated with CD22 immunotoxin (RFB4-dgRTA) in AIM-V media at 37°C for 24–48 h in 96-well plate in triplicate. The % of live cells was determined by flow cytometric analysis following propidium iodide staining. Error bars are SD values of triplicates. Student's *t*-test is used for statistical analysis. The differences seen at 1–2 pM concentrations are statistically significant ($p < 0.005$). The differences seen at 5 pM concentration is statistically significant ($p < 0.05$). This is one representative experiment out of four.

Thus it appears that antibody binding to CD22 enhances endocytosis via the same or different mechanisms that are involved in the constitutive process. Even if the mechanisms are different, the antibody-mediated process is of practical significance, because antibodies and antibody-based immunotoxins are being investigated for the treatment of CD22-expressing lymphomas and leukemias (Herrera *et al.*, 2000, 2003; Mansfield *et al.*, 1997; Messmann *et al.*, 2000; Pagel *et al.*, 2002; Tuscano *et al.*, 2003). We therefore asked whether CD22 interactions with cell surface sialylated ligands have an impact on antibody-mediated endocytosis.

As shown in Figure 4A, sialidase-treated cells showed a higher rate of antibody-induced CD22 endocytosis than untreated cells. The differences, though small, are reproducible (the differences at 30, 60, and 90 min are statistically significant). A result consistent with this was also seen in an immunotoxin killing assay. RFB4-dgRTA is an immunotoxin (kindly provided by Dr. Ellen S. Vitetta) consisting of a CD22 antibody coupled to the ricin A chain (Shen *et al.*, 1988). Because this toxin is delivered via CD22 endocytosis after binding via the antibody portion (Chan *et al.*, 1998), we tested the toxin sensitivity of cells in which CD22 was either masked or unmasked. Sialidase treatment requires serum-free media, and we found that the cells were intrinsically more sensitive to the immunotoxin killing in serum-free media than in serum-containing media (data not shown). Regardless, the overall killing efficiency was higher in sialidase-treated cells in comparison with buffer-incubated controls (Figure 4B, the differences seen at 1–5 pM concentrations are statistically significant, $p < 0.05$). Taken together, these findings negate the hypothesis that binding of sialylated *cis* ligands is a major factor regulating the cell-surface half-life of CD22 under native conditions. However, the antibody-induced CD22 endocytosis, and therefore CD22 immunotoxin killing can be altered by the presence of Sia.

Surface IgM cross-linking inhibits antibody-induced CD22 endocytosis and CD22 immunotoxin killing

Previous studies showed that activation of human B cells results in a small amount of CD22 unmasking on some B cells from human peripheral blood by as yet unknown mechanisms (Razi and Varki, 1998). We therefore tested the endocytosis rate of CD22 following BCR stimulation with anti-IgM antibodies, a functional surrogate for the process of B cell activation by antigen. We found that sIgM cross-linking had no effect on the constitutive rate of CD22 endocytosis (data not shown). Interestingly, sIgM cross-linking resulted in a much slower rate of CD22 antibody-induced CD22 endocytosis (BCR activation was proven by calcium influx measurement on these cells, data not shown). A similar finding was recently reported by another group (John *et al.*, 2003). To further explore this finding, cell surface Sias on BCR-activated cells were removed by sialidase treatment, which did not cause any further change in CD22 endocytosis rate in comparison to BCR activation only (Figure 5).

An immunotoxin killing assay was also performed on sIgM cross-linked cells, as shown in Figure 6. At a low concentration of immunotoxin in the presence of an anti-IgM antibody, cell survival is slightly reduced compared to that without the antibody, possibly as a result of anti-IgM induced apoptosis (Chan *et al.*, 1990; Chaouchi *et al.*, 1995). However with increasing amounts of immunotoxin, the presence of anti-IgM prevents cells from being killed by immunotoxin, which is possibly the result of the anti-IgM reducing immunotoxin uptake. This result is consistent with the one seen with anti-IgM in the endocytosis assay (Figure 5). Thus the final efficiency of CD22 immunotoxin killing must be influenced by multiple factors, including the activation state of the cell.

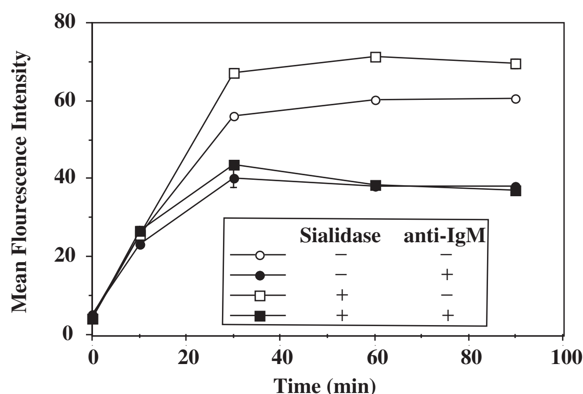

Fig. 5. BCR stimulation inhibits antibody-mediated CD22 turnover but simultaneous sialidase treatment has no further effect. Daudi cells were surface labeled with FITC-conjugated anti-human CD22 in cold and incubated at 37°C for 0–90 min to allow CD22 endocytosis. At each time point, 10 volumes of ice-cold media were used to stop the process, and 0.2 M glycine (pH 2.5) was then applied to remove remaining surface bound antibody. Cells were then subjected to flow cytometric analysis for endocytosed CD22. *A. ureafaciens* sialidase and 1 μg/ml goat anti-human IgM, Fc_{5u} specific antibody were added right before incubation at 37°C. Error bars are SD values of triplicates.

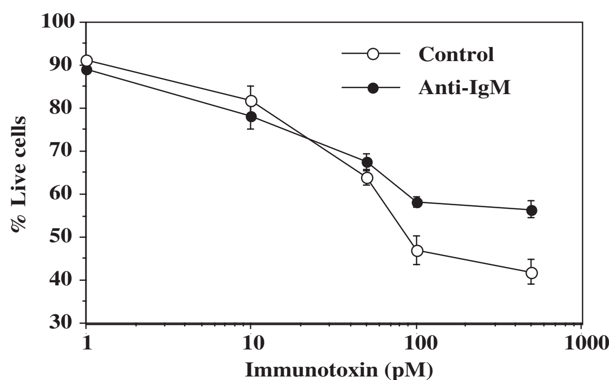

Fig. 6. BCR stimulation diminishes the killing efficiency of CD22 immunotoxin on Daudi cells. Daudi cells treated with or without 1 μg/ml anti-IgM were studied for CD22 immunotoxin killing. Daudi cells were incubated with CD22 immunotoxin (RFB4-dgRTA) at 37°C for 24–48 h in 96-well plate in triplicate. The % of live cells was determined by flow cytometric analysis following propidium iodide staining. Error bars are SD values of triplicates. This is one representative experiment out of three.

Discussion

In this article we explored two possible mechanisms of how *cis* Sia ligand binding might be important in modulating CD22 physiological functions: (1) that *cis* binding helps maintain CD22 partners on the cell surface; and (2) that *cis* binding affects CD22 turnover and thus changes its cell surface levels. We report a novel approach for studying the natural *cis* ligands of CD22 on B cells, and the role of Sias in these interactions. Simultaneous cross-linking and surface labeling in the cold of rapidly chilled cells provides a picture likely to be much closer to the native state. This approach shows that a major fraction of total cell surface CD22 exists in complexes with other cell surface proteins such as CD45 and sIgM. Up to 16% of cell surface CD22 could be associated with sIgM, which is much higher than the 1–2% previously reported by others (Law *et al.*, 1994; Leprince *et al.*, 1993). Likewise, the fraction of the total sIgM associated with CD22 was 8%, again much higher than the 0.2–2% reported in previous studies (Peaker and Neuberger, 1993). Furthermore, we found that 30% of the cell surface CD45 was associated with CD22. Conversely 5.6% of cell surface CD22 and 0.6% of sIgM were found in a CD45 immunoprecipitate.

Overall, our conclusion is that the steady-state associations of CD22 with sIgM and CD45 occur at much higher levels than previously reported. This is presumably because our approach of simultaneous labeling and cross-linking rapidly chilled cells is more likely to detect relatively labile noncovalent complexes. We also excluded other factors that could have biased the result. First the content of potentially biotinylated lysine residues in the extracellular domain of CD22, CD45, and sIgM (μ) C region are 5.8%, 5.8%, and 4.8%, respectively. Second, the overall efficiency of first pass immunoprecipitation was similar for CD22, CD45, sIgM (μ) (30%, 25%, and 36%, respectively, the numbers probably reflect the fact that not all molecules are accessible for immunoprecipitation after such major cross-linking). Therefore the disparity with previous reports is unlikely to be a result of major differences in biotinylation or immunoprecipitability of these molecules in our experiment. Rather it is likely due to the sensitivity of the protein complexes to detergent lysis and/or surface labeling steps that are typically done as a separate step, prior to cross-linking. Our new approach should be of value in many other biological situations where there are multiple loosely associated components in such cell surface complexes. Potentially, it could even be used to study intracellular protein complexes by simultaneously adding the biotinylation and cross-linking reagents along with a detergent or cell-permeabilizing reagent.

As mentioned before, there are two proposed models regarding CD22 *cis* ligand binding (Collins *et al.*, 2002) indicating that Sia binding is the major force either keeping CD22 bound to sIgM or away from it (by binding to other sialylated proteins, e.g., CD45). Our result does not support either model, because no major difference was found in the protein components within the CD22-related complex between wild-type and arginine-mutated molecules or when treating cells with sialidase. Our results instead show that Sia binding is not required for mediating the primary interactions of CD22 with CD45 and sIgM (and some other unidentified proteins). Thus we propose that these associations are primarily mediated via protein–protein interactions. Of course because CD45 and sIgM can themselves carry N-glycans with α2-6 Sias, initial associations with CD22 via protein–protein interactions may then give them an advantage over other cell surface molecules in developing secondary interactions via Sias. On the other hand, we cannot rule out the possibility that some other minor cell surface molecules associate with CD22 exclusively in a Sia-dependent matter. CD22 may also be masked via low-level interactions with many or all of the α2-6 sialylated molecules on the cell surface, in a dynamic equilibrium, without

requiring a favored interaction with a specific ligand. Results consistent with this conclusion come from a very recent publication, in which the authors showed that the degree of CD22 masking on cell surface is affected by local concentration of the ligands and that CD45 is not requisite for CD22 masking (Collins *et al.*, 2004).

As with all coimmunoprecipitation and cross-linking approaches, ours is based on the vicinity of the proteins to one another. If a Sia residue that binds to CD22 is located on a protein that is physically further away from CD22 than the length of the cross-linker, then that association could be missed using our method. It is also possible that an interacting protein could carry glycan groups that are bulky enough to prevent the access of our cross-linker. In this article, we focused mainly on the two proteins that have been proposed as ligands for CD22 and found their associations with CD22 are not solely based on Sia binding. Defining all of the possible ligands for CD22 remains to be done, but this is outside the scope of the present study.

BCR stimulation is a process during which CD22–Sia binding is known to have effects on calcium response, SHP-1 recruitment, CD22 phosphorylation, and so on. Thus it is possible that CD22–Sia binding may have different functions following activation. We have done some preliminary experiments using anti-IgM stimulation without seeing any major effects on the associations between CD22 and sIgM or CD45 (Figure 3C). We cannot rule out some very subtle changes that are not detectable with our method, of course. However, our results do not support an extensive change in major partners as a mechanism for how Sia binding affects CD22 function during activation. Fully exploring this issue will require a lot of additional work.

Another point of interest is that the protein–protein interactions between CD22, sIgM, and CD45 occur even when human CD22 is transfected into mouse B cells. Thus the domains and amino acid residues involved in these interactions have remained conserved since the common ancestor of humans and mice more than 50 million years ago. Given the 55–70% conservation of the amino acid sequences of the extracellular domains of CD22 between humans and mice (Torres *et al.*, 1992), it should be possible eventually to define the specific domains and amino acid residues involved in these interactions.

Also of note is the fact that CD22 itself is the main component in the high-molecular-weight immunoprecipitated complexes both in Daudi cells and in transfected J2-44 cells (~ 60% as calculated by fluorescence intensity). This fits with previous findings suggesting that CD22 may form multimers (Powell *et al.*, 1995). If such multimers do exist, the fact that the high-molecular-weight complexes were seen even with the arginine-mutated CD22 indicates again that protein–protein interactions rather than Sia recognition are primarily involved in generating them. Of course, considering the relatively low single-site affinity of CD22 for Sias, a multimeric complex mediated by protein–protein interactions would facilitate any secondary binding avidity via Sias and/or facilitate possible self-masking due to the α2-6-linked Sias carried by N-glycans on CD22 itself. Because of the very high molecular weight of all the cross-linked complexes, we could not accurately analyze whether such CD22 clustering is further assisted by Sia-dependent interactions.

In the second part of the article we tested an alternative hypothesis regarding how *cis* binding might affect CD22 function. The question we ask is whether the Sia-based interactions of CD22 with other cell surface molecules might restrict its ability to be cleared from the cell surface by endocytosis. We found that although Sia did not affect constitutive CD22 endocytosis and turnover, it did have some effect on antibody-induced CD22 endocytosis. Removing cell surface Sia enhances the rate of antibody-induced CD22 endocytosis. Although the overall effect is modest, it is reproducible and could be of practical relevance in the use of immunotoxins for treatment of lymphomas, where the therapeutic margin (efficacy over toxicity) is likely to be narrow. The transfected J2-44 cells discussed could not be used for a similar study, because toxin sensitivity is critically dependent on cell surface target density, and it was difficult to precisely match the cell surface expression of the wild-type and arginine-mutated CD22.

It is possible that antibody-induced endocytosis is representative of the situation when CD22 is being cross-linked, for example, by natural *trans* ligands after exposure during activation. More work needs to be done to explore this potential connection. Beyond this possibility, the critical role of Sia recognition by CD22 in controlling B cell activation state remains unexplained. Finally, we found that anti-IgM cross-linking, a process involved not only in BCR activation but also in CD22 unmasking, results in a slower rate of antibody-induced CD22 turnover and a less efficient CD22 immunotoxin killing. Whether these effects are due to cell activation, stoichiometric change in associated protein partners, or other mechanisms is not clear. Further studies on this topic are needed to better understand the relationship between BCR activation state and CD22 turnover, and the results could be relevant to clinical usage of CD22 immunotoxin.

Materials and methods

Cells and culture

Daudi cells, a human B lymphoblast (from ATCC) was cultured in RPMI 1640 media supplemented with 10% fetal calf serum, 2 mM glutamine. In experiments involving sialidase treatment, serum-free lymphocyte media (AIM-V) was used instead. The CD22-/- J2-44 mouse B cell line was a generous gift from Dr. Henry Wortis (Jin *et al.*, 2002). It was maintained in RPMI 1640 media supplemented with 20% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 0.1 mM nonessential amino acids, 20 mM HEPES, and 50 μM β-mercaptoethanol. Transfected J2-44 cells were maintained with the presence of 1 μg/ml puromycin. cDNA for human CD22 was obtained from Dr. Ivan Stamenkovic (Stamenkovic *et al.*, 1991), and transferred into the pcDNA 3.1(-) vector. An arginine mutation (R120A) was introduced into CD22 by a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used were sense primer: 5′ GGTC AGCTGGGGCTG<u>GCC</u>ATGGAGTCCAAGACTGAG3′,

and anti-sense primer: 5′ CTCAGTCTTGGACTCCAT-GGCCAGCCCCAGCTGACC3′ (mutation site was as underscored). The mutation was confirmed by sequencing. The cDNA was subcloned into pMSCVpuro vector. Retrovirus was produced through introducing hCD22/MSCV and ecotropic packaging constructs into 293T cells by calcium phosphate transfection (Invitrogen, Carlsbad, CA). J2-44 cells were infected by retrovirus as described (Sykes and Kamps, 2001). Briefly, 1 ml retrovirus supernatant was used to infect J2-44 cells by spinoculation ($2500 \times g$, 1 h, 25°C) with the presence of 1:1000 Lipofectamine (Invitrogen).

Simultaneous cross-linking and biotinylation and CD22 immunoprecipitation

DTSSP and EZ-Link Sulfo-NHS-Biotin were purchased from Pierce (Rockford, IL). Daudi cells (10×10^6) or transfected J2-44 cells were harvested, washed three times in ice-cold phosphate-buffered saline (PBS), pH 8.0, and resuspended to 25×10^6 cells/ml in ice-cold PBS, pH 8.0, containing 1 mM each freshly prepared DTSSP and Sulfo-NHS-Biotin. Cross-linking and biotinylation were allowed to proceed at 4°C for 30 min with gentle shaking. Cells were then pelleted and washed three times in ice-cold PBS, pH 8.0. The pellet was lysed in lysis buffer (PBS, 1% NP-40, 1% Triton X-100, 1 mM ethylenediamine tetra-acetic acid, 2 μg/ml each of leupeptin, aprotinin, and pepstatin A) for 30 min at 4°C with inverting. Supernatants were collected after centrifuging for 30 min at $20{,}000 \times g$ at 4°C. Aliquots of the lysate were precleared with protein A–Sepharose (Amersham Biosciences, Little Chalfont, UK) for 1 h at 4°C on a rotating wheel, and protein quantity was determined using the BCA protein assay kit (Pierce). The precleared lysate was subjected to immunoprecipitation with protein A–Sepharose conjugated with antibodies to CD22 (clone To15, Dako, Denmark), IgM (clone G20-127, BD Biosciences Pharmingen, San Diego, CA), CD45 (clone HI30, BD Biosciences Pharmingen) or isotype-matched mouse IgG (BD Biosciences Pharmingen) as control. Immunoprecipitation was done at 4°C for 2 h on a rotating wheel, and the beads were washed three times with lysis buffer and one time with PBS. Immunoprecipitates were boiled in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer with or without 5% β-mercaptoethanol (2-ME) for 5 min, run on 7.5% SDS–PAGE gels, transferred, blocked, and stained by 1:300 Cy5-conjugated streptavidin (Jackson Immuno Research, West Grove, PA). Visualization and quantification were performed using Storm 860 scanner (Amersham Pharmacia Biotech) and ImageQuant software.

Sialidase treatment and BCR stimulation

Arthrobacter ureafaciens sialidase (Sigma, St. Louis, MO) 10 mU was used to unmask CD22 on 1×10^6 CD22-expressing cells. For α2-6 Sia probe binding experiments, sialidase pretreatment was performed in sialidase treatment buffer (20 mM HEPES, 140 mM NaCl) at room temperature for 15 min with shaking. Cells were washed four times in FACS staining buffer (PBS containing 1% bovine serum albumin, 0.02% sodium azide) before staining with the probe. For simultaneous cross-linking and biotinylation of Daudi cells following sialidase treatment, sialidase was used in sialidase treatment buffer for 1 h at 4°C with gentle shaking. For the other experiments, sialidase was added into serum-free culture media at the same concentration. For BCR stimulation, $F(ab')_2$ fragments of goat anti-human IgM, Fc_{5u} specific antibody (Jackson ImmunoResearch) were added to the cell culture media at 1 μg/ml.

Antibody-induced CD22 turnover assay

The whole experiment was done at 4°C unless otherwise indicated. Cells were pelleted, washed with serum-free AIM-V media, and resuspended at 10×10^6/ml in AIM-V media containing 1:100 fluorescein isothiocyanate (FITC)-conjugated anti-human CD22 (clone 4KB128, from Dako). The incubation was done in the dark for 30 min with gentle shaking. Cells were then washed three times to remove unbound antibody and resuspended in culture media with or without serum, depending on the experiment. After being incubated at 37°C for 0–90 min, 10 volumes of ice-cold media were added to the cells, which were then washed twice, followed by incubation with 0.2 M glycine, pH 2.5, for 5 min to remove any remaining surface-bound antibody. Cells were then washed, and subjected to flow cytometric analysis. FACSCalibur (BD Biosciences) and Flowjo software were used to collect and analyze the data.

Flow cytometric analysis on transfected J2-44 cells

Cells (1×10^6) were collected, washed, and suspended in FACS staining buffer (PBS containing 1% bovine serum albumin, 0.02% sodium azide). 1:100 FITC-conjugated anti-human CD22 (clone 4KB128, from Dako) was used to stain cell surface CD22 for 1 h at 4°C. α2-6-Sialyllactose-PAA-biotin (biotinylated polyacrylamide arrays from GlycoTech [Rockville, MD], carrying multiple copies of α2-6-sialyllactose and biotin) was used to detect Sia binding by CD22. α2-6-Sialyllactose-PAA-biotin (10 μg/ml) was incubated with cells for 1 h at 4°C, washed, and followed by 1:100 R-phycoerythrin-conjugated streptavidin (Jackson ImmunoResearch) staining for 30 min at 4°C. FACSCalibur (BD Biosciences) and Flowjo software were used to collect and analyze the data.

Immunotoxin killing assay

CD22 immunotoxin (RFB4-dgRTA) was a kind gift from Dr. Ellen S. Vitetta (Shen *et al.*, 1988). Cells (5×10^4) were incubated with RFB4-dgRTA 10^{-12} to 10^{-9} M and cultured at 37°C for 24–48 h in 96-well plate in triplicate. Cells were harvested, washed, and resuspended in PBS containing 1% bovine serum albumin, 0.02% sodium azide. Propidium iodide (Roche, Indianapolis, IN) was added to the suspension immediately before analysis to a final concentration of 2 μg/ml. Samples were subjected to flow cytometric analysis.

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Abbreviations

2-ME, β-mercaptoethanol; BCR, B cell receptor; dgRTA, deglycosylated ricin A chain; DTSSP, 3,3′-dithiobis (sulfo-succinimidylpropionate); FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Sia, sialic acid.

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