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MINI-REVIEW

The Sialoadhesins – A family of sialic acid-dependent cellular recognition molecules within the immunoglobulin superfamily

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For many years evidence has accumulated that sialic acids function in cellular interactions either by masking or as a recognition site. However, receptors or adhesion molecules mediating such functions between eukaryotic cells were unknown until about 5 years ago, when it was found that the members of the Selectin family mediate adhesion of leukocytes to specific endothelia through binding to sialylated glycans like sialyl Lewis^x. More recently, the Sialoadhesin family of sialic acid-dependent adhesion molecules was defined within the superfamily of immunoglobulin-like molecules. So far, it has been shown that sialoadhesin (Sn), CD22, CD33, the myelin-associated glycoprotein (MAG) and the Schwann cell myelin protein (SMP) belong to this family. In contrast to the Selectins, these proteins are associated with diverse biological processes, i.e. hemopoiesis, neuronal development and immunity. In this review their properties, carbohydrate specificities and potential biological functions are discussed. Finally, we provide perspectives with respect to the nature of ligands, implications of sialic acid modifications and future research.

Keywords: sialic acid, cell adhesion, immunoglobulin, signalling, protein family

Abbreviations: IgSF, immunoglobulin superfamily; MAG, myelin-associated glycoprotein; Sia, sialic acid; SMP, Schwann cell myelin protein; Sn, sialoadhesin.

Introduction

Sialic acids (Sia) contribute significantly to the structural diversity of cell surface glycans mainly in two aspects. (1) They are found in nature in about 40 modifications [1–3], the most common Sia in mammalia being *N*-acetylneuraminic acid (Neu5Ac), *N*-acetyl-9-*O*-acetyl-neuraminic acid (Neu5,9Ac$_2$) and *N*-glycolylneuraminic acid (Neu5Gc) (Fig. 1A); and (2) they occur in terminal positions linked to other sugars like Gal, GalNAc, GlcNAc and Sia itself on glycoproteins and glycolipids. Some of the most common oligosaccharide structures containing Sia are shown in Fig. 1B. Specific patterns of oligosaccharides are regulated by the cell type specific expression of glycosyltransferases, particularly of sialyltransferases [4, 5]. Thus, striking differences have been found in the glycosylation patterns of cells during development, activation and oncogenic transformation. Based on these findings many functional roles have been proposed, as extensively reviewed by Varki [6].

The discovery of the Selectin family has drawn a lot of attention towards Sia-dependent receptors in cell adhesion, as reviewed by Lasky [7]. Recently, the Sialoadhesin family of Sia-dependent adhesion receptors was defined within the immunoglobulin superfamily (IgSF) [8]. To date, members of this family are sialoadhesin (Sn) [9], found on specific subsets of macrophages, CD22 [10–12], a B cell specific protein, CD33, a molecule

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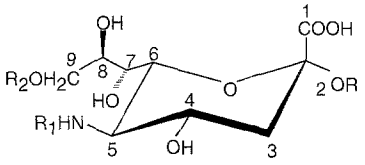
A



Sialic acid	R_1	R_2
Neu5Ac	acetyl	H
Neu5,9Ac_2	acetyl	acetyl
Neu5Gc	glycolyl	H

B

Structure	Abbreviation
Siaα2→3Galβ1→3GalNAc-R	(3-O)
Siaα2→3Galβ1→3(4)GlcNAc-R	(3-N)
Siaα2→6Galβ1→4GlcNAc-R	(6-N)

Figure 1. Sialic acids and sialylated glycans on glycoconjugates. A) Structure of sialic acids with the most common modifications at positions 5 and 9. B) Sialylated oligosaccharides frequently found as terminal sequences of glycans linked to Asn (N) or Ser/Thr (O) on glycoproteins.

expressed by myeloid progenitor cells [13, 14], myelin-associated glycoprotein (MAG), found only in myelin membranes of oligodendrocytes and Schwann cells [8, 15] and Schwann cell myelin protein (SMP) [16], a MAG-like protein found in quail brain. Studies on the adhesion properties and specificities of these proteins have shown that they can all mediate cell adhesion through recognition of sialylated cell surface glycans (Table 1) [8, 14] (Tropak, personal communication and unpublished observations).

The aim of this paper is to review recent progress made in the characterization of these Sia-dependent receptors. Further aspects discussed will be the Sialoadhesin family as a distinct family of adhesion proteins within the IgSF, the nature of potential ligands for these receptors and the modulatory role of Sia modifications.

Sialoadhesin (Sn)

Apart from the Selectins, Sn was the first characterized adhesion molecule that binds to specific sialylated glycans on cell surfaces [17] and is the eponymous member of the Sialoadhesin family. It was originally described as a sheep erythrocyte receptor [18] and subsequently shown to be a 185 kDa immunoglobulin-like membrane protein found on specific macrophage subpopulations. Sn is a type I transmembrane protein with 17 extracellular Ig-like domains, comprising 16 C2-set domains and one unusual V-set domain which contains an intrasheet disulfide bridge (Fig. 2) [9]. The most closely related molecules are MAG [15], SMP [16], CD22 [10] and CD33 [13] (Fig. 2). Whereas most studies on Sn were done in the mouse, the existence of a homologous protein in the rat was demonstrated in spleen and lymph node macrophages [19]. In addition, reports on the specificity of a ganglioside binding activity on rat alveolar macrophages [20–22] point to the possibility that Sn may be present in these macrophages, although murine alveolar macrophages express only relatively low amounts of the receptor [23].

Using resialylated erythrocytes, glycoproteins and glycolipids with defined glycan structures it was shown that Sn recognizes the sequence Neu5Acα2,3Galβ1, 3GalNAc and Neu5Acα2,3Galβ1,3(4)GlcNAc on glycoproteins and glycolipids [8, 17].

In the mouse, highest levels of Sn are found in resident bone marrow macrophages of hemopoietic clusters, marginal zone macrophages in the spleen and macrophages in the subcapsular sinuses and medullary cord of lymph nodes [24, 25]. A striking distribution of Sn on the ultrastructural level was observed in bone marrow, where the receptor is highly enriched at contact sites between the macrophages and developing myeloid cells [26]. In contrast, no staining was observed at contact sites of the same macrophages to erythroblasts.

Possible functions for Sn have been implicated in the development of myeloid cells in bone marrow and in the trafficking of leukocytes in lymphatic organs [17, 19, 25, 27]. Evidence for this hypothesis comes from the distribution of the receptor in bone marrow (see above) and from cell binding experiments. A preference for myeloid cells at all stages of development could be

Table 1. Occurence, structure of glycans bound and target cells of members of the Sialoadhesin family

Molecule	*Distribution*	*Glycans bound*	*Target cells*
Sialoadhesin	Macrophage subpopulations	Siaα2,3Galβ1,3GalNAc and Siaα2,3Galβ1,3/4GlcNAc	Myeloid cells
Myelin-associated Glycoprotein (MAG) and SMP	Myelin of oligodendrocytes and Schwann cells	Siaα2,3Galβ1,3GalNAc	Neurons, oligodendrocytes
CD22	B cells	Siaα2,6Galβ1,4GlcNAc	Lymphocytes
CD33	Myelomonocytic cells	Siaα2,3Galβ1,3GalNAc and Siaα2,3Galβ1,3/4GlcNAc	Myelomonocytic cells

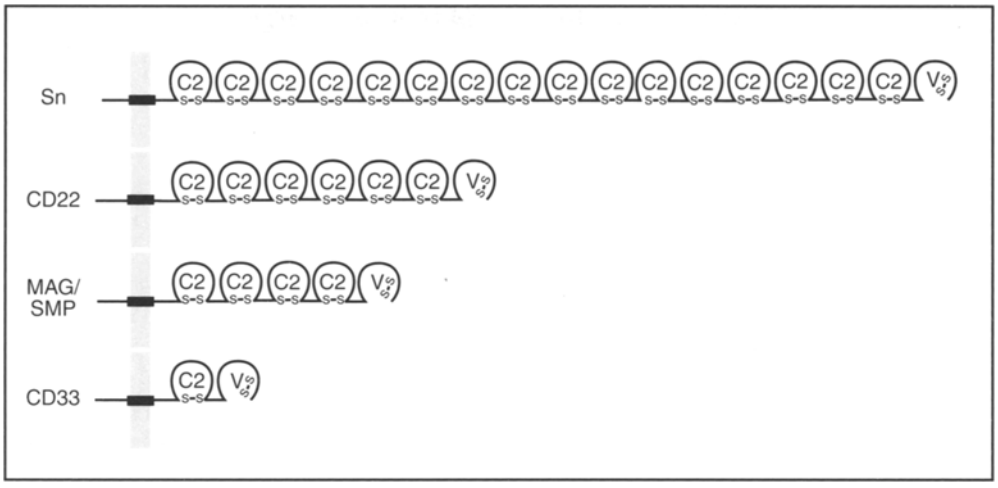


Figure 2. Domain structures of sialoadhesins. The members of the Sialoadhesin family share a similar topology with a variable number of extracellular C2-set domains and one N-terminal V-set domain.

demonstrated in experiments with purified Sn, recombinant Fc-chimeras and macrophages expressing the receptor (Fig. 3) [27]. In contrast, binding to lymphocytes was low and was barely detectable with murine erythrocytes. Single cell analysis of the cells bound by Sn from total bone marrow revealed that more than 90% were myeloid cells, whereas more than 90% of the cells bound by CD22 from the same cell population were lymphocytes [27]. However, in binding assays with frozen sections of spleen and lymph nodes Sn could also mediate the adhesion of lymphocytes and lymphoma cell line TK1 [19]. In quantitative binding assays activated T cells bound better than resting cells and lowest binding in this cell lineage was found with thymocytes. In contrast, binding to activated B cells was not higher than to resting cell populations. In summary, these findings suggest that Sn functions in interactions of specific macrophages with myeloid cells and possibly subsets of T lymphocytes, although granulocytes bind Sn much better than any lymphocyte population tested.

CD22

CD22 is a 140 kDa cell surface molecule with 7 extracellular IgSF domains (Fig. 2) which is exclusively found on B cells [10, 11, 28]. Other forms of CD22 lacking domains 3 and 4 or domain 4 were also described in Daudi cells [10, 29]. However, whether these are expressed on the surface of normal B cells is unclear. First evidence for Sia-dependent binding came from a study reporting that CD22 interacts with CD45RO on T cells and CD75 on B cells [11], an epitope which depends

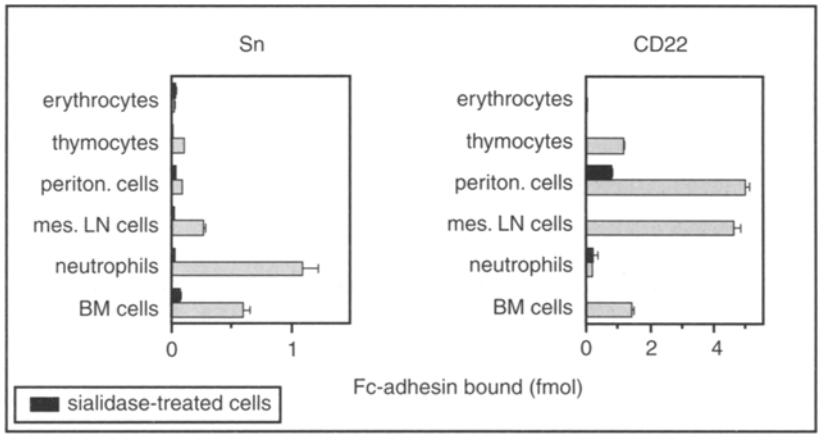


Figure 3. Selective binding of Sn and CD22 to murine cells. Anti-Fc complexed radioiodinated Fc-chimeras containing the N-terminal four domains of Sn or the N-terminal 3 domains of murine CD22 were assayed for binding to the murine cells indicated. Binding assays were performed with glutardialdehyde-fixed cells (mes. LN, mesenteric lymph node; BM, bone marrow). Peritoneal cells (periton. cells) consist of 70% lymphocytes and 30% macrophages (extracted from [8]).

on the expression of α-2,6 sialyltransferase in these cells [12, 30, 31]. The requirement for α-2,6-linked Sia on branched N-linked oligosaccharides on ligands for CD22 was unambiguously demonstrated and the specificity of CD22 towards sialylated glycans was described in detail in a series of elegant studies [32–35]. In summary, the minimal structural requirement for CD22 binding is Siaα2,6Hex(NAc), in which Hex could be Gal or Glc [35]. However, branched oligosaccharides carrying two or more Sia residues are bound with higher avidity, probably by interacting with more than one CD22 binding site. Although these studies were done with recombinant Fc-chimeras containing two CD22 binding sites, it seems likely that on a cell surface CD22 is expressed also in an oligomeric state [35].

Regarding the biological function, CD22 is the best studied member of the Sialoadhesin family. The four areas where most progress has been made are: (1) CD22 as a cell adhesion molecule; (2) CD22 in signalling events in B cells; (3) signalling events in T cells mediated through CD22 binding to CD45; and (4) the regulation of CD22 activity by α2,6 sialyltransferase activity.

(1) Recombinant soluble CD22 constructs and CD22 transfected COS cells were used to analyse its specificity to different blood cells [8, 12, 27, 36, 37]. In summary, these studies demonstated that CD22 binds most strongly to lymphocytes and to a lesser extent to neutrophils, monocytes and erythrocytes (Fig. 3). Whereas these data suggest that CD22 functions in interactions of B cells with other lymphocytes, CD22 could also mediate adhesion to activated endothelia which express high levels of α2,6 sialyltransferase [38, 39].

(2) Recent reports have provided evidence that CD22 plays a role in B cell signal transduction. The cytoplasmic domain of CD22 contains six tyrosine residues which are conserved between the human and murine homologues [36]. Some CD22 molecules are associated with the surface IgM (sIgM) B cell receptor complex [40, 41] and sIgM crosslinking leads to rapid phosphorylation of tyrosine residues in CD22 molecules [40–43]. Furthermore, it has been demonstrated that phosphorylated CD22 binds and activates SHP, a protein tyrosine phosphatase that negatively regulates signalling through the sIgM-complex [43]. Based on these findings, it has been proposed that crosslinking of CD22 enhances the sensitivity of B cells towards antigen activation by sequestration of SHP away from the sIgM-complex [43]. In another study it was shown that crosslinking of CD22 enhances antigen-induced apoptosis [44]. Whether these short and long term effects of CD22 crosslinking are related and triggered by the same signalling events remains to be elucidated. Whereas in these studies CD22 was crosslinked with anti-CD22 antibodies, *in vivo* these events would be expected to be triggered by binding of glycoconjugates expressing appropriate α2,6-linked Sia. These could be molecules on the B cell itself, on opposing cells, i.e. T cells or endothelia, or soluble molecules in the environment (see also discussion below).

(3) Since the first report on CD22 as a T cell binding protein, CD45 has been the most prominent ligand candidate for CD22 on T cells. This receptor-like phospho-tyrosine phosphatase has been proposed to be involved in T cell receptor-mediated signalling, suggesting that CD22 may also regulate signalling events in T cells [11, 45–48]. However, at present it is unclear how a ligation of CD45 by CD22 affects T cell metabolism. For example, recently it was shown that ligation of CD45 by CD22 modulates early signalling events in Jurkat T cells like phosphorylation of phospholipase Cγ1 [48], whereas in an earlier study with normal T cells and CEM T cells the opposite effect was described [45]. Certainly, further investigations are needed to clarify these apparently conflicting observations.

(4) In B cells a specific promoter of the α2,6 sialyltransferase gene regulates the cell type specific expression of this enzyme during B cell development [49], leading to high levels of binding sites for CD22 on activated B cells. These glycans of glycoproteins on the same cell surface as CD22 itself may mask the binding activity of CD22 by occupation of binding sites in *cis* position [39, 50] (see also discussion further down under Ligands). It is tempting to speculate that the association of CD22 with the sIgM-complex (see above) could be mediated through such binding of glycans carrying α2,6-linked Sia on the complex by CD22. Furthermore, the role of serum in the regulation of CD22 activity must be considered, since many serum glycoproteins carry α2,6-linked Sia. Accordingly, a recent study by Hanasaki *et al.* [51] revealed that IgM and haptoglobin are the two main proteins from human serum which bind to CD22.

CD33

CD33 is the smallest member (67 kDa) of the Sialoadhesin family to date with only two extracellular Ig-like domains (Fig. 2) [13, 14]. It is exclusively expressed by myelomonocytic progenitors, monocytes and tissue macrophages [52].

Since molecular cloning revealed that CD33 is a member of the IgSF, it has been suspected to function in cell-cell interactions [13]. However, the binding properties of CD33 remained obscure for many years. No cell adhesion could be detected in COS cells transfected with CD33, since its binding sites are occupied by glycoconjugates on the cell surface and sialidase treatment of the transfected cells is necessary to demonstrate that CD33 binds to both Neu5Acα2,3Galβ1,3GalNAc and Neu5Acα2,3Galβ1,3(4)GlcNAc, similar to Sn [14].

The expression of CD33 is tightly regulated during myelopoiesis. Whereas it is not found on hemopoetic stem cells, all myelomonocytic precursor cells are CD33-positive. It is then downregulated on mature granulocytes cells but persists on monocytes and tissue macrophages. Because of its expression pattern, CD33 became an important marker for the diagnosis of acute myeloid leukaemias (AML), especially of the more immature forms which cannot be distinguished from lymphomas by morphological criteria but require a different therapy [53–55]. In addition, mAbs against CD33 have been used in preliminary therapeutic trials [56–59].

To date, no potential biological function for CD33 has been demonstrated. The distribution pattern of CD33 suggests a role during the maturation of myeloid cells in the bone marrow. Like Sn, CD33 binds with high preference to myeloid cells [14]. The simultaneous expression of CD33 on the same cell as its ligands could lead to regulation of CD33 binding activity and even prevent CD33 from functioning as a cellular interaction molecule *in vivo*. Possible modulatory functions of *cis*-acting ligands for the members of the Sialoadhesin family are also discussed below. As in the case of CD22 and MAG, the presence of a potential tyrosine phosphorylation site in the cytoplasmic domain also suggests a role for CD33 in signalling events [60].

Myelin-associated glycoprotein (MAG)

MAG contains 5 extracellular Ig-like domains and is expressed only on myelinating oligodendrocytes and Schwann cells [61]. It is found in two forms of 72 kDa (L-MAG) or 67 kDa (S-MAG) polypeptide size with identical extracellular domains but different cytoplasmic domains [15, 62]. Whereas L-MAG is expressed transiently during development and is the main form found at the onset of myelination, S-MAG is expressed later in development and persists in adult animals [63, 64]. As a result of variable glycosylation MAG migrates on SDS-PAGE as a broad smear of about 100 kDa. Although the role of MAG as a cell adhesion molecule has been under investigation for many years and binding activities to various extracellular components have been reported [65, 66], the binding specificity has remained obscure. Since MAG is one of the molecules in the nervous system carrying the LNK-1 carbohydrate epitope, this was also considered as the recognition marker for MAG-dependent cellular interaction [67]. However, binding studies with resialylated erythrocytes, the neoganglioprotein $GT1_b$-BSA and glycolipids have shown that MAG recognizes Neu5Acα2,3Galβ1,3GalNAc glycans on glycoproteins and glycolipids [8] (unpublished observations) (Refer to note added in proof, Ref [A]). In addition, recent experiments have demonstrated that the previously reported binding of MAG-containing liposomes to neuronal cells, particularly to axons and to oligodendrocytes [65], is mediated through the recognition of sialylated glycans (Hillenbrand *et al.*, unpublished observations).

Biological functions of MAG in myelination, axonal growth regulation and signal transduction have been supported by a number of studies. *In vitro* experiments indicated that MAG plays a crucial role in the early steps of myelination [61, 68, 69]. However, in transgenic mice lacking MAG ($MAG^{-/-}$), the degree of myelination is essentially normal, although some minor abnormalities were described [70, 71]. However, in adult $MAG^{-/-}$ animals more drastic histological changes occur, suggesting that MAG is important for the maintenance of myelin/axon organization [72, 73].

MAG can influence neuronal growth in opposite ways *in vitro*. On the one hand, MAG promotes neurite outgrowth in dorsal root ganglion (DRG) neurons [74, 75] and on the other hand, MAG exhibits an inhibitory effect on neurite growth of neurons from cerebellum, adult DRG [75] or neuroblastoma cells [76]. These studies suggest that glycoconjugates on the neuronal cells if ligated by MAG could induce opposing biological effects (Refer to note added in proof, Ref [B].). An interesting question is whether the same ligand(s) for MAG transmits these signals or whether different signal transduction molecules carrying the appropriate sialylated glycans are involved. Therefore, the next important step will be to identify these glycoconjugate ligands for MAG in different cell types.

A number of studies point also to a role of MAG as a signal transducing molecule itself. Already a few years ago it was noticed that MAG is phosphorylated on the cytoplasmic domain, mainly on serine and tyrosine residues [77–80]. As mentioned above, MAG is expressed in two forms (L-MAG and S-MAG). Interestingly, a tyrosine phosphorylation site (Tyr-620) is found only in L-MAG, which was shown to interact with the SH2 domain of phospholipase Cγ [81]. Furthermore, the Fyn tyrosine kinase, which can phosphorylate Tyr-620, associates with L-MAG and is activated by crosslinking MAG with anti-MAG antibodies [82]. It is tempting to speculate that *in vivo* this crosslinking is mediated by glycoconjugate ligands of MAG.

Schwann cell myelin protein (SMP)

SMP is a protein closely related to MAG, which has been characterized from quail and chicken brains [16]. Despite its close homology to MAG, it has been proposed to be distinct from the avian MAG homologue [16]. However, binding studies with COS cells transiently expressing SMP and with stably transfected CHO cells gave evidence that the binding specificities of MAG and SMP are identical (Tropak, personal communication and our own unpublished observation). In conclusion, it remains unclear

whether SMP is a distinct fifth member of the Sialoadhesin family or the avian homologue of mammalian MAG; this issue may be resolved if another avian MAG analogue or a mammalian SMP homologue is found.

The Sialoadhesin family

For all the proteins discussed above the specificities for sialylated glycans are well defined and in all cases it has been shown that the sialic acid residues are recognized with high specificity. Therefore, they represent true sialic acid-dependent cell interaction molecules.

They belong to the IgSF, by far the largest superfamily of cell surface proteins. All members of the IgSF contain a variable number of extracellular IgSF domains which are characterized by sequence similarities over about 100 amino acids forming distinct structural units of β-strands arranged in a sandwich of β-sheets stabilized by a conserved cysteine pair (for review see [83–85]). The overall sequence similarities between members of the IgSF are 20–25%, with the highest degree of homology within the β-strands and a high variability in the loops [86]. Based on the number of amino acids in the domain and sequence similarities between the β-strands, different types of IgSF domains, like the C2-set and the V-set, were assigned [83, 85]. The three dimensional structures of several such IgSF domains have been elucidated showing a similar overall architecture [86]. This structural design seems to be the basis for the high diversity of molecules recognized by Ig-like molecules. Where studied, the face consisting of the G-, F-, and C-strands (GFC face) seems to be most important for the binding of heterologous ligands by IgSF domains [87–89].

Whereas most members of the IgSF function through protein-protein interactions, it should also be kept in mind that many antibodies recognize specific oligosaccharide structures. There is evidence that in principle the IgSF domains are well suited for the discrimination of cell surface carbohydrates. However, in immunoglobins the CDR loops are most important for binding. In contrast, recent mutagenesis studies support the idea that for Sn and CD22 the GFC face of domain 1 contains the binding site for sialylated glycans (refer to note added in proof, Vinson *et al.*, 1996 Ref [C] and van der Merwe *et al.*, 1996 Ref [D]). Besides the sialoadhesins, some other IgSF members, like NCAM, have been shown to bind specific carbohydrate structures. For these proteins the term I-type lectins was introduced by Powell and Varki [90]. The adhesion molecules discussed here are distinct from all other members of the IgSF including the other I-type lectins in a number of aspects as discussed below. Therefore, the sialoadhesins can be considered as a distinctive family [8].

The homologies shared between the members of this family are highest in the N-terminal 4 (or 2 for CD33) IgSF domains with over 45% sequence similarity (Fig. 4) [9]. One of the most striking features of all members of the Sialoadhesin family is the unusual distribution of highly conserved cysteine residues in the first two N-terminal domains. These were predicted to give an intrasheet disulfide bridge within the V-like first domain and an interdomain disulfide bridge between domains 1 and 2 [83, 85]. These features are not found in the other I-type lectins [90]. In addition, it should be mentioned that all proteins with these structural features found until today by means of sequence similarity were shown to function as Sia-dependent adhesion molecules. To date, it is most likely that the N-terminal two domains contain the complete binding site for specific sialylated glycans. CD33 contains only these two extracellular domains and can function as an adhesion molecule recognizing specific sialylated glycans [14]. Recent binding studies in our and other laboratories using recombinant proteins containing only the N-terminal two domains of Sn or CD22 have shown that these have the same specificity for sialylated glycans as the native proteins [29, 91]. In addition, for Sn the V-set IgSF domain alone is sufficient for binding glycans with the same specificity as full length Sn [91]. Further studies will show which structural elements compose the binding site for sialylated glycans and whether a similar Sia binding pocket is shared by all sialoadhesins.

Despite the common structural features of the Sialoadhesin family, the overall homology between the members is relatively low [9]. Furthermore, even for homologous proteins from different species the sequence similarity can be rather low. For example, only 62% of the amino acids are identical between human and murine CD22, with the highest homology in the extracellular domain 7, the transmembrane and the cytoplasmic domain (71, 68 and 67% identity, respectively) [36]. Despite the numerous differences in the N-terminal two domains (Fig. 4), both the human and the murine homologue require α2,6-linked Sia, but differ in the type of Sia recognized, as discussed below [8, 34, 92] (unpublished results). Also the murine and human homologues of CD33 have only 60% sequence identity [60]. In contrast, MAG is much more conserved between species, suggesting a very high evolutionary pressure on this protein. Even the potential avian homologue SMP has a high degree of sequence similarity with MAG (66% amino acid identity in domain 1, Fig. 4) [16] and a very similar binding specificity (unpublished).

The overall structure, as reflected in the number of IgSF domains, also varies significantly within the Sialoadhesin family, with Sn as the largest (17 domains) and CD33 the smallest (two domains) member. Nevertheless, these two proteins show very similar specificity for sialylated glycans [14]. Although the biological significance of these differences in molecular size is

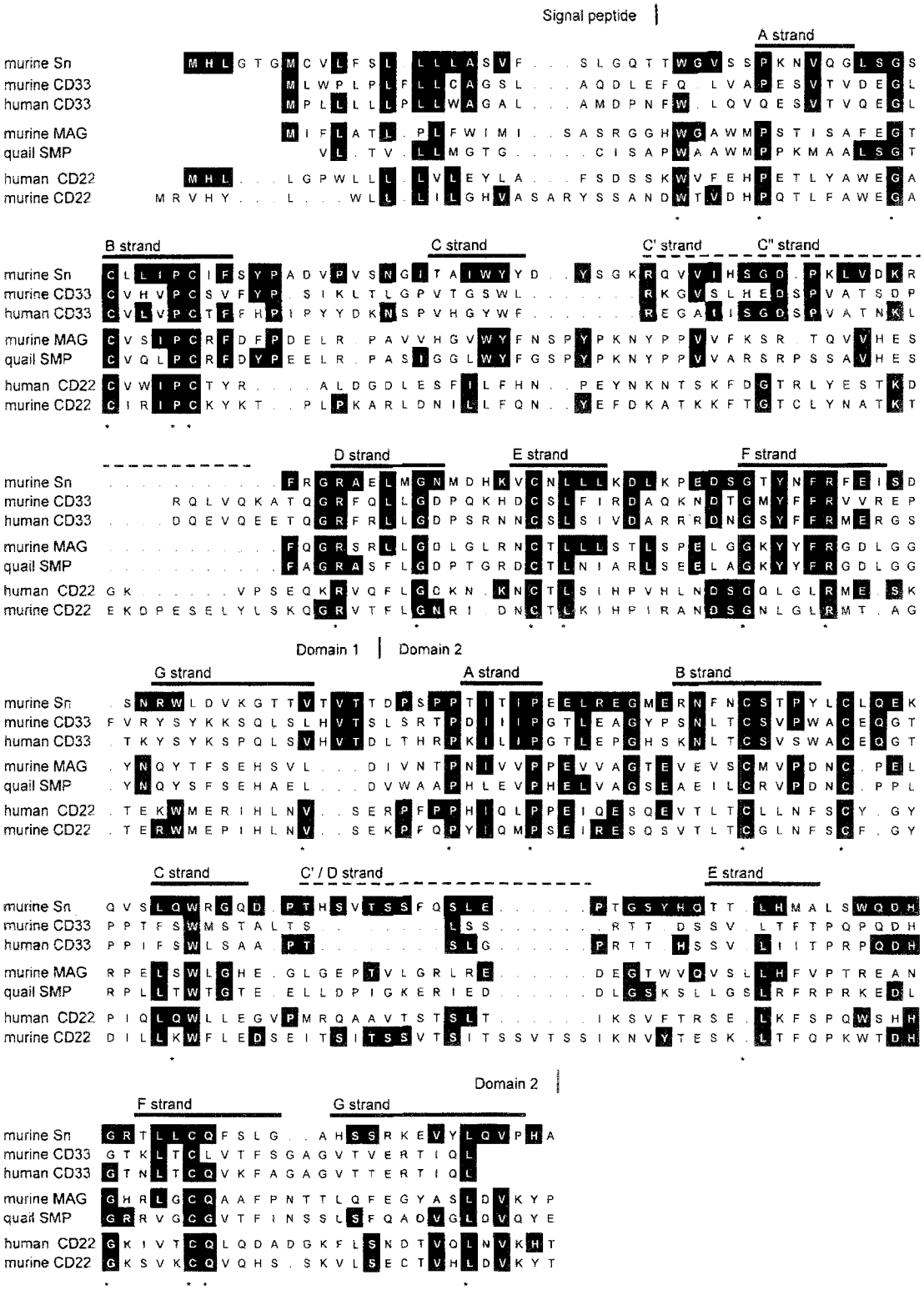


Figure 4. Homologies within the Sialoadhesin family (domains 1–2). The amino acid sequences shown are from the murine or human homologues except for SMP which is from quail. For murine CD22 different alleles have been found. The sequence shown is the allele cloned from BALB/c mice [36]. Amino acids which are identical to Sn are on black background, positions which are identical in all proteins are marked by an asterix. The predicted beta-strands of domains 1 and 2 are marked with a black bar on top of the sequences. They were assigned based on alignments of domains 1 with the V-set domain of human CD2, for CD22, or CD8α, for Sn, and of domains 2 with the C2-set second domain of VCAM-1, both proteins of known crystal structure (refer to note added in proof, P.A. van der Merwe *et al.*, 1996, ref [D] and M. Vinson *et al.*, 1996, ref [C]). The C′ and C″ strands of domain 1 and the C′/D strand of domain 2 are marked with broken lines, since no precise assignments could be made on this basis.

not clear at the moment, two potential roles for the size of Sn have been proposed [9]. Sn could have developed this size either in order to escape the occupation of binding sites by glycans in the glycocalyx of the same cell or in order to reach small glycoconjugates like glycolipids on the opposing cell.

The genes of the sialoadhesins have the overall genomic organization typical for non-neuronal IgSF proteins, for each IgSF domain is contained in a separate exon [15, 62, 93–95]. The genes of CD22, CD33 and MAG map to the same genomic locus in human (chromosome 19) and mouse (chromosome 7), suggesting that the genes of these proteins may have arisen from the same ancestral gene [93, 94]. Interestingly, Sn maps differently to chromosome 20 in human and to chromosome 2 in mouse [95], suggesting an early divergence during evolution, possibly before the development of mammals.

For four members of the Sialoadhesin family, Sn, CD22, CD33 and MAG, the existence of alternatively spliced forms was demonstrated. These changes could involve both the extracellular domains, as in Sn [9] and CD22 [10, 11, 28, 29], or the cytoplasmic domain as in MAG [15, 62] and CD33 [60]. Whereas the changes in the extracellular domains do not seem to influence the binding specificity towards sialylated glycans, they can affect the presentation of the binding site on the cell surface leading to different cell adhesion properties as in CD22 [10, 11, 28, 29]. Although for CD22 only the N-terminal two IgSF domains are required for binding of sialylated glycans [91], CD22 lacking domains 3 and 4 expressed in COS cells showed a different binding specificity [10, 28, 37]. Also secreted forms of the protein can be generated as shown for Sn [9]. In contrast, modifications of the intracellular domain could alter signalling functions of the molecule, e.g. by removing or adding protein phosphorylation sites as in MAG [15, 62, 82] and murine CD33 [60].

A number of characteristic differences between the Selectin and the Sialoadhesin families should also be discussed. In contrast to the Selectins, members of the Sialoadhesin family do not contain the cation-dependent carbohydrate recognition domain (CRD) defined by Drickamer [96] and they do not require divalent cations for binding. Whereas all three Selectins described so far are involved in the initiation of leukocyte binding to specific endothelia, the members of the Sialoadhesin family are associated with very diverse biological processes like hemopoiesis, neuronal development and immunity. Furthermore, the specificity of Sia residue recognition is strikingly different. The Selectins accept considerable structural modifications of the sugar molecule, e.g. shortening of the glycerol side chain [97–99] and even sulfate as a replacement for Sia [99–102]. In fact, the specificity towards the other monosaccharides of sialyl-Lewis^x or sialyl-Lewis^a i.e. fucose, is more pronounced [99]. In contrast, the members of the Sialoadhesin family are quite sensitive to modifications of the Sia residue [92] (unpublished results) as will be discussed below.

Ligands

Considerable progress has been made in the characterization of ligands for CD22 [32, 33]. From B and T cells as well as from lymphoma cell lines several surface glycoproteins including CD45 could be isolated on CD22-columns, which is in contrast to the very limited number of ligands for selectins (see also below). The number and size of these glycoproteins was dependent on the type of cell used as source for CD22 ligands [32], supplying evidence that for CD22 different cell type specific carrier proteins (counter receptors) with probably similar ligand determinants can exist. Furthermore, from serum, two glycoproteins bound with high affinity to CD22, haptoglobin and IgM [51]. Since serum contains many glycoproteins with glycans carrying α-2,6-linked Sia, these findings suggest that appropriate presentation of the oligosaccharide is very important for high affinity binding to CD22. Since the first reports of CD22 binding specifically to lymphocytes, the protein tyrosine phosphatase CD45RO on T cells has been studied intensively as a molecule transducing signals by interacting with CD22 [11, 45], as discussed above.

For Sn, MAG or CD33, only little information is available on their ligands. Sn bound to specific ganglioside bands in TLC overlay assays with glycolipid extracts from inflammatory neutrophils or bone marrow cells. In addition, treatment of bone marrow cells or inflammatory neutrophils with proteases including O-sialoglycoprotease [103] had little if any effect on binding (unpublished observation). Although this is not definite proof that glycolipids are the ligands on these cells, it supports this possibility. However, Sn binds specifically to glycoproteins from erythrocytes [17, 92] and from several cell lines (unpublished observation). CD33 binding to myeloid cells is reduced after trypsin treatment of the cells, suggesting a proteinaceous ligand [14].

Besides ligands on opposing cells, glycoconjugates on the same cell could interact with sialoadhesins. Such *cis*-interactions (Fig. 5) were found to regulate and even mask the binding sites for CD22 [39, 50], CD33 [14] and MAG (unpublished observation). In principle, this feature emerged from experiments in which these molecules were expressed in the plasma membrane of cells with a glycocalyx containing the sialylated glycans recognized. Under these conditions, the binding activities could only be detected if the cells were pretreated with sialidase to destroy the *cis* ligands. Especially for CD22 or CD33, binding ligands in *cis* may be biologically more

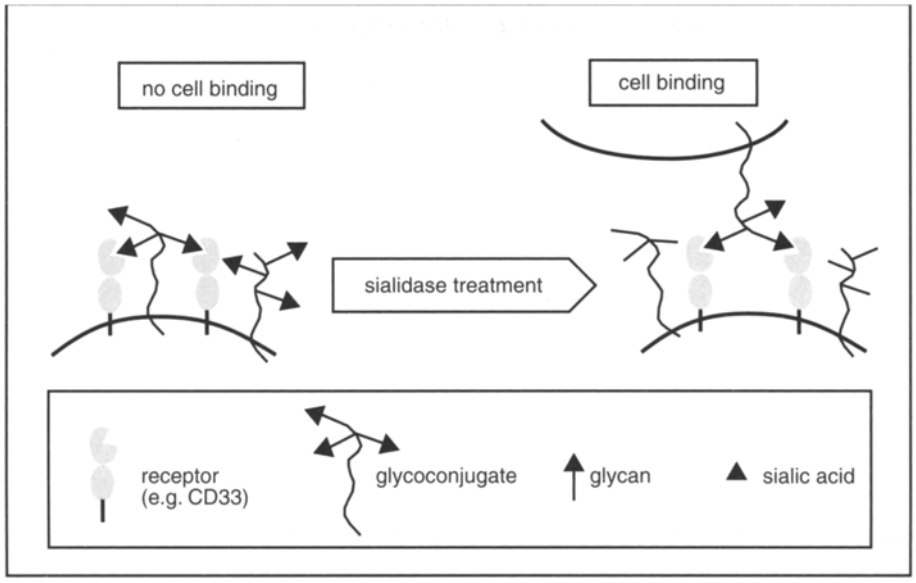


Figure 5. Model for the influence of *cis* interacting ligands of sialoadhesins. Glycoconjugates within the same plasma membrane carrying ligand determinants occupy the binding sites for sialoadhesins (e.g. CD33). After sialidase treatment of the cells, the binding sites become available for interaction with glycans on other cells.

important than cell adhesion, since the cells expressing these proteins also carry high levels of binding sites. Furthermore, no data have been presented to date showing that these proteins function as cell adhesion molecules in their native environment on B cells or myeloid cells, respectively. One could also speculate that *cis*-interactions may be relevant for the formation of complexes with other plasma membrane components (e.g. sIgM-complex on B cells). In contrast, for Sn [27] and MAG (unpublished observation) Sia-dependent cell adhesion could be shown, if these proteins are expressed in their natural environment on macrophages or oligodendrocytes, respectively. It is important to note that native Sn is an extended molecule of about 50 nm [17], which is much larger than the other members of this family. One possibility is that this unique structure developed during evolution to escape *cis*-interactions and to function in *trans*-interactions with neighbouring cells.

Since our knowledge on specific ligands for members of the Sialoadhesin family is still limited, the following discussion deals with some theoretical aspects on glycoconjugate ligands. Whereas the protein backbone is a linear gene product, glycans of glycoconjugates are the result of the concerted action of several enzymes which in part compete for the same substrates. Therefore, glycoforms of the same protein exist ('microheterogeneity'), dependent on the glycosylation machinery of the cell producing it. Since the oligosaccharide determines whether a glycoconjugate can serve as a ligand, we could call the oligosaccharide 'ligand determinant' by analogy to the 'receptor determinant' for virus binding [104]. This means that one glycoform may be bound with high affinity, whereas on other cells a different glycoform of the same protein may not be recognized. In addition, distinct molecules can serve as ligands, if they carry the appropriate glycans. All receptors discussed here recognize specific carbohydrate structures containing terminal Sia residues. Whereas the restrictions towards these structures are more or less stringent depending on the receptor, different glycoconjugates can carry identical oligosaccharides, which of course could trigger different signals when ligated by the same adhesion molecule. For example, this could be an explanation for the seemingly contradictory effects of MAG on neurite outgrowth, as discussed above.

Another feature of carbohydrate recognition is that only a few functional groups of the oligosaccharide structure are required for binding, as has been shown in many examples of carbohydrate-protein interactions. Therefore, on some of these groups relatively major changes are tolerated, whereas others cannot be modified at all. Due to the large variability of naturally occurring oligosaccharide structures, sometimes related structures are recognized as well, as discussed above for the Selectins. Considering these aspects, we cannot necessarily expect precise receptor-ligand pairs involving simple kinetics in Sia-dependent interactions. Studies dealing with the 'specificity' of these receptors have to take this into account, especially since experimental approaches used often involve artificial presentation of the molecules investigated. One example is the TLC overlay technique, where the oligosaccharides are presented in a rather

unnatural way including high clustering of single molecules which might not occur on cell surfaces. Also the expression of the receptor in transfected cells leads to high levels of these molecules on the cell surface not always found in the natural environment. Although these conditions might lead to the adhesion of cells not found with normal expression levels, usually, the observed specificity reflects the flexibility of protein-carbohydrate interaction, which allows also binding to low affinity ligands, if the density is high enough. One example for this is the binding of soluble P-selectin to HL-60 cells compared to CHO cells expressing sLe^x following transfection with fucosyltransferase [105]. Whereas on HL-60 cells P-selectin bound to a low number of high affinity sites, on transfected CHO cells P-selectin bound to a high number of low affinity sites. Despite the obvious artificial situation in the CHO cells, we cannot rule out that such high number of low affinity interactions may have some biological significance for example in tumour metastasis formation. *In vivo*, one specific glycoprotein can be relevant, as recently shown for the rolling of neutrophils on activated endothelia, which can be blocked by a monoclonal antibodies against PSGL-1, a high affinity ligand of P-selectin [106]. An important goal of future studies will therefore be the characterization of biologically relevant ligands for the Sialoadhesin family.

Sialic acid modifications

Sia occur in a variety of modifications. All members of the Sialoadhesin family investigated to date distinguish different Sia modifications in a specific fashion [92, 107]. This is in contrast to the Selectins which seem not to be sensitive to Sia modifications [97, 99].

Experiments with various glycoconjugates and cells demonstrated that Sn and MAG bind Neu5Ac with much higher affinity than Neu5Gc or $Neu5,9Ac_2$ [92] (unpublished observation). For human CD22, is has been demonstrated that the glycerol side chain is an essential structural element [32, 33] and an *O*-acetyl group at position 9 as in $Neu5,9Ac_2$ prevents the recognition [107]. In contrast to Sn and MAG, the murine homologue of CD22 binds to Neu5Gc with much higher affinity than to Neu5Ac [92]. Interestingly, the human homologue of CD22 recognizes Neu5Ac as well as Neu5Gc [107] (unpublished observations). This is of biological significance, since both human and murine CD22 bind with high preference to lymphocytes [8, 27, 36, 37] and Neu5Gc is not found in normal human tissues in contrast to most other higher animals [1]. Therefore, in order to bind glycans on human cells, CD22 had to evolve an affinity for Neu5Ac. Since human CD22 binds to Neu5Gc as well as to Neu5Ac, it seems likely that the ability of CD22 to bind Neu5Ac evolved later without the loss of affinity to Neu5Gc. In this context, the specificity of avian CD22, if it exists, would be interesting to know, since birds also do not normally express Neu5Gc [1].

Based on these observations, it seems feasible to develop synthetic Sia analogues with modifications at C-5 which will bind with high affinity to specific members of the Sialoadhesins family [8] (unpublished observations). Similar to the studies on the influenza A virus haemagglutinin [108], such inhibitors will be useful to determine the contribution of contacts in the binding site to the affinity to Sia residues.

The metabolic pathways leading to Neu5Gc and *O*-acetylated Sia and their intracellular locations (Fig. 6) are well documented [1, 2, 109]. With respect to cellular interactions, some of their characteristics are of particular interest. Neu5Ac bound to glycoconjugates is *O*-acetylated probably in the trans Golgi network (Fig. 6A) [110]. This opens the possibility that only specific glycans or glycoconjugates are modified by the enzyme(s) involved, as has been shown in rat liver [110]. In addition, the transport of glycoconjugates through this compartment

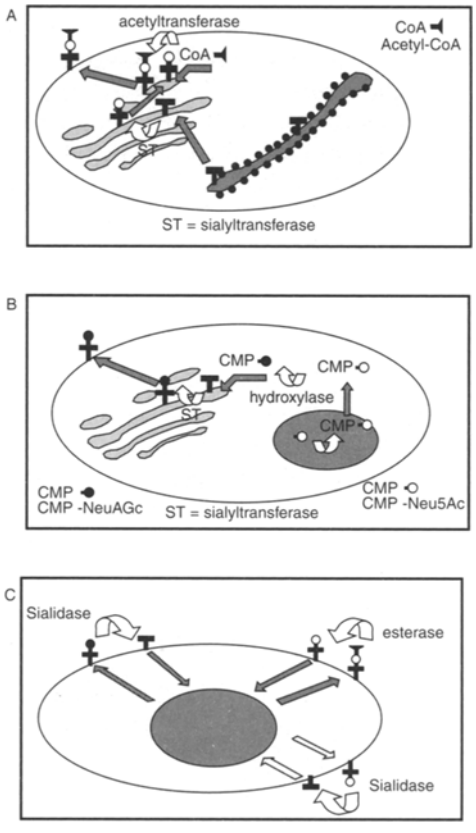


Figure 6. Influence of sialic acid modifications on ligands for sialoadhesins (for details see text). (A) Metabolic pathway leading to cell surface $Neu5,9Ac_2$. (B) Metabolic pathway leading to cell surface Neu5Gc. (C) External enzymes modifying cell surface Sia.

could have an impact on the relative amount of Neu5,9Ac$_2$ on the cell surface. On the other hand, CMP-Neu5Ac is converted to CMP-Neu5Gc by a cytoplasmic monooxygenase system (Fig. 6B) [111–114]. Since transport to the Golgi apparatus and transfer to the glycoconjugates does not seem to be specific for either donor substrate, the amount of Neu5Gc in glycoconjugates on the cell surface would mainly be regulated by the ratio of CMP-Neu5Ac to CMP-Neu5Gc generated by the monooxygenase [112]. Another characteristic difference between Neu5,9Ac$_2$ and Neu5Gc is their catabolism. Whereas intra- or extracellular esterases can remove the 9-*O*-acetyl group from Neu5,9Ac$_2$ on cell surface glycoconjugates leading to cell surface bound Neu5Ac (Fig. 6C), no enzymatic system is known to convert Neu5Gc to Neu5Ac.

This leads to the following possible modulatory situation for Sia modifications. For example, cells expressing the ligand determinant for Sn, Neu5Acα2,3Galβ1,3GalNAc or Neu5Acα2,3Galβ1,3(4)GlcNAc, could mask their ligands by acetylation. However, this mask could be removed by extracellular 9-*O*-acetylsialate-esterases allowing interactions of the ligands with Sn. In contrast, Neu5Gc can only be removed by sialidases. However, this would lead to the asialooligosaccharides, which are not ligand determinants for Sn. A cell expressing Neu5Gc on the surface could only interact with Sn after consumption of the intracellular pool of CMP-Neu5Gc and exchange of the cell surface Sia. This model represents an example how modifications of Sia could modulate molecular and cellular interactions and how this is affected by their different metabolic pathways.

Perspectives

Several aspects of research on the Sialoadhesin family will be of main interest in future. Important questions to answer will be: (1) What are the biological roles; do sialoadhesins function as cell adhesion molecules, in signal transduction or both? (2) Which biologically relevant ligands are involved and how do they induce biological signals? (3) Which amino acids form the binding site and how do they interact with the sialylated glycans? (4) Do further members of this family exist?

(1) Evidence has been presented that members of the Sialoadhesin family can mediate cell adhesion and trigger signalling events *in vitro*. Certainly, *in vivo* experiments are required to answer the question for biological relevance. Although experiments with transgenic animals can be expected to give important insights, knocking out the gene of interest is often not sufficient, since the interpretation of unexpectedly small effects is often very difficult due to redundancy and backup systems. Complementary strategies will use both the aberrant expression of adhesion molecule and specifically targeted alterations of cell surface glycans in transgenic animals. Also specific inhibitors would be useful reagents for both *in vitro* and *in vivo* studies. Preliminary experiments with synthetic sialic acid analogues provide hope that the development of such inhibitors is possible.

(2) The rather broad specificity towards different ligands (see above) resulted in a wide range of affinity for ligands to occur on cell surfaces. Theoretically, this adds a new potential in regulating and fine tuning cellular interactions not possible with highly specific receptor-ligand systems. Therefore, besides the characterization of high affinity ligands, an important aspect of future research will be to examine whether these low affinity ligands have a biological function and how this is accomplished or whether they are just side products of a glycosylation machinery necessary to produce the high affinity ligands. An aspect of increasing interest is the function of *cis*-interacting ligands. Do they just regulate the availability of binding sites or are they (also) important for the assembly of protein complexes in the plasma membrane? To answer this question, it will be necessary to investigate these complexes in cells with altered glycosylation machinery, for example by suppressing α-2,6 sialyltransferase activity in B cells.

In addition, an increasing knowledge on mechanisms like clustering of ligand determinants, e.g. on mucin-like molecules, leading to high affinity ligands will be helpful in the design of inhibitors specific for certain receptor-mediated interactions. One example is the increasing effort to develop carbohydrate-based drugs as inhibitors for Selectins for the treatment of undesired inflammatory reactions.

(3) The binding site for the sialoadhesins is contained in the first two domains, probably only in the V-set domain at the N-terminus. Site-directed mutagenesis experiments in combination with structural studies, e.g. from crystallographic and NMR studies, can be expected to give exciting insights into the molecular mechanisms of protein-Sia interactions.

(4) The discovery of the Sialoadhesin family and the close localization on the genome for most of them raises the intriguing possibility that further members exist. However, finding these might be a difficult task, since the homology is rather low, making low homology screens of cDNA libraries unlikely to work. Besides Sn, all members identified so far have been characterized after their primary sequence became available. This would mean that such proteins may be found only by coincidence. Certainly, the human genome project will be very helpful in answering this question. In addition, the characterization as Sia-dependent adhesion molecules could be difficult, since the binding activity could be masked by *cis* interactions and possibly only be detected,

if the appropriate glycans recognized by the protein are included in the investigation.

Whereas until now protein-protein interactions have been implicated in the vast majority of cellular interaction and signalling, the discovery of the Sialoadhesin family has demonstrated that also the recognition of sialylated glycans is involved in quite diverse biological processes. We can expect that progress on the Sialoadhesin family will provide much insight into the roles of protein-carbohydrate interactions.

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Note added in proof: while this manuscript was in process, the following reports were published:

A. Debellard ME, Tang S, Makhopadhyay G, Shen YJ, Filbin MT (1996) *Mol Cell Neurosci* **7**: 89–101.
B. Yang LJS, Zeller CB, Shaper NL, Kiso M, Hasegawa A, Shapiro RE, Schnaar RL (1996) *Proc Natl Acad Sci USA* **93**: 814–18.
C. Vinson M, van der Merke PA, Kelm S, May A, Jones EY, Crocker PR (1996) *J Biol Chem* **271**: 9267–72.
D. van der Merwe PA, Crocker PR, Vinson M, Barclay AN, Schauer R, Kelm S (1996) *J Biol Chem* **271**: 9273–80.