Sialoadhesin and CD22 are members of a recently characterized family of sialic acid-dependent adhesion molecules belonging to the immunoglobulin superfamily. Sialoadhesin is a macrophage-restricted receptor containing 17 extracellular Ig-like domains which recognizes oligosaccharides terminating in NeuAcα2-3Gal in N- and O-linked glycans. CD22 is a B cell-restricted receptor with seven Ig-like domains which selectively recognizes oligosaccharides terminating in NeuAcα2-6Gal in N-glycans. Sequence similarity between these proteins is highest within their first four amino-terminal Ig-like domains. Here we identify the domain(s) containing the binding sites of both molecules by generating a series of extracellular domain deletion mutants fused to the Fc portion of human IgG1. Binding activity was analyzed by solid phase cell adhesion assays and also by surface plasmon resonance using purified glyco-phorin and CD45 as ligands for sialoadhesin and CD22, respectively. For sialoadhesin, the amino-terminal V-set Ig-like domain was both necessary and sufficient to mediate sialic acid-dependent adhesion of the correct specificity. In contrast, for murine CD22, only constructs containing both the V-set domain and the adjacent C2-set domain were able to mediate sialic acid-dependent binding. These results are consistent with the sialic acid binding site for both proteins residing in the membrane distal V-set domain, but for CD22 a direct contribution in binding from the neighboring C2-set domain cannot be excluded.
for sialoadhesin, the amino-terminal V-set Ig domain is both necessary and sufficient for sialic acid-dependent binding to cellular and molecular ligands. In contrast, murine CD22 appears to require both the V-set Ig domain and the adjacent C2-set domain as a "minimal unit" for mediating sialic acid-dependent adhesion. These results are consistent with the sialic acid binding site for both proteins residing in the membrane distal V-set domain, but for CD22 a direct contribution in binding from the neighboring C2-set domain cannot be excluded.

EXPERIMENTAL PROCEDURES

Materials—Unless specified otherwise, all reagents and chemicals were purchased from Sigma (Poole, U.K. or St. Louis, MO). For resialylation experiments the following sialyltransferases were used: Galβ1-4GlcNAcβ1-2-sialyltransferase, giving NeuAcα2-6Galβ1-3GlcNAcα (6-9, purified from rat liver (25); Galβ1-3GalNAcβ1-2-sialyltransferase, giving NeuAcα2-3Galβ1-3GalNAcα (3-O), purified from porcine liver (26); recombinant Galβ1-3(GlcNAcβ1-2-sialyltransferase (27), giving NeuAcα2-3Glcβ1-3Galα (N-3), was kindly donated by J. C. Paulson at Cytel Inc., La Jolla, CA, 2-7-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) was purified from Molecular Probes Inc. (Eugene, OR). Vibrio cholerae sialidase was from Calbiochem. Microtiter plates (Immulon 3) were from Dynatech (Chantilly, VA). COS-1 cells were obtained from the Imperial Cancer Research Fund Cell Bank, Clare Hall, U.K. CD45 was purified from rat thymus as described (28). Antibodies—Purified IgG and F(ab')2 fragments of 36G (IgG2a) and 1C2 (IgG2b) rat monoclonal antibodies (mAbs) directed against mouse sialoadhesin (17) were prepared as described previously for SER-4 (29). T18146 (rat anti-mouse B220) mAb was generously provided by Dr. J. M. Austyn (Nuffield Department of Surgery, University of Oxford), and 265 (rat anti-mouse CD11b) was a gift of Professor S. Gordon (Sir William Dunn School of Pathology, University of Oxford). Rat anti-mouse CD22 antibodies N1M6R (13) (tissue culture supernatant) and mouse anti-mouse CY34 (30) (purified IgG) were kindly provided by Drs. M. Parkhouse and F. Symington, respectively. Mouse anti-human IgG mAb R10Z8E9 (31) was obtained from Professor R. J effers and Dr. M. Goodall (Department of Immunology, University of Birmingham). This mAb is available from Recognition Sciences, University of Birmingham Science Park, Birmingham B15 2SQ, U.K.

Recombinant Protein Constructs—Sn(d3)Fc, Sn(d2–3)Fc, Sn(d2–7)Fc, and Sn(d1–7)Fc were generated by PCR amplification from full-length mouse CD22 cDNA in pcDNA I/Amp (5) and the Fc expression vector, pIG1 (32). Similarly, CD22(d1)Fc, CD22(d1–7)Fc, and CD22(d2–3)Fc were obtained by PCR using the following forward primers (5’-3) with the appropriate reverse primers (above) and the following forward primers (5’-3):

- Sn(d2): AAGATCTCATTACCAGTCGATTCGTCACTAGTGCACTGCTGTG.
- Sn(d3): ACAAGCTTATTACCTGTTGCAGGTGACGTGTTG.
- Sn(d7): ACAAGCTTATTACCTGTTGGGACTTGTTGGAGTACACAC.
- Sn(d17): ACAAGCTTATTACCTGTTGGGACTTGTTGGAGTACACAC.
- CD22(d1): GATCGGATCCACTTACCTGTCTTGCCGATGGTCTCTGGACTGTA.
- CD22(d2): ACAAGATCTGACCATTCCTGAGGAGCTGCGTGAA.
- CD22(d3): ACAAGATCTACTTACCTGTGGTATACTTAACATCCAGATGCAC.
- CD22(d7): ACAAGCTTACTTACCTGTTGCGGGGTTCATTTTGACTTCAGC.
- CD22(d17): ACAAGCTTACTTACCTGTTGCACTTGCAGGTAAAC.

PCR products were sequenced and cloned into the mammalian expression vector pEF-BOS (34).

Production of Recombinant Proteins—COS-1 cells were transfected at 70–80% confluency by the DEAE-dextran method using 20 μg of plasmid DNA/15-cm diameter plate. 24 h post-transfection, the medium was changed to Dulbecco's modified Eagle's medium with 0.5% fetal calf serum that had been depleted of IgG with protein A-Sepharose. In the case of Sn(d1), medium was changed to Dulbecco's modified Eagle's medium plus 1% fetal calf serum. The COS cell supernatants were harvested 7–10 days post-transfection, and insoluble material was removed by centrifugation and filtration. The Fc chimeras were purified on protein A-Sepharose as described (32). The Sn(d1) protein was purified by affinity chromatography on the 36G mAb matrix (2). Preparation of CD11b—Human erythrocytes were freshly prepared and stored in Alsever's solution. Mouse bone marrow cells were flushed from excised femora and dissociated into a single cell suspension by gentle pipetting in RPMI 1640 + 20 mM HEPES followed by passage through a 25-gauge needle. Mouse mesenteric lymph node cells were obtained by gentle dissociation of the tissue in RPMI 1640 + 20 mM HEPES. Sialidase treatment of bone marrow cells, mesenteric lymphocytes, and erythrocytes was carried out with 0.1 unit/ml V. cholerae sialidase in RPMI 1640 + 20 mM HEPES for 3 h at 37°C followed by three washes in phosphate-buffered saline (PBS). Human erythrocytes were derivatized to contain sialic acid in different linkages as described previously (23).

Solid Phase Binding Assays—Fc chimeras at varying concentrations were adsorbed for 3 h at 37°C to wells of microtiter plates that had been coated overnight at 4°C with goat anti-human IgG at 15 μg/ml in 1× bicarbonate buffer, pH 9.6. For binding assays with Sn(d1) protein, microtiter plates were coated as above with 40 μg/ml F(ab')2 fragments of purified 36G (IgG2a) followed by varying amounts of purified Sn(d1), 107 mouse bone marrow cells or mesenteric lymphocytes were labeled by polymer chain reaction (PCR) amplification of the corresponding cDNA fragments from full-length sialoadhesin cDNA in pcDNA I/Amp (2). After cutting with appropriate restriction enzymes, the fragments were cloned into the Fc expression vector, pl G1 (32). Similarly, CD22(d1)Fc, CD22(d1–2)Fc, CD22(d1–7)Fc, and CD22(d1–7)Fc were generated by PCR amplification from full-length CD22 cDNA in pcDNA I/Amp (5) and cloned into plG1 vector. The T7 primer (5’-3’), AATACGACTCAGTATAG, was used as the forward primer with the following reverse primers (5’-3’):

- Sn(d1): AAGATCTCATTACCAGTCGATTCGTCACTAGTGCACTGCTGTG.
- Sn(d2): ACAAGCTTATTACCTGTTGCAGGTGACGTGTTG.
- Sn(d3): ACAAGCTTATTACCTGTTGGGACTTGTTGGAGTACACAC.
- Sn(d7): ACAAGCTTATTACCTGTTGGGACTTGTTGGAGTACACAC.
- CD22(d1): GATCGGATCCACTTACCTGTCTTGCCGATGGTCTCTGGACTGTA.
- CD22(d2): ACAAGATCTGACCATTCCTGAGGAGCTGCGTGAA.
- CD22(d3): ACAAGATCTACTTACCTGTGGTATACTTAACATCCAGATGCAC.
- CD22(d7): ACAAGCTTACTTACCTGTTGCGGGGTTCATTTTGACTTCAGC.

Following coupling, noncovalently bound antibody was removed by sequential washing with 0.1% glycine HCl, pH 2.5, and 50 mM NaOH. Specific interactions of Sn-Fc and CD22-Fc constructs with their respective ligands were carried out at a continuous flow rate of 1 μl/min in
Mapping the Binding Sites of Sialoadhesin and CD22

HBS buffer. All injected proteins were diluted in HBS. Ligand binding to the sensor surface is recorded as an increase in response units where 1,000 response units corresponds approximately to the binding of 1 ng of protein/mm².

RESULTS

Production of Truncated Sn-Fc and CD22-Fc Chimeras—Previous studies have shown that recombinant forms of sialoadhesin and CD22 containing the first three amino-terminal Ig-like domains (d1–3) fused to the Fc portion of human IgG1 mediate cell adhesion in a sialic acid-dependent manner (3, 4, 6, 13, 17, 23, 24). To localize the binding site further, we made a set of three additional domain truncation mutants. For both sialoadhesin and CD22 the chimeric domain constructs made were (d1)Fc, (d1–2)Fc, (d2–3)Fc, and (d1–3)Fc (Fig. 1A). In addition, Sn(d1–17)Fc and CD22(d1–7)Fc are the wild type constructs containing all of the extracellular Ig-like domains. Purified preparations of each form were obtained by protein A-Sepharose affinity chromatography using supernatants of transiently transfected COS cells (Fig. 1B).

Comparison of the Binding Properties of Sn-Fc Chimeras with Human Erythrocytes and CD22-Fc Chimeras with Mouse Lymphocytes—It was shown previously that human erythrocytes and mouse mesenteric lymphocytes bind at high levels to sialoadhesin and CD22, respectively (6, 17, 22). These cellular ligands were used in solid phase adhesion assays to characterize the binding activities of Sn-Fc and CD22-Fc domain truncation mutants. Sn(d1–17)Fc, Sn(d1–3)Fc, Sn(d1–2)Fc, and Sn(d1)Fc exhibited concentration-dependent adhesion to human erythrocytes (Fig. 2A). Adhesion to all constructs was sialic acid-dependent as pretreatment of erythrocytes with sialidase completely abolished binding (data not shown). Both Sn(d1)Fc and Sn(d1–3)Fc were able to mediate similar levels of binding. A lower level of binding was observed with Sn(d1–2)Fc and Sn(d1–17)Fc. In contrast, no binding was observed with Sn(d2–3)Fc. Since this construct was engineered to contain the CD33 leader peptide, we also made a form of Sn(d1–3)Fc with the same leader peptide, designated Sn(d1–3L)Fc (Fig. 1B). The binding activity of this construct was identical to that of Sn(d1–3)Fc, showing that introduction of the CD33 leader peptide does not affect subsequent binding activity (data not shown). These results therefore suggest that for sialoadhesin, domain 1 is both necessary and sufficient for sialic acid-dependent binding.

For CD22, mesenteric lymphocytes bound at high levels to CD22(d1–7)Fc and CD22(d1–3)Fc with intermediate binding to CD22(d1–2)Fc, but no binding was observed with CD22(d1)Fc or CD22(d2–3)Fc (Fig. 2B). To exclude the possibility that the failure of CD22(d1)Fc to mediate binding was due to denaturation during purification (see BIAcore experiments below), binding assays were also carried out with unpurified Fc chimeras in the form of tissue culture supernatants. Under these conditions, no binding of lymphocytes to CD22(d1)Fc was observed, whereas high and equivalent levels of binding were seen with CD22(d1–2)Fc and CD22(d1–3)Fc (data not shown). Binding of lymphocytes to CD22 was sialic acid-dependent as pretreatment of cells with sialidase abolished binding with all constructs (data not shown). Since the CD22(d1)Fc construct may be incorrectly folded, these results are not inconsistent with the possibility that, like sialoadhesin, the binding site of CD22 lies in domain 1.

Binding of Bone Marrow Cells to Truncated Sn-Fc and CD22-Fc Chimeras—Previous single cell analyses with bone marrow cells have shown that sialoadhesin binds preferentially to developing granulocytes, whereas CD22 binds selectively to B lymphocytes (17). To test the cellular specificity of the truncation mutants, solid phase binding assays were performed with bone marrow cells, and the bound cells were characterized by immunocytochemistry using anti-mouse myeloid cell mAb 5C6 (anti-CD11b) and the anti-mouse B lymphocyte mAb TIB146 (anti-CD45 ABC/B220). The rank order of binding observed for Sn-Fc and CD22-Fc domain constructs with bone marrow cells (Table I) was similar to that observed with human erythrocytes and mouse lymphocytes, respectively (Fig. 2). No binding was observed with NCAM-Fc used as a negative control. Single cell analysis showed that the Sn-Fc constructs selectively bound cells of the myeloid lineage, whereas CD22-Fc constructs showed a strong preference for B lymphocytes (Table I). These results demonstrate that the cellular binding preferences of the domain constructs were preserved.

![Fig. 1. Domain deletion series of Sn-Fc and CD22-Fc chimeric proteins.](image-url)
Sialic Acid Linkage Specificity of Sialoadhesin—To confirm that domain 1 of sialoadhesin preferred NeuAcα2-3Gal in N- and O-linked glycans over NeuAcα2-6Gal in N-glycans, we compared the binding of Sn(d1)Fc and Sn(d1-3)Fc to a panel of derivatized human erythrocytes carrying a single type of sialylated glycan. These cells are generated by removing all terminal sialic acids and then reconstituting only specific sialylated glycans using purified sialyltransferases (35). This strategy has been used successfully to characterize the binding specificity of several sialic acid-dependent receptors including sialoadhesin, CD22, MAG, and CD33 (6, 7, 22, 23). Sialidase-treated erythrocytes were reconstituted to give exclusively 3-O, 3-N, or 6-N. Both Sn(d1)Fc and Sn(d1-3)Fc bound erythrocytes containing either 3-O or 3-N, but not the 6-N structure (Fig. 3). In a previous study full-length sialoadhesin exhibited a similar sialic acid specificity when expressed on the surface of transiently transfected COS cells (5). Thus, the sialic acid specificity of domain 1 is determined independently of domains 2 and 3.

To show unequivocally that domain 1 of sialoadhesin contains the sialic acid binding site we engineered a soluble form of domain 1, Sn(d1), lacking the Fc portion. The purified recombinant protein migrated on SDS-polyacrylamide gel electrophoresis with an apparent Mr of 12,500 (Fig. 4), close to the calculated Mr of 13,302. Solid phase binding assays were carried out by immobilizing Sn(d1) onto microtiter plates coated with F(ab')2 fragments of 3D6 mAb. Sn(d1) was able to bind human erythrocytes at high levels (Fig. 4). Binding was sialic acid-dependent as sialidase treatment completely abolished binding (data not shown).

Analysis of Binding Using Surface Plasmon Resonance—Previous studies have shown that sialoadhesin binds selectively to glycoporphin on human erythrocytes, whereas CD22 binds to CD45 on lymphocytes (13, 22, 36). We next carried out binding assays with these defined molecular ligands for sialoadhesin and CD22 using the BIAcore biosensor, which employs surface plasmon resonance to detect protein interactions in real time (37). In a BIAcore experiment one of the proteins is immobilized onto a dextran matrix, and the other protein ligand is injected over the matrix. Protein binding is detected optically and measured as arbitrary units (response units, as defined earlier). In all BIAcore experiments the Fc chimeras were immobilized indirectly onto the sensor chip via a covalently coupled mAb (R10Z8E9), which is directed specifically against the Fc portion of human IgG. For both sialoadhesin and CD22, only those constructs that bind cellular ligands in solid phase binding assays were selected for the BIAcore experiments.

For sialoadhesin, we used BSA and asialoglycoporin to control for nonspecific and nonsialic acid-dependent binding to glycoporphin, respectively. As shown in Fig. 5, A, C, and E, when the protein samples were injected before the binding of Sn(d1-3)Fc to the surface, the response rose quickly to a plateau level and remained at that level until the injection ended, after
which it dropped quickly to the same level as before the injection. In contrast, when glycophorin was injected following the immobilization of Sn(d1–3)Fc, the response did not rise to a plateau level but instead continued to increase during the injection, indicating that glycophorin was binding to the Sn(d1–3)Fc protein. Following the injection, the response initially dropped quickly as the injected sample was washed out of the flow cell. However, the response remained elevated after the injection, indicating that glycophorin remained bound to the Sn(d1–3)Fc and slowly dissociated (see Fig. 5 inset). In comparison, following immobilization of the Sn(d1–3)Fc protein, no changes in response were seen with the control proteins, asialoglycophorin and BSA.

Because of the variability in absolute responses between experiments, binding to glycophorin of CD22(d1–3)Fc, Sn(d1–17)Fc, and Sn(d1)Fc was compared with Sn(d1–3)Fc in separate experiments (Fig. 5). Sn(d1)Fc and Sn(d1–17)Fc bound glycophorin as indicated by a small but clearly increased response during and immediately after glycophorin injection, whereas the response remained elevated after the injection, indicating that glycophorin remained bound to the Sn(d1–3)Fc and slowly dissociated (see Fig. 5A, inset). In comparison, following immobilization of the Sn(d1–3)Fc protein, no changes in response were seen with the control proteins, asialoglycophorin and BSA.

Figure 4.
Solid phase binding assay of Sn(d1) with human erythrocytes. Sn(d1) protein at varying concentrations was adsorbed onto microtiter plates that had been coated with 40 μg/ml F(ab)2 fragments of 3D6 mAb. Binding assays were carried out with human erythrocytes as described in the legend to Fig. 2. Data show the mean values of duplicate well at each point; similar results were obtained in three independent experiments. The inset shows SDS-polyacrylamide gel electrophoresis of 5 μg of purified Sn(d1) protein under reducing conditions, stained with Coomassie Blue.

Figure 5.
Analysis of glycophorin binding to truncated Sn-Fc chimeras. Purified forms of the indicated Fc chimeras were injected into a flow cell in which an anti-human IgG mAb had been immobilized onto the sensor surface. At the end of the injection the response is higher than the baseline, which represents bound Fc chimera. BSA (0.5 mg/ml), asialoglycophorin (aG, 0.5 mg/ml) and glycophorin (G, 0.5 mg/ml) were all injected for 3 min (short bars) before and after the Fc chimera was bound. An increase in the response both during and following the injection of glycophorin reflects binding to that particular Fc chimera. The inset in panel A shows a magnified view of glycophorin binding before (dotted lines) and after (solid lines) injection of Sn(d1–3)Fc. Results of three experiments are shown. In each experiment binding of Sn(d1–3)Fc is compared with CD22(d1–3)Fc (panels A and B), Sn(d1–17)Fc (panels C and D), and Sn(d1)Fc (panels E and F). Similar results were obtained for each construct in at least three independent experiments.

Figure 6.
Analysis of CD45 binding to truncated CD22-Fc chimeras. Panel A, purified forms of the indicated Fc chimeras were injected (long bars) into a flow cell in which an anti-human IgG mAb had been immobilized onto the sensor surface. BSA (0.5 mg/ml) and purified rat thymus CD45 (30 μg/ml) were injected before and after (short bars) the Fc chimera was bound. Panel B, tissue culture supernatants (TCS) containing the indicated Fc chimeras were injected (long bars) into a flow cell in which an anti-human IgG mAb had been immobilized onto the sensor surface. BSA (10 μg/ml) and CD45 (10 μg/ml) were injected before and after (short bars) the Fc chimera was bound. An increase in the response both during and following the injection of CD45 reflects binding to that particular Fc chimera. Similar results were obtained for each construct in at least three independent experiments.
Sn(d1–3)Fc.

One explanation for why CD22(d1–2)Fc bound weakly to CD45 might be that a large fraction of the protein had been denatured during affinity purification of the protein which involves elution at pH 3.0. To test this possibility, we compared binding of unpurified CD22(d1–2)Fc and CD22(d1–3)Fc present in COS cell supernatants. As shown in Fig. 6B, unpurified CD22(d1–2)Fc and CD22(d1–3)Fc bound at equivalent levels to CD45, suggesting that CD22(d1–2)Fc had undergone selective denaturation during low pH elution. Binding of the CD22 constructs to CD45 was sialic acid-dependent because sialidase treatment of immobilized CD45 prevented subsequent binding (data not shown).

Epitope Mapping of mAbs Directed against Sialoadhesin and CD22—The availability of a panel of truncated Sn-Fc and CD22-Fc chimeras allowed us to map the epitopes recognized by mAbs to each protein using the BIAcore. For sialoadhesin, we have recently described a panel of mAbs which define at least two distinct epitopes, one recognized by the cross-competing mAbs 3D6 and 1C2 and another recognized by SER-4 (17). Both 3D6 and 1C2 bound to Sn(d1)Fc, Sn(d1–2)Fc, and Sn(d1–3)Fc (Fig. 7), showing that the epitope recognized by these antibodies is present within domain 1. However, binding of both antibodies to Sn(d1–2)Fc was lower than to the other two proteins, suggesting that a fraction of Sn(d1–2)Fc may have been partially denatured. SER-4 mAb bound to Sn(d1–3)Fc but not to Sn(d1)Fc or Sn(d1–2)Fc (Fig. 7A). Therefore the epitope of SER-4 may lie in domain 3 or in a region shared between domains 2 and 3.

Two mAbs, CY34 (30) and NIMR6 (15), have been shown to recognize mouse CD22. Using the BIAcore with unpurified forms of the CD22-Fc constructs, CY34 mAb bound to CD22(d1–2)Fc, CD22(d1–3)Fc, and CD22(d1–7)Fc but not to CD22(d1)Fc (Fig. 8). Since it is possible that the CD22(d1)Fc construct was incorrectly folded, these results suggest that the epitope recognized by CY34 mAb may lie in either domain 1 or domain 2 or in a region shared between both of these domains. In contrast, NIMR6 mAb bound CD22(d1–3)Fc and CD22(d1–7)Fc but not CD22(d1–2)Fc (Fig. 8). This shows that the epitope recognized by this antibody is either in domain 3 or is in a region shared between domains 2 and 3.

**DISCUSSION**

By using a series of domain deletion constructs we have mapped the sialic acid binding sites of sialoadhesin and CD22.
as well as epitopes recognized by mAbs specific to each molecule. We demonstrate that for sialoadhesin, the amino-terminal V-set Ig-like domain (domain 1) is both necessary and sufficient for binding in all assays. Domains 2 and 3 do not appear to contribute significantly to the adhesive functions since binding of Sn(d1–2)Fc was at levels comparable to that of Sn(d1–3)Fc. However, a recombinant construct that contained the first two domains displayed reduced binding as compared with the construct containing domain 1 on its own or domains 1–2–3. Since mAbs 3D6 and 1C2 bound to Sn(d1–2)Fc at reduced levels compared with Sn(d1)Fc and Sn(d1–3)Fc, this suggests that a fraction of Sn(d1–2)Fc was partially denatured, which could therefore explain its reduced binding activity. Similar observations were made with CD22(d1–2)Fc, and in this case it was found that full activity could be restored if binding assays and epitope mapping were carried out with unpurified forms of the Fc proteins. Since the routine purification of Fc proteins involves an elution step at pH 3.0, these results indicate that the Sn- and CD22(d1–2)Fc constructs are unusually susceptible to acid denaturation. In this respect it is interesting that our attempts to generate purified MAG(d1–2)Fc have been unsuccessful so far.2

In contrast to sialoadhesin, our results show that murine CD22 requires at least the first two domains for binding to cellular and molecular ligands. These observations are consistent with a recent report3 demonstrating that for human CD22 expressed in COS cells the presence of the first two amino-terminal domains together is essential for sialic acid-dependent adhesion. In view of the similarities in sequence and domain organization between the amino-terminal domains of sialoadhesin and CD22 it might be expected that, similar to sialoadhesin, the binding site of CD22 would reside in the first amino-terminal Ig-like domain. Indeed, recent mutagenesis screens of sialoadhesin and CD22 have shown that domain 1 of both molecules contains residues that are critical for sialic acid-dependent binding.4 We therefore favor the idea that the sialic acid binding site of CD22 lies in domain 1 and that domain 2 is required for correct folding of domain 1. Indirect support for this interpretation is provided by the observation that human CD22 constructs lacking domain 2 show reduced reactivity with mAbs that are directed against domain 1 and/or domain 2 (13, 38), thereby suggesting that domain 2 is critical for the correct conformation of domain 1. Likewise, in the present study it is possible that the failure of the CY34 mAb to bind CD22(d1)Fc is a result of incorrect folding. Generation of more mAbs and/or production of chimeric constructs between appropriate mouse strains (12) will be required to resolve this issue. Unpublished observations in our laboratory with MAG and CD33 show that, like CD22, domain 1 on its own is unable to mediate sialic acid-dependent adhesion.5 Within the sialoadhesin family, therefore, it appears that sialoadhesin is unusual in that domain 1 can function independently of domain 2. It is possible that formation of a disulfide bond between domains 1 and 2 (9, 10) could be important for the correct folding of domain 1 in the cases of CD22, MAG, and CD33, but not in the case of sialoadhesin.

For the majority of Ig superfamily adhesion molecules involved in heterophilic interactions, structure-function studies have shown that the dominant binding sites are localized to the first amino-terminal Ig-like domain (reviewed in Ref. 1). In general, it can be expected that the amino-terminal domain is more accessible for mediating interactions with corresponding counterreceptors. The binding activities of the membrane proximal domains are less defined, but they may be important in making the binding site more accessible. Thus, the binding site on ICAM-1 for lymphocyte function-associated molecule-1, rhinovirus, and malaria-infected erythrocytes is localized in the first amino-terminal domain, but removal of the membrane proximal domains results in decreased ligand binding when ICAM-1 is expressed in monkey COS cells (39, 40). In certain cases where domain 1 has been shown to contain the binding site, it has also been shown that there is a conformational dependence on domain 2. With ICAM-1 and CD4, it has not been possible to express domain 1 in the absence of domain 2 (39, 40). This conformational dependence is thought to be a result of the close packing of the first two domains as shown in the crystal structure of CD4 (41, 42). Thus, it appears that for several adhesion molecules belonging to the Ig superfamily, the ligand binding site is located in the first amino-terminal domain, but an adjacent domain is required for correct conformation.

Several cell adhesion molecules of the Ig superfamily exhibit a dual homophilic/heterophilic adhesion activity, including NCAM, CD31, and CEA family proteins (1). In these molecules, the homophilic and heterophilic binding sites appear to be distinct. For example, homophilic NCAM-NCAM binding interactions in trans appear to be mediated by a 10-amino acid sequence within the third Ig-like domain (43). This is distinct from its heterophilic glycosaminoglycan binding region in the second amino-terminal domain. NCAM can also mediate carbohydrate-dependent cis interactions with L1 via a region located in its fourth Ig-like domain (44). CD31-CD31 homophilic interactions involve a more extensive area over multiple domains than NCAM homophilic interactions. It is proposed that domains 2 and 3 on one CD31 molecule interact in an antiparallel fashion with domains 5 and 6 on an apposing CD31 molecule (45). A similar mechanism has been proposed for CEA homotypic interactions, which involve reciprocal interactions between antiparallel CEA molecules aligned in trans (46). It is therefore evident that cell adhesion molecules containing several Ig-like domains can bind multiple ligands using distinct domains.

The Sialoadhesin family represents a new class of carbohydrate-binding proteins in which an Ig fold has acquired the capacity to mediate sialic acid-dependent adhesion. The overall molecular organization of sialoadhesins bears several similarities to selectins, arguably the best characterized mammalian lectins involved in cell-cell interactions (reviewed in Ref. 47). Selectins contain an amino-terminal C-type lectin domain that functions in carbohydrate binding. Our results show that for sialoadhesin, the lectin region is within the amino-terminal V-set domain. We anticipate that the same region is used by MAG and CD33, but not in the case of sialoadhesin.

2 P. R. Crocker, unpublished observations.

3 M. Vinson and P. A. van der Merwe, unpublished observations.

4 S. D. Freeman, unpublished observations.
inter-β-sheet disulfide between β-strands B and E of the V-set domain (9) and a predicted disulfide between the V- and C2-set domains (10). Since the V-set domain of sialoadhesin can function independently of the C2-set domain, a direct role of Vinson for assistance in the preparation and analysis of Fc chimeras. The role in sialic acid-dependent binding of the conserved inter-β-sheet disulfide within the V-set domain of sialoadhesins awaits further investigation.

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