

## **Identification of the Ligand-binding Domains of CD22, a Member of the Immunoglobulin Superfamily that Uniquely Binds a Sialic Acid-dependent Ligand**

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### **Summary**

CD22 is a B cell-restricted member of the immunoglobulin (Ig) superfamily that functions as an adhesion receptor for leukocytes and erythrocytes. CD22 is unique among members of the Ig superfamily in that it has been suggested to bind a series of sialic acid-dependent ligands, potentially through different functional domains expressed by different splice variants of CD22. In this study, the epitopes identified by a large panel of function-blocking and non-function-blocking CD22 monoclonal antibodies were localized to specific Ig-like domains, revealing that all function-blocking monoclonal antibodies bound to the first and/or second Ig-like domains. Consistent with a single ligand-binding region, the two amino-terminal domains were the functional unit that mediated CD22 adhesion with lymphocytes, neutrophils, monocytes, and erythrocytes. The predominant cell surface species of CD22 was a full length 140,000 relative molecular mass seven Ig-like domain glycoprotein and a minor 130,000 relative molecular mass form lacking the fourth domain. While the two amino-terminal Ig-like domains of CD22 are structurally similar to those found in other members of the Ig superfamily involved in cell adhesion and containing an amino acid sequence motif associated with integrin recognition, site-directed mutagenesis of critical residues surrounding this motif did not disrupt CD22-mediated adhesion. These results demonstrate that the unique ligand-binding properties of CD22 are distinct from those of other members of the Ig superfamily involved in integrin-mediated cell adhesion.

**I**g superfamily members most commonly interact with other members of the Ig superfamily or members of the integrin family through protein-protein interactions. In contrast, the CD22 adhesion molecule is a member of the Ig superfamily that binds cell surface ligands through a sialic acid-dependent mechanism (1-4). CD45RO and CDw75 were initially proposed to be specific ligands for CD22 (1). However, subsequent studies have not confirmed these findings (2, 5, 6). A soluble fusion protein composed of the extracellular domain of CD22 and Ig has been reported to bind to multiple glycoproteins, including all isoforms of CD45, generating the conclusion that CD22 may have multiple ligands (3, 7). Nonetheless, all studies have confirmed that sialic acid is an essential component of the CD22 ligand, as neuraminidase treatment of target cells eliminates CD22-mediated adhesion (1-4). Therefore, the ligand-binding activity of CD22 is unprecedented among members of the of Ig superfamily, leading to the notion that CD22 represents a new class of Ig superfamily members which function as mammalian lectins (3, 4).

CD22 is a B lineage-restricted phosphoglycoprotein expressed in the cytoplasm of pre-B cells and on the surface

of mature B lymphocytes (8, 9). The binding of antibodies to CD22 in vitro augments both the increase in intracellular free  $Ca^{++}$  and the proliferation induced after cross-linking of cell surface Ig (10, 11). The cytoplasmic tail of CD22 contains a region of homology with the antigen-receptor recognition homology 1 motif that is found in the cytoplasmic domains of several signal transduction molecules (12, 13). A fraction of cell surface CD22 has also been reported to associate with the surface Ig receptor complex (12, 13). Therefore, ligand-binding by CD22 is likely to play a significant role in B cell activation.

The first isolated CD22 cDNA encoded an extracellular region composed of an amino-terminal V-type Ig-like domain, followed by four C-type Ig domains (14). Subsequently, a CD22 cDNA that encoded two additional C-type Ig-like domains (domains 3 and 4) was cloned (15). The five Ig domain form, termed CD22 $\alpha$ , was reported to bind erythrocytes and monocytes (1, 14), while the seven Ig domain form (CD22 $\beta$ ) mediates binding of B and T lymphocytes, monocytes, neutrophils, and erythrocytes (2, 14, 15). CD22 can be immunoprecipitated from B lymphocytes and some B cells lines as a dominant 140,000-M<sub>r</sub> protein, with a modest 130,000-

*M<sub>r</sub>* protein species (2, 8, 16). The above structural and functional data imply that CD22 contains, at least, two distinct ligand-binding sites and that the third and/or fourth Ig-like domains play a selective role in CD22 binding to ligands present on B and T lymphocytes (1). In contrast to this hypothesis, studies using a panel of CD22 mAb indicate that a single region of CD22 is involved in binding of lymphocytes, monocytes, neutrophils, and erythrocytes (2). To further determine the components of CD22 responsible for its unique sialic acid-binding activity and to examine the differential function of the CD22 forms, the ligand-binding domains of CD22 were mapped using mAb and domain deletion mutants. These studies demonstrate that the first two domains of CD22 form a functional unit that mediates adhesion.

## Materials and Methods

**Antibodies.** The HB22 mAb reactive with CD22 were used as hybridoma tissue culture supernatant fluid or as purified mAb at 5  $\mu$ g/ml, as described (2). The remaining CD22 mAb were from the Fifth International Leukocyte Differentiation Antigen Workshop (Boston, MA) and were used as purified mAb at 5  $\mu$ g/ml.

**Production of Mutant CD22 cDNA.** Truncated CD22 cDNA were produced using restriction sites naturally present at the boundaries between Ig-like domains and/or by introducing new unique restriction sites by PCR (GeneAmp; Perkin-Elmer Cetus, Norwalk, CT), using a full-length CD22 cDNA template (15), as described (2). Domain deletions were guided by previous assignment of domain boundaries (15). All PCR products were subcloned, sequenced, ligated with the appropriate CD22 cDNA fragments, and subcloned into the pMT2 expression vector (Genetics Institute, Cambridge, MA). Different oligonucleotide primers were used, which changed amino acids D<sup>50</sup>, D<sup>52</sup>, L<sup>53</sup>, E<sup>54</sup>, and I<sup>56</sup> to alanine. The amino acids F<sup>55</sup>IL were also changed to L<sup>55</sup>AR. PCR products containing point mutations were swapped with the corresponding sequence, using a unique HindIII site close to the mutation site.

**Adhesion Assays.** COS cells were transfected by the DEAE-dextran method with full-length CD22 cDNA in the CDM8 expression vector (15), or with the CD22 cDNA mutants. After transfection (24 h), the cells were trypsinized and transferred to 35-mm tissue culture dishes and cultured for an additional 24 h. Adhesion assays were as described (2).

**Staining of cDNA-transfected COS Cells with mAb.** Transfected COS cells were stained with different CD22 mAb 48 h after transfection, using a peroxidase-conjugated rabbit anti-mouse Ig antiserum (Dako, Glostrup, Denmark) and a substrate containing amino ethyl carbazole (Sigma Chemical Co., St. Louis, MO) and dimethyl formamide (Sigma Chemical Co.). None of the mAb stained untransfected COS cells, except the HB22.33 and S-HCL1 mAb, which gave light staining in some experiments.

**Radiolabeling and Immunoprecipitation Analysis.** Daudi cells were surface labeled ( $2 \times 10^7$  cells in 200  $\mu$ l) with <sup>125</sup>I, as described (2). Immunoprecipitations were as described (17). Immunoprecipitates were electrophoresed under reducing conditions on an 8.5% SDS-PAGE gel, dried, and autoradiographed. *M<sub>r</sub>* were determined, using prestained standard mol wt markers (Gibco-BRL, Gaithersburg, MD).

## Results

**Epitopes Identified by mAb that Block CD22-mediated Adhesion.** A panel of 30 CD22 mAb was tested for the ability

to block the attachment of B cell lines (Raji and Daudi), a T cell line (Jurkat), erythrocytes, neutrophils, and monocytes to COS cells transfected with a full length CD22 cDNA (COS-CD22). 4 mAb completely blocked (80–100%) the binding of all cells to COS-CD22 cells (Table 1). 4 additional mAb partially blocked adhesion (20–80%). The remaining 22 mAb had little or no effect on cell binding, except 1 mAb, IS7, which partially blocked erythrocyte adhesion.

The domains identified by the CD22 mAb were determined using truncated forms of CD22 cDNA that lacked domain 1 (CD22 $\Delta$ 1), domain 2 (CD22 $\Delta$ 2), domains 3 and 4 (CD22 $\Delta$ 3,4), domain 4 (CD22 $\Delta$ 4), and domains 2, 3, and 4 (CD22 $\Delta$ 2–4). All truncated forms of CD22 were identified by the HB22-12 and BC-8 mAb, and all cDNA were expressed with an intensity similar to that observed for wild-type CD22 (Table 1). The binding of most CD22 mAbs was dependent on the expression of individual Ig-like domains, whereas 12 of the CD22 mAb recognized epitopes dependent on the presence of both the first and the second Ig-like domains (Table 1). The epitopes recognized by mAb HD239, HD39, HB22.27, and S-HCL1 were also dependent on expression of the first two domains, but reduced expression was detected on CD22- $\Delta$ 2 cells, suggesting that these mAb recognize an epitope primarily localized within the first domain. These results suggest that the first and second domains interact to achieve correct folding of this portion of the molecule. Importantly, each of the mAb that blocked CD22-mediated adhesion was reactive with the first and/or second Ig-like domains (Table 1).

Since the conformation of the first Ig-like domain of CD22 may be affected by the loss of the second Ig-like domain, the second domain of mouse CD22 was substituted for that of human CD22. Essentially, this domain substitution had an effect on CD22 mAb binding similar to that induced by deletion of the second human Ig-like domain, except low level binding of the HB22-196 and HI22 mAb was preserved (Table 1). None of the anti-human CD22 mAbs were reactive with mouse CD22.

**CD22 Ig-like Domains 1 and 2 Mediate Cell Adhesion.** COS cells transiently transfected with the truncated CD22 cDNA constructs were tested in direct binding assays with erythrocytes, B cell lines (Raji and Daudi), monocytes, neutrophils, and the Jurkat T cell line. All cDNA constructs containing domains 1 and 2 conferred cell binding, and no binding was observed when either domain 1 or domain 2 was absent (Fig. 1). COS cells transfected with CD22 $\Delta$ 3-4 and CD22 $\Delta$ 4 cDNA supported adhesion to equivalent levels as wild-type CD22. Ligand-binding activity of the second domain of mouse CD22 was completely absent. Thus, CD22-mediated adhesion requires both the first and second Ig-like domains, but not subsequent domains.

**Mutational Analysis of the First Ig-like Domain.** A conserved amino acid motif is present in the first domain of several members of the Ig superfamily that represents an essential primary binding site for integrins (17, 18). Since this motif is similar in location and sequence to regions in the first domain of human and mouse CD22, the aspartic acid residue at position 50 was changed to an Ala (D<sup>50</sup>/A) as well as

**Table 1.** Adhesion-blocking Activity and Domain Reactivity of CD22 mAb

mAb Name	Blocking activity*		Reactivity with CD22 cDNA-transfected cells†						
	Lym.	RBC	Δ1	Δ2	Δ3,4	Δ4	Δ2-4	m2	Domain
HB22-2	-	-	++	++	-	++	-	+	3
HB22-5	+	++	-	-	++	++	-		1-2
HB22-7	++	++	-	-	++	++	-		1-2
HB22-12	-	-	++	++	++	++	++	++	5-7
HB22-13	+	++	-	-	++	++	-		1-2
HB22-15	-	-	-	-	++	++	-		1-2
HB22-17	-	-	-	-	++	++	-		1-2
HB22-18	-	-	-	-	++	++	-		1-2
HB22-19	-	-	-	-	++	++	-		1-2
HB22-22	++	++	-	-	++	++	-	-	1-2
HB22-23	++	++	-	-	++	++	-		1-2
HB22-25	-	-	++	++	-	-	-	++	4
HB22-27	-	-	-	+	++	++	-	+	1
HB22-33	++	++	-	-	++	++	-		1-2
HB22-196	+	++	-	-	++	++	-	+	1-2
3G5	-	-	++	++	-	++	-		3
4KB128	-	-	++	-	++	++	-	-	2
BL-3C4	-	-	++	-	++	++	-	-	2
G28-7	-	-	++	++	-	-	-		4
HD239	-	-	-	+	++	++	-	+	1
HD39	-	-	-	+	++	++	-	+	1
HD6	-	-	++	++	-	++	-		3
IS7	-	+	++	-	-	++	-		2-3
To15	-	-	++	++	-	-	-	++	4
3H4	-	-	++	-	-	++	-		2-3
OKB22A	-	-	++	++	-	++	-		3
HI22	-	-	-	-	++	++	-	+	1-2
BU59	+	++	++	-	++	++	-		2
BC-8	-	-	++	++	++	++	++		5-7
S-HCL1	-	-	-	+	++	++	-		1

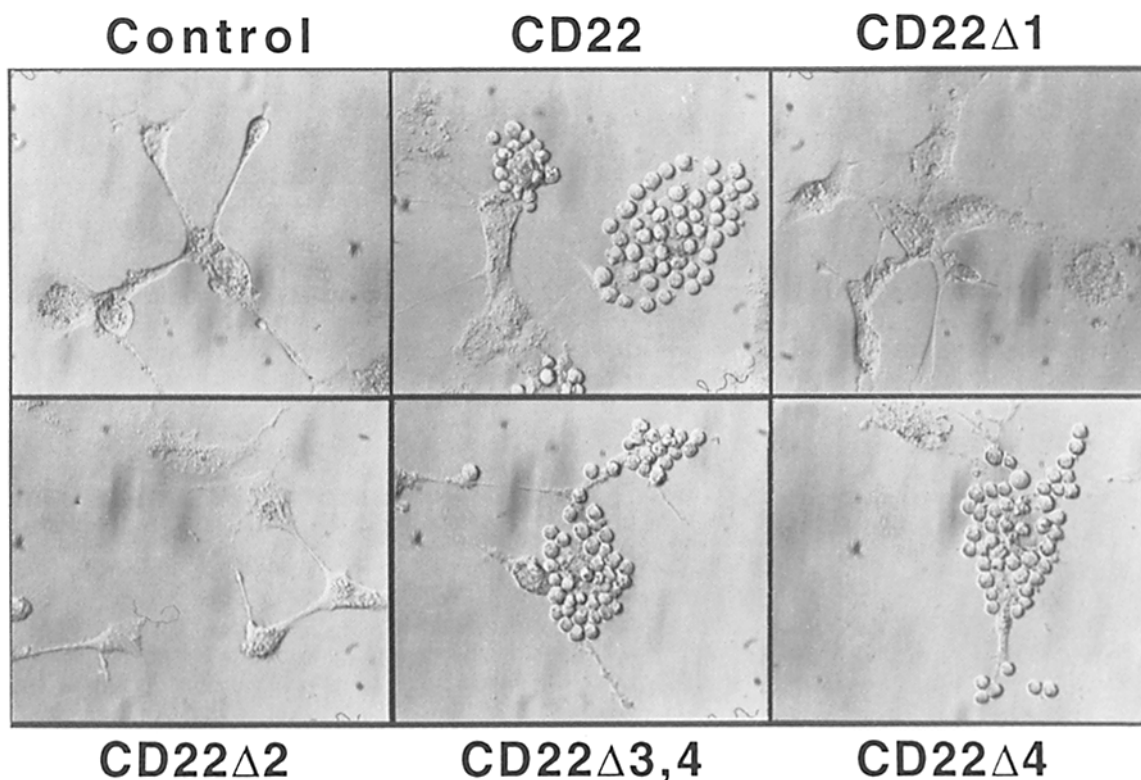
\* Values represent the amount of adhesion inhibited by the mAb: -, <20% blocking; +, 20-80% blocking; ++, 80-100% blocking. Similar results were obtained with Raji, Daudi, and Jurkat cells (Lym). These results are representative of those obtained in at least two independent experiments.

† Results represent: -, staining identical to background; +, distinct positive staining; ++, bright staining intensity, as determined by immunohistochemical analysis.

other residues surrounding and within the motif, D<sup>52</sup>/A, L<sup>53</sup>/A, E<sup>54</sup>/A, and I<sup>57</sup>/A (Fig. 2). Proper folding of CD22 was maintained, since staining of these mutant receptors with each mAb directed against the first and/or second Ig-like domains was not affected. Similarly, none of the introduced amino acid changes had an effect on the binding of B cells, T cells, or erythrocytes. One mutation, in which the amino acids F<sup>55</sup>IL in the motif were changed to L<sup>55</sup>AR, abrogated adhesion of all cell types analyzed. However, this change disrupted the overall conformation of CD22, since domains 1

and 2 mAb did not react with this mutant. Thus, this region of CD22 was not critical for ligand-binding activity.

**Analysis of CD22 Isoforms.** The structural basis for the size difference in cell surface CD22 species was examined by immunoprecipitating CD22 with mAb that recognize domains 1 (HD39), 1-2 (HB22-7), 2 (BU59), 2-3 (I57), 3 (3G5), 4 (G28-7 and To15), and 5-7 (BC-8). All CD22 mAb, except those recognizing the fourth domain, immunoprecipitated two bands of 140,000 and 130,000 M<sub>r</sub> (Fig. 3). Antibodies that bind to the fourth domain precipitated only the

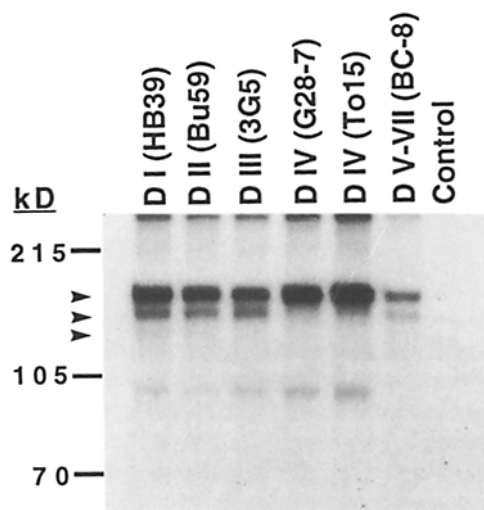


**Figure 1.** Binding of Raji cells to mock-transfected COS cells (*Control*), or COS cells transfected with full-length, or mutants of, CD22 lacking the indicated Ig-like domains. CD22-mediated adhesion was verified by blocking test cell attachment with the HB22-23 mAb (5  $\mu$ g/ml). Results represent those obtained with RBC, neutrophils, T cell lines, and monocytes.

		I / L - D / E - S / T - P / X - L	
		* * *	# # #
<b>human CD22 D1</b>	CVWIPCTYRALDGD--	<b>LESFI</b>	LFH
human ICAM-1 D1	CSTS-CDQPKLLG---	<b>IETPL</b>	PKK
human ICAM-2 D1	CSTT-CNQPEVGG---	<b>LETSL</b>	NKI
human ICAM-3 D1	CSTD-CPSEKIA---	<b>LETSL</b>	SKE
human VCAM-1 D1	CSTTGCESPFFSWRTQ	<b>IDSPL</b>	NGK
human VCAM-1 D4	CSVMGCESPFSWRTQ	<b>IDSPL</b>	SGK
<b>mouse CD22 D1</b>	CIRIPCKYKTPLPKAR	<b>LDNIL</b>	LFQ
mouse ICAM-1 D1	CSSS-CKEDLSLG---	<b>LETQW</b>	LKD
mouse ICAM-2 D1	CSTN-CAAPDMGG---	<b>LETPT</b>	NKI
mouse VCAM-1 D1	CSTTGCESPFWSWRTQ	<b>IDSPL</b>	NAK
mouse VCAM-1 D4	CAAIGCDSPFSWRTQ	<b>TDSPL</b>	NGV
mouse MAdCAM-1 D1	CSMSCDEGVARVHWRG	<b>LDTSL</b>	GSV

**Figure 2.** The first Ig-like domain of CD22 contains an amino acid motif characteristic of that found in integrin ligands. Amino acid sequence alignment of members of the Ig superfamily with known integrin receptors showing the C-X<sub>3-4</sub>-C motif and the motif that directs integrin binding. Sequences from Ig-like domain 1 (D1) and domain 4 (D4) are indicated. Dashes indicate spaces introduced in the sequence to provide optimal alignment. The bold and boxed amino acids represent the conserved residues necessary for integrin binding; # indicates amino acids essential for VCAM-1 and ICAM-1 binding, and \* indicates amino acids in CD22 that were mutated to Ala.

140,000  $M_r$  band, indicating that the smaller isoform of CD22 results from the selective loss of domain 4. A very minor protein species of  $\sim$ 120,000  $M_r$  was detectable with long exposure of some immunoprecipitations with domains 1 and 2 mAb (Fig. 3). However, this band was not precipitated by domain 4 mAb and was not convincingly detectable



**Figure 3.** Immunoprecipitation of CD22 from surface-labeled Daudi B cells, using mAb that identify different CD22 Ig-like domains.

with domain 3 mAb, suggesting that it may be composed of CD22 forms lacking domains 3 and 4.

## Discussion

The first and second domains of CD22 were both essential for CD22-mediated adhesion of T cells, B cells, monocytes, neutrophils, and erythrocytes, and neither domain could function alone (Fig. 1, Table 1). Also, all CD22 adhesion-blocking mAbs bound epitopes located within the first and/or second Ig-like domains (Table 1). Thus, CD22 is similar to other adhesion receptors, CD2, CD4, intercellular adhesion molecule (ICAM)-1, ICAM-3, and vascular cell adhesion molecule (VCAM)-1, which contain ligand-binding sites in the two amino-terminal Ig-like domains. The amino-terminal Ig-like domain of CD22 is structurally similar to that of several integrin ligands; ICAM-1, ICAM-2, ICAM-3, VCAM-1, and mucosal addressin-1, which each contain extra cysteine residues generating the C-X<sub>3-4</sub>-C motif. Integrin binding to these proteins depends on the integrity of a critical five-amino acid sequence motif proximal to the C-X<sub>3-4</sub>-C motif (Fig. 2), while additional nonconserved sequences confer specificity for binding of the appropriate integrin (18, 19). Although a similar amino acid motif is present within human and mouse CD22, mutagenesis of the critical residues of this region did not affect CD22-mediated adhesion or the binding of function-blocking CD22 mAb (Fig. 2). These results argue that, despite the structural similarities between CD22 and other adhesion molecules of the Ig-superfamily that bind integrins, CD22 adhesion in the cases examined may not be integrin mediated.

Expression of most CD22 mAb-defined epitopes within the first two domains of CD22 was dependent on the presence of both the first and second Ig-like domains, suggesting that these two domains are conformationally linked and function cooperatively as a ligand recognition unit. The two amino-terminal Ig-like domains of CD22 share the most significant amino acid sequence homologies with CD33 and myelin-associated glycoprotein (MAG) (14, 15), and these proteins all share a common chromosome location, 19q13.1 (20). The

two amino-terminal domains of CD22, CD33, and MAG also contain one additional cysteine residue per domain located at the same relative position. In fact, these two residues form a novel disulfide linkage between the first two Ig-like domains in MAG (21). Since this potential interdomain disulfide bridge is a unique feature of this small subset of Ig superfamily members, these three proteins may identify a new class of adhesion receptors that have similar specificities for sialylated ligands.

Previous studies have suggested that CD22 is expressed in two functionally distinct forms on the cell surface, full length 140,000 M, CD22 $\beta$  and a smaller 130,000 M, CD22 $\alpha$  that lacked Ig-like domains 3 and 4 (1). However, deletion of either the first or second domain of CD22 completely eliminated all cell adhesion, despite high-level expression of the truncated proteins (Table 1). Also, COS-CD22 that lacked the third and fourth domains had the same binding capacity as full-length CD22. Thus, all adhesion is mediated by the first and second Ig-like domains of CD22. Immunoprecipitation of CD22 with mAb that bind different domains demonstrated that the 130,000 M form of CD22 is lacking just the fourth domain (Fig. 3), which is consistent with earlier suggestions. A CD22 mRNA splice variant lacking domain 4 has already been identified from different B cell lines, and the relative levels of expression of this mRNA species correlates with the level of 130,000 M, CD22 protein precipitated from the cell surface (2). Although a CD22 protein of the size predicted for a form lacking two domains (~120,000 M) has not been previously reported, there was a very minor protein species observed in this study that could correlate with this mRNA species. Since there are no binding capacity differences between the splice variants, the functional significance of the smaller CD22 species remains unknown.

In conclusion, these studies demonstrate that ligand binding occurs through a single region of CD22 that is completely dependent on the presence of both the first and second Ig-like domains. The rare presence of a potential interdomain disulfide bridge between domains 1 and 2 suggests a new subclass of Ig superfamily receptors which may mediate adhesion through a sialic acid-dependent mechanism.

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