Molecular analysis of sialoside binding to sialoadhesin by NMR and site-directed mutagenesis

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The molecular interactions between sialoadhesin and sialylated ligands have been investigated by using proton NMR. Addition of ligands to the 12 kDa N-terminal immunoglobulin-like domain of sialoadhesin result in resonance shifts in the protein spectrum that have been used to determine the affinities of sialoadhesin for several sialosides. The results indicate that α2,3-sialyl-lactose and α2,6-sialyl-lactose bind respectively 2- and 1.5-fold more strongly than does α-methyl-*N*-acetylneuraminic acid (α-Me-NeuAc). The resonances corresponding to the methyl protons within the N-acetyl moiety of sialic acid undergo upfield shifting and broadening during titrations, reflecting an interaction of this group with Trp^2 in sialoadhesin as observed in co-crystals of the terminal domain with bound ligand. This resonance shift was used to measure the affinities of mutant and wild-type forms of sialoadhesin in which the first three domains are fused to the Fc region of human IgG1. Substitution of Arg^{97} by alanine completely abrogated measurable interaction with α-Me-NeuAc, whereas a conservative substitution with lysine resulted in a 10-fold decrease in affinity. These results provide the first direct measurement of the affinity of sialoadhesin for sialosides and confirm the critical importance of the conserved arginine in interactions between sialosides and members of the siglec family of sialic acid-binding, immunoglobulin-like lectins.

Key words: lectins, receptor, sialic acid, sialyl-lactose, siglecs.

INTRODUCTION

Mammalian carbohydrate-binding proteins (animal lectins) have been classified into different groups on the basis of structural features of the lectins themselves and the types of carbohydrate ligand that are recognized [1]. The I-type lectins are part of the immunoglobulin superfamily. The best-characterized I-type lectins are a distinct subset of membrane proteins that are sialic acid-binding, immunoglobulin-like lectins, designated 'siglecs' [2]. Mammalian siglecs comprise sialoadhesin (siglec-1) [3], CD22 (siglec-2) [4], CD33 (siglec-3) [5], myelin-associated glycoprotein (siglec-4a) [6] and siglec-5 [7]. The siglecs appear to recognize sialic acids as their dominant ligands, rather than protein determinants more commonly recognized by members of the immunoglobulin superfamily [6]. A striking feature of the siglecs is that they are expressed in a highly tissue-specific manner, indicating that they perform distinct, non-overlapping functions in both the haemopoietic (sialoadhesin, CD22, CD33 and siglec-5) and nervous systems (myelin-associated glycoprotein). For example, CD22 functions as a negative regulator of B-cell activation [8], sialoadhesin is a macrophage adhesion molecule [9] and myelin-associated glycoprotein is involved in myelin–axon interactions [10].

Each siglec exhibits a characteristic preference for both the type of sialic acid recognized and its linkage to the subterminal sugar. For example, none of the siglecs examined so far are able to bind 9-O-acetylated NeuAc, a commonly found sialic acid in mammalian tissues [11]. Sialoadhesin, CD33 and myelin-associated glycoprotein bind preferentially to *N*-acetylneuraminic acid (NeuAc) in α2,3 linkage [6] and CD22 binds preferentially to NeuAc in α2,6 linkage [12], whereas siglec-5 appears to bind NeuAc in either linkage [7]. These differences in sugar-binding specificities are likely to be important in the cellular-recognition functions of these proteins.

Siglecs consist of a membrane-distal V-set immunoglobulin-like domain followed by a variable number of C2-set domains that ranges from one in CD33 to 16 in sialoadhesin [13]. Considerable progress has been made recently in elucidating the molecular basis for carbohydrate recognition by these proteins using a combination of approaches that include domain deletion analysis [14], site-directed mutagenesis [15] and, most recently, X-ray crystallography [16]. These studies have demonstrated that the N-terminal V-set domain is necessary and sufficient for sialic acid binding and that most of the relevant interactions are made with a discrete set of amino acids within this domain. The crystal structure of the N-terminal fragment of sialoadhesin complexed with 3′-sialyl-lactose has revealed that the carboxylate, N-acetyl and glycerol substituents of sialic acid interact with a conserved arginine residue and two tryptophan residues that are located on the F, A and G β-strands respectively.

Proton-NMR spectroscopy has previously been used to analyse the interaction of soluble lectins with their carbohydrate ligands in solution, including the sialic acid-binding proteins influenza-virus haemagglutinin [17] and wheat-germ agglutinin [18]. In the work presented here, proton NMR was used to analyse the molecular interactions between sialoadhesin and sialic acid-containing ligands. The results provide direct measurements of the affinities of a ligand-binding fragment of sialoadhesin with

Abbreviations used: NeuAc, *N*-acetylneuraminic acid; α-Me-NeuAc, α-methyl-*N*-acetylneuraminic acid; β-Me-NeuAc, β-methyl-*N*-acetylneuraminic acid; siglecs, sialic acid-binding, immunoglobulin-like lectins.

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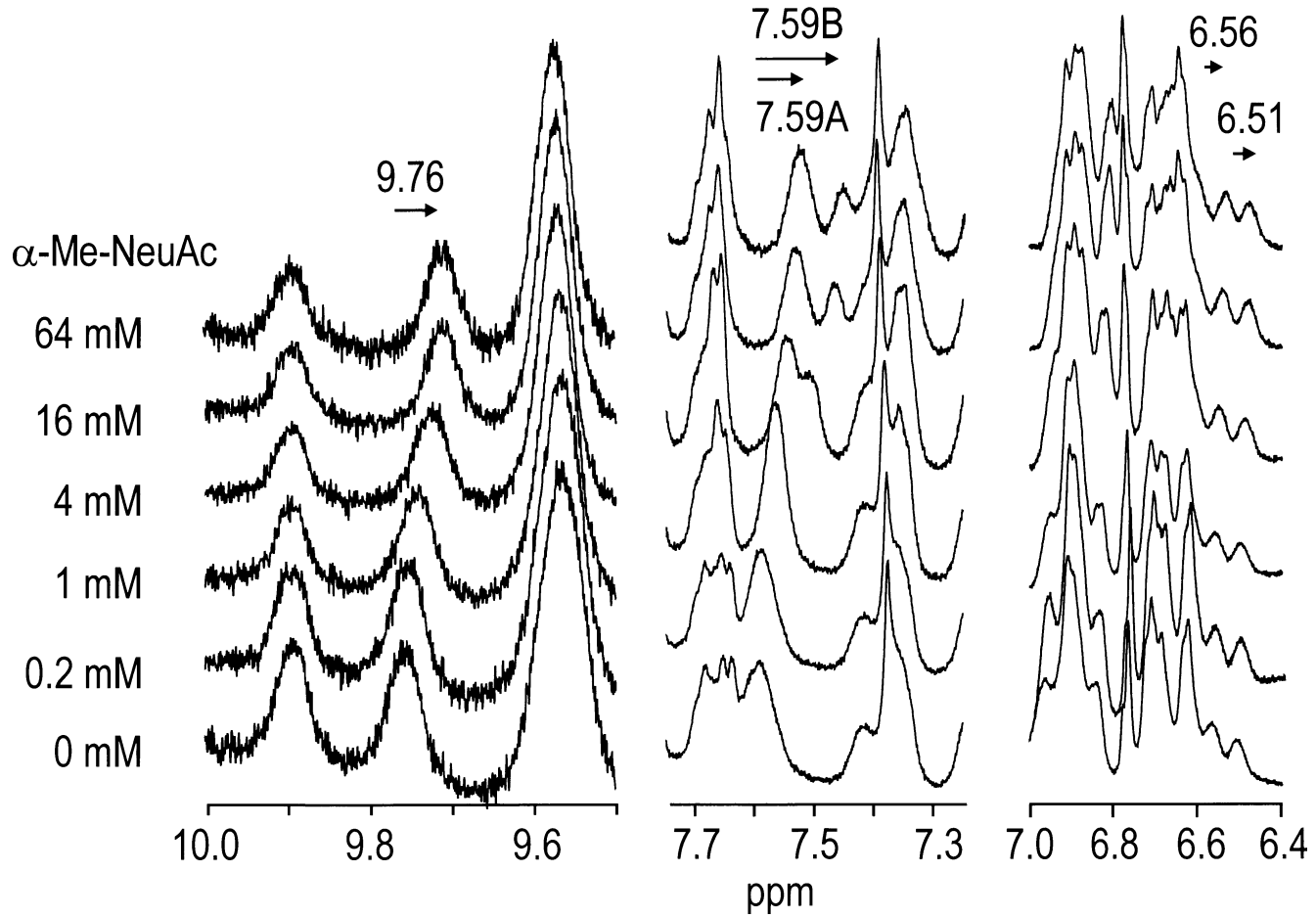

Figure 1 Changes in the proton NMR spectrum of the sialoadhesin terminal domain in the presence of α-Me-NeuAc

The terminal domain was present at a concentration of 0.5 mM. Data from every second spectrum collected (512 scans each) are shown.

different sialosides. The roles of two key residues, implicated in binding by X-ray crystallography, have also been probed by mutagenesis.

EXPERIMENTAL

Preparation of proteins

The N-terminal V-set immunoglobulin-like domain of sialoadhesin was prepared and purified as described previously [19]. DNA constructs encoding wild-type and mutant forms of sialoadhesin fused to the Fc portion of human IgG1 were prepared as described in [15]. To produce the quantities of protein required for NMR sugar titrations, Chinese-hamster ovary cell lines stably secreting the transfected proteins were prepared using the glutamine synthetase expression system [20]. Fc–sialoadhesin fusions were purified from cell supernatants using Protein A–Sepharose chromatography. Sialic acids that might interfere with the NMR spectra from recombinant Fc fusion proteins were removed by sialidase digestion. The proteins eluted from Protein A–Sepharose were adjusted to 5 mg/ml and dialysed against 50 mM sodium acetate buffer, pH 5.5, containing 9 mM $CaCl_2$. *Vibrio cholerae* sialidase (Calbiochem, La Jolla, CA, U.S.A.) was added at 0.1 unit/ml and proteins digested overnight at 37 °C. Following digestion, proteins were generally re-purified by Protein A–Sepharose chromatography and dialysed into 20 mM Tris chloride, pH 8.0. In certain cases, proteins used for one NMR titration were re-used for another following extensive dialysis in 20 mM Tris chloride, pH 8.0. Prior to the NMR titrations, proteins at approx. 10 mg/ml were desalted into 25 mM [^{2}H]Tris chloride, pH 8.0, in [^{2}H]water, and adjusted to the desired concentration prior to analysis by NMR. [^{2}H]Tris was obtained from Cambridge Isotope Laboratories, Andover, MA, U.S.A.

Sugar ligands

β-Methyl-*N*-acetylneuraminic acid (β-Me-NeuAc) was kindly provided by Dr. Reinhard Brossmer (Institut für Biochemie II, Universität Heidelberg, Heidelberg, Germany), who also provided some of the α-methyl-*N*-acetylneuraminic acid (α-Me-NeuAc). Additional α-Me-NeuAc was obtained from Sigma Chemical Co. α2,3-Sialyl-lactose and α2,6-sialyl-lactose were obtained from Oxford GlycoSciences, Abingdon, Oxon., U.K., and from Glycoseparations, Moscow, Russia. The concentrations of ligands were assayed by a 'micro' version of the orcinol/$FeCl_3$ procedure [21].

Collection of NMR spectra

All spectra were obtained on a Varian Unity 500 NMR spectrometer at 30 °C without spinning. The internal water peak was

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used as a reference assigned as 4.78 p.p.m. In most cases, 512 scans were recorded and averaged. Measurements of peak positions were obtained on spectra that had been zero-filled before Fourier transformation. Protein concentrations were determined by the BCA (bicinchoninic acid) method (Pierce Chemical Co.) and were adjusted to 6 mg/ml for the N-terminal domain and 5 mg/ml for the Fc fusion proteins.

Data-fitting procedures

For titrations of terminal domain, the changes in proton resonances in the protein spectrum were fitted to the simple binding equation:

$$\delta = \delta_{free} + \Delta\delta_{bound} \cdot [ligand]/(K_D + [ligand])$$

in which δ_{free} is the chemical shift of a particular proton in the absence of sugar. K_D, the concentration for half-maximal binding, and $\Delta\delta_{bound}$, the change in chemical shift when sugar is bound, were determined by non-linear least-squares fitting using the program SigmaPlot.

For titrations of Fc chimaeras, data were fitted by a linearized equation:

$$[Ligand] = [protein]\Delta\delta_{bound}/(\delta - \delta_{free}) - K_D$$

in which δ is the observed chemical shift for the N-acetyl protons, δ_{free} is the chemical shift in the absence of protein and $\Delta\delta_{bound}$ is the change in chemical chift when NeuAc is bound to the protein. The parameters $[protein]\Delta\delta_{bound}$, δ_{free} and K_D were obtained using SigmaPlot to fit the observed data.

RESULTS

Quantification of binding to the terminal domain of sialoadhesin

In an initial investigation of the interaction of sialoadhesin with its sialic acid-containing ligands, the proton NMR spectrum of the isolated terminal domain was measured in the presence of increasing concentrations of α-Me-NeuAc. Comparison of the spectra reveals a number of resonances that are shifted in the presence of ligand (Figure 1). The resonance at 9.76 p.p.m. falls in the amide proton region of the spectrum, whereas the remaining four resonances correspond to protons on aromatic residues. The peak initially observed at 7.59 p.p.m. clearly represents two different protons with similar chemical shifts, which are resolved into two distinct resonances in the presence of sugar ligand.

Quantitative analysis of the observed changes in chemical shift (Figure 2) make it possible to determine binding constants for the ligand–protein interaction under solution conditions. Fitting the data to a simple first-order binding equation produces several independent estimates of the dissociation constant for the ligand–protein interaction (Table 1). The average K_D value of approx. 3 mM is consistent with the K_D estimated from the ability of this ligand to compete in solution-phase binding assays [22].

In contrast with the interaction of α-Me-NeuAc with the terminal domain, a similar titration with β-Me-NeuAc failed to show any significant effect on the proton NMR spectrum of the domain, even when added to a concentration of 32 mM. This result demonstrates the specific nature of the interaction with the α-anomer of sialic acid, which has also been observed in cell-binding assays [22].

Titrations were also performed using 3′-sialyl-lactose and 6′-sialyl-lactose as ligands. Similar shifts in all of the proton resonances of the terminal domains were obtained with all three ligands. Quantification of the dissociation constants derived from these shifts is summarized in Table 1. For each ligand, there

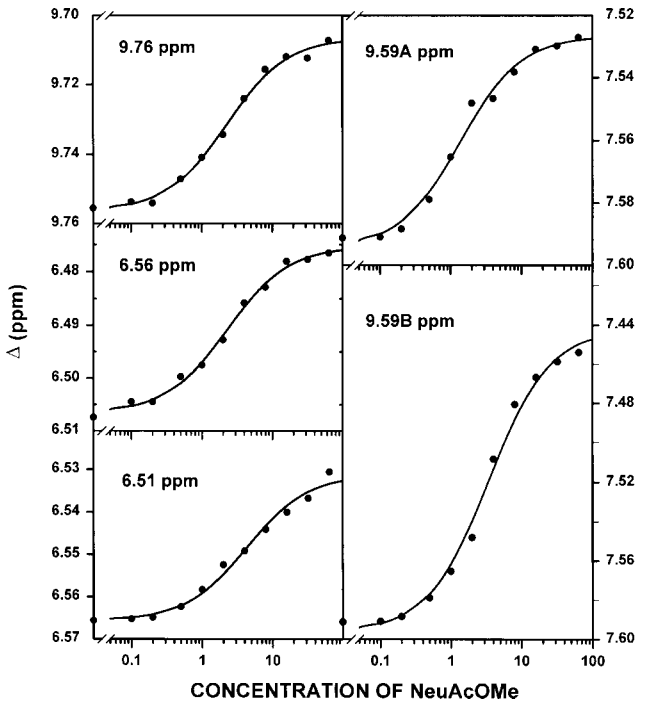

Figure 2 Quantification of ligand binding to the sialoadhesin terminal domain

Chemical shifts of five proton resonances were quantified and the data were fitted to a simple first-order binding equation. ●, Measured data; ——, fitted curve.

is variability in the K_D estimated from each of the changes in different proton resonances. However, the pattern of the variability is similar for each ligand. Relative affinities calculated by comparing the K_D values for 3′- and 6′-sialyl-lactose to the K_D for α-Me-NeuAc yield much more consistent results for each proton resonance quantified. On the basis of these results, the affinity for 3′-sialyl-lactose was found to be approximately twice the affinity for α-Me-NeuAc, while binding to 6′-sialyl-lactose is only marginally tighter than binding to the methyl glycoside.

Other estimates of the relative affinities of sialoadhesin for these ligands have been obtained largely from haemagglutination and erythrocyte-binding assays [6]. These results show preferential binding of sialoadhesin to cells displaying α2,3-linked sialic acid. Although the difference in affinity between the α2,3- and α2,6-linked ligands observed in the present studies is only 1.6-fold, such a modest difference can be magnified substantially by the highly multivalent interaction between cell surfaces and could readily account for the differences observed previously.

The structure of sialoadhesin in complex with 3′-sialyl-lactose reveals that most of the contacts are with the terminal sialic residue [16]. However, an additional hydrogen bond between the 6-hydroxy group of galactose and residue Tyr^{44} of sialoadhesin as well as hydrophobic packing of the apolar B face of the galactose and Leu^{107} might be expected to contribute to increased affinity for this ligand compared with α-Me-NeuAc. The observation that the change $Tyr^{44} \rightarrow Ala$ does not affect binding to erythrocytes suggests that the hydrophobic contact may be more important than the hydrogen bond [16]. This interpretation is also supported by the finding that removal of the 6-hydroxy group from the galactose residue in the 3′-sialyl-lactose ligand does not affect the potency of the ligand in inhibition assays [23]. The packing interaction with Leu^{107} would not be possible in a

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Table 1 Binding of NeuAc glycosides to sialoadhesin measured by NMR

Changes in chemical shifts of protein resonances were quantified and fitted to a binding equation as shown in Figure 2. For the N-acetyl groups in sialosides, dissociation constants were derived using the linearization procedure illustrated in Figure 6. Results are means ± S.D. Abbreviation: SL, sialyl-lactose.

Resonance	$K_{\alpha\text{-Me-NeuAc}}$ (mM)	$K_{3'\text{-SL}}$ (mM)	$K_{6'\text{-SL}}$ (mM)	$K_{3'\text{-SL}}/K_{\alpha\text{-Me-NeuAc}}$	$K_{6'\text{-SL}}/K_{\alpha\text{-Me-NeuAc}}$	$K_{3'\text{-SL}}/K_{6'\text{-SL}}$
Protein resonances						
9.76 p.p.m.	2.3	1.2	1.7	0.52	0.74	0.71
7.59A p.p.m.	1.4	0.7	1.0	0.50	0.71	0.70
7.59B p.p.m.	3.5	2.1	2.9	0.60	0.82	0.73
6.56 p.p.m.	4.3	1.4	2.3	0.32	0.53	0.60
6.51 p.p.m.	2.3	1.4	2.2	0.61	0.96	0.64
Mean...	2.8 ± 1.1	1.4 ± 0.4	2.0 ± 0.6	0.5 ± 0.1	0.8 ± 0.1	0.68 ± 0.05
Sugar resonances						
N-acetyl	1.7	0.8	2.1	0.5	1.2	0.4

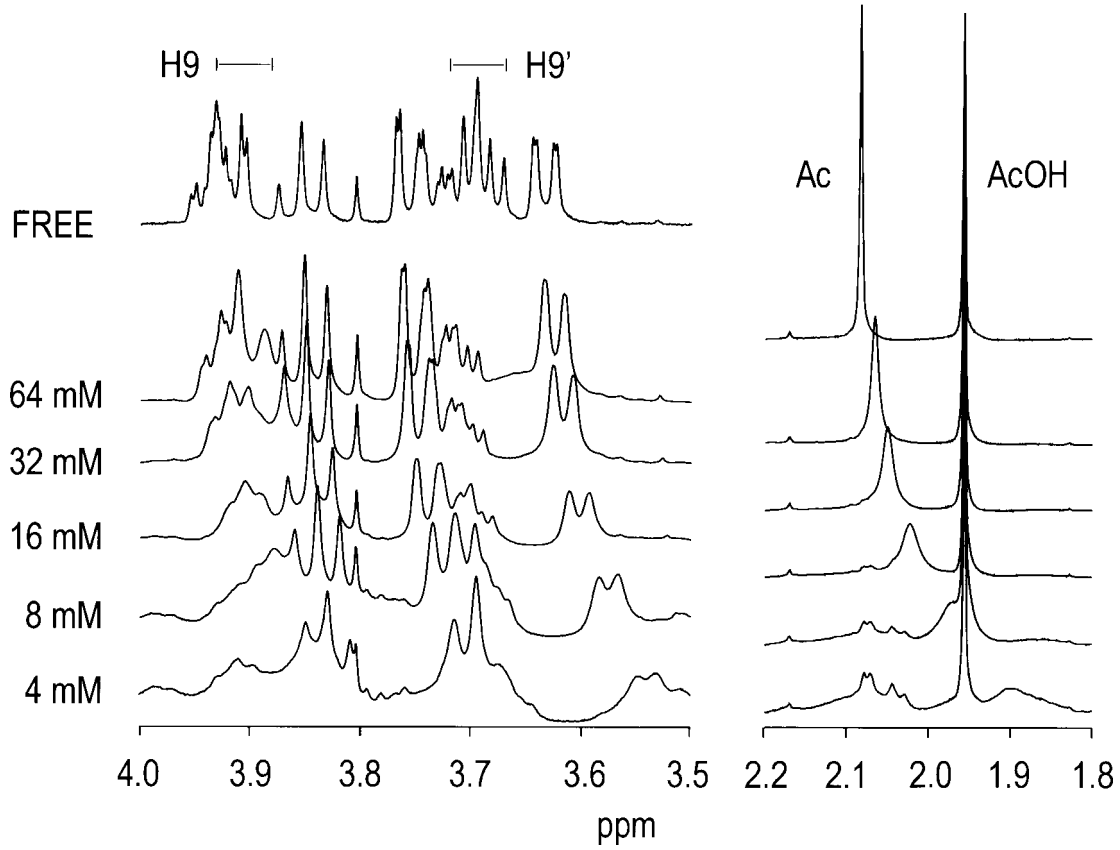

Figure 3 Changes in the proton NMR spectrum of α-Me-NeuAc in the presence of the sialoadhesin terminal domain

The spectrum of the free ligand preparation at the top reveals that acetate was present in the ligand preparation at a concentration approximately equal to the α-Me-NeuAc. Normalization to this peak allows comparison of the sugar spectra at similar intensities and reveals the selective broadening and shifting of certain resonances. The concentrations of sugar indicated are nominal concentrations based on amounts of stock solutions added, prior to correction of stock concentration based on the sialic acid assay.

complex with 6′-sialyl-lactose, suggesting that this contact provides at least a partial explanation for the modest ligand-binding selectivity of the domain.

Structural aspects of sialoadhesin–ligand interactions

Regions of the NMR spectrum corresponding to the sugar resonances in the titration with α-Me-NeuAc were also examined. As shown in Figure 3, the results can be viewed as a reverse titration, in which the ratio of sugar to protein increases from a starting condition of free sugar. Several major changes in the α-Me-NeuAc resonances are observed as the ratio of protein to ligand increases. The most notable effect is broadening and shifting of the well-resolved peak corresponding to the methyl protons of the N-acetyl substitutent.

Broadening of selected resonances of a ligand usually reflects a transition from fast to intermediate exchange of protons that are affected by nearby aromatic residues when bound to the

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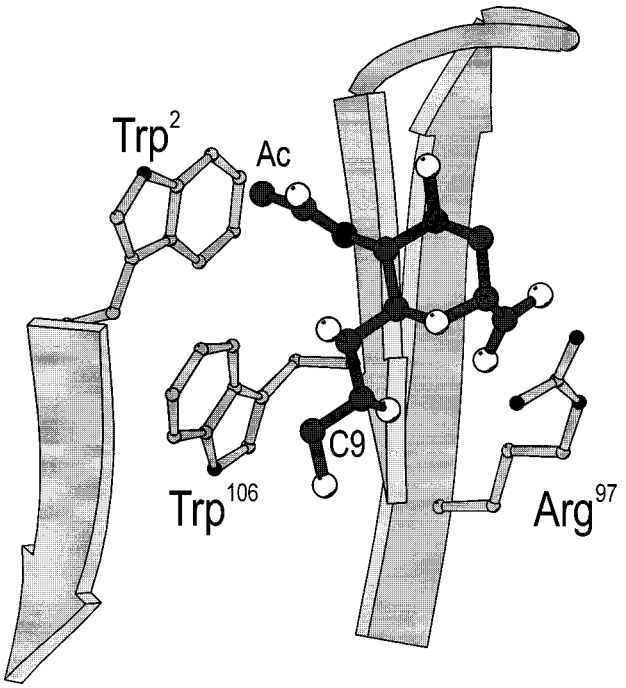

Figure 4 Structure of the NeuAc-binding site in the sialoadhesin terminal domain

Positions of key residues near the NeuAc residue in the crystal structure of the sialoadhesin terminal domain complexed with 3′-sialyl-lactose are shown. Oxygen atoms are shown in white, nitrogen in black and carbon in *grey*. The NeuAc residue is shaded dark grey. The co-ordinates were derived from the Brookhaven Protein Database (1qfo) and the Figure was prepared with Molscript [25].

protein [24]. The ring current associated with the residues can result in substantial changes in the resonances associated with protons located above or below the aromatic ring. When this change approaches the rate of the association–dissociation reaction, broadening due to intermediate exchange is observed.

Although the proton resonances for the ring and glycerol side-chain protons of α-Me-NeuAc are less well resolved than the α-methyl and N-acetyl protons, selective effects on the H-9 and H-9′ protons can be observed in Figure 3. The H-9′ proton, represented by a quartet of resonances, is relatively well resolved in the free sugar spectrum and is already largely suppressed at the highest ratio of sugar to protein (64 mM sugar). The H-9 proton, also represented by a quartet of resonances, is not well resolved from the H-8 proton which is observed as a partially overlapping octet of resonances centred slightly further downfield. Both the H-8 and H-9 protons are affected by the presence of protein, but the H-9 proton resonances appear to be affected at lower ratios of protein to sugar.

The structural basis for the observed interactions is illustrated in Figure 4. In the sialoadhesin terminal domain complexed with 3′-sialyl-lactose, the methyl carbon atom in the N-acetyl substituent and the terminal C-9 in the glycerol side chain make van der Waals contacts with tryptophan residues that project from β-strands A and G (Trp^2 and Trp^{106} respectively). Protons attached to these carbon atoms would be located above the tryptophan rings in regions where they would be deshielded. The acetyl proton resonance is clearly shifted upfield, a finding which is consistent with this deshielding effect, although the complexity of the remaining spectrum makes it difficult to confirm the details of the C-9 shift.

These NMR results provide strong evidence that the binding of sialic acid-containing ligands to the terminal domain of sialoadhesin observed in the crystal complex closely mimics the binding in solution. The results also emphasize a common aspect to many sialic acid-binding sites, in which the N-acetyl substituent packs against a tryptophan residue [17,18]. The packing of the terminal portion of the glycerol side chain against a tryptophan residue is more specific to sialoadhesin.

Binding of sialoadhesin–immunoglobulin Fc chimaeras

It is of interest to utilize NMR to analyse solution-phase ligand binding by mutant forms of sialoadhesin, although the difficulty of preparing isolated terminal domain is a limiting factor for such studies. The purification process can be greatly enhanced by use of immunoglobulin Fc chimaeras as a tag for affinity chromatography of a fragment of sialoadhesin containing the three terminal immunoglobulin-like domains. However, the resulting multidomain dimeric chimaera has a native molecular mass of approx. 200 kDa. A molecule of this size is not suitable for obtaining a high-resolution proton NMR spectrum.

Nevertheless, it is still possible to measure perturbation of the sugar spectrum in the presence of such proteins and to use this approach as an alternative way of quantifying binding. In order to eliminate interference from sialic acid attached to the three N-linked glycosylation sites in the Fc–sialoadhesin chimaera, the protein was desialylated. The results for α-Me-NeuAc are illustrated in Figure 5. The resonance for the methyl protons of the N-acetyl substituent is well resolved from the resonance from the *N*-acetylglucosamine that remains covalently associated with the protein. Upfield shifting and broadening of this resonance at low sugar concentration is evident. The protein concentration was approx. 10-fold lower in this experiment than in the studies with the free terminal domain, so the effects on the sugar spectrum appear at lower concentrations of ligand. Changes similar to those observed for the free domain are also seen for the H-9′ and H-9 resonances, confirming that a similar mode of binding is being observed.

Changes in the N-acetyl proton resonance of α-Me-NeuAc were measured as a means of quantifying binding to the Fc chimaera. Under conditions of low protein concentration, the binding can be analysed by a simple linearization procedure [18]. The estimated K_D value of 1.7 mM obtained from the projected x-intercept of such a plot (Figure 6) compares favourably with the value of 2.8 mM obtained from analysis of the free terminal domain. Experiments with 3′- and 6′-sialyl-lactose yielded K_D values of 0.8 mM and 2.1 mM respectively (Table 1). These results reflect preferential binding to the α2,3-sialyl-lactose which is similar to that observed in the direct titration experiments.

Analysis of ligand binding by mutant forms of the terminal domain

Modelling and mutagenesis studies followed by more recent structural analysis of the terminal domain of sialoadhesin have led to the identification of key residues involved in ligand binding [15,16]. As shown in Figure 4 and demonstrated by the NMR experiments described above, Trp^2 makes van der Waals contact with the N-acetyl group of NeuAc, whereas Arg^{97} is involved in ionic interactions with the carboxy group of the ligand. The importance of these two residues was investigated further using the NMR approach.

Changing Trp^2 to glutamine in the context of the terminal domain-Fc chimaera results in complete loss of binding activity

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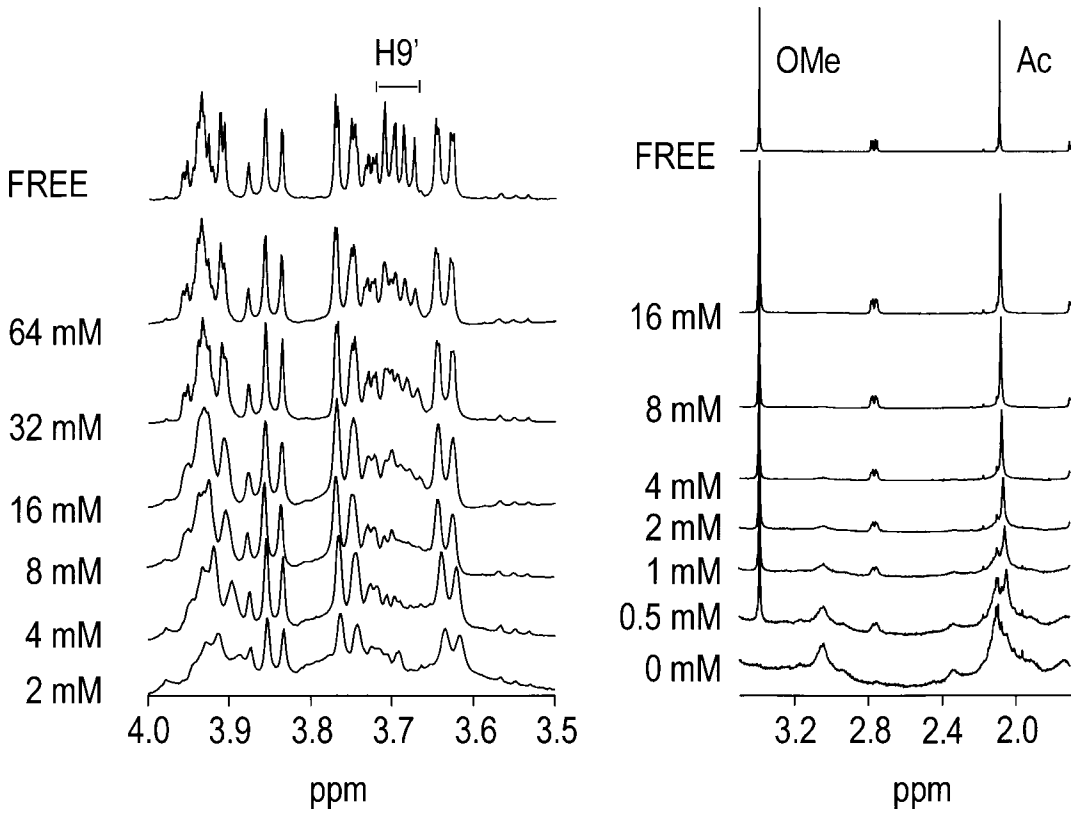

Figure 5 Changes in the proton NMR spectrum of α-Me-NeuAc in the presence of sialoadhesin–Fc chimaera

Spectra were normalized using the O-methyl protons of the ligand so that changes in the N-acetyl methyl protons in the glycerol side chain are evident. The concentrations of sugar indicated are nominal concentrations based on amounts of stock solutions added, prior to correction of stock concentration based on sialic acid assay.

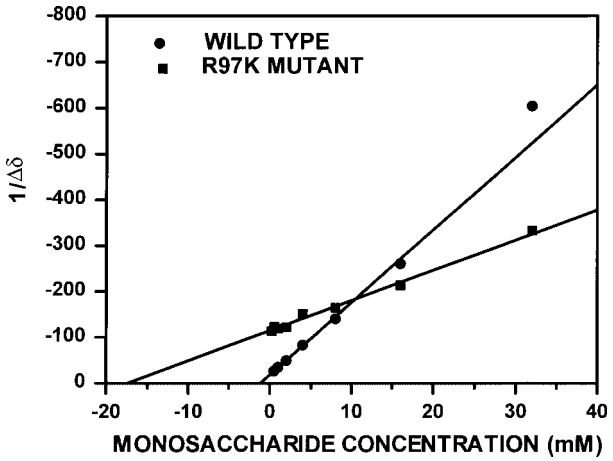

Figure 6 Quantification of sialoside interaction of sialoadhesin–Fc chimaera

Data for changes in chemical shifts of sugar proton resonances were analysed using the linearization protocol described in the Experimental section. ■,●, Experimental data; ——, fitted curve.

as assessed by the inability to detect any changes in the sugar spectrum even at the highest ratios of protein to sugar that could be tested. Similarly, changing Arg^{97} to alanine results in the loss of detectable interaction with α-Me-NeuAc. However, replacement of Arg^{97} with lysine results in a binding constant of approximately 15 mM (Figure 6), which represents nearly a 10-fold loss in affinity compared with the wild-type protein. These quantitative results are consistent with haemagglutination assays performed with these same mutants, since the $Trp^2 \rightarrow Gln$ and $Arg^{97} \rightarrow Ala$ mutants show no haemagglutination activity [15,16] and the $Arg^{97} \rightarrow Lys$ mutant shows only very weak activity that is difficult to quantify [15].

Analysis of ligand binding to other siglec family members

In an attempt to compare the interactions of different siglecs with sialoside ligands, Fc chimaeras with terminal domains from CD22, myelin-associated glycoprotein and CD33 were titrated with α-Me-NeuAc. Each desialylated protein was tested for sialic acid-dependent binding activity to human erythrocytes [5,6] and found to be fully active (results not shown). Titrations were monitored at the resonance associated with the N-acetyl protons, since this is the best resolved resonance which undergoes the most readily quantified shift in sialoadhesin. Surprisingly, only very minor changes were observed, roughly 10-fold smaller than the changes seen during titration of sialoadhesin. Further, the shift with CD22 appeared to be downfield rather than upfield as in the case of sialoadhesin. Titrations of CD22 with 6′-sialyllactose and of myelin-associated glycoprotein with 3′-sialyllactose produced no systematic effects on the sugar spectra.

One potential explanation for these findings is that the affinity of these interactions is too high to be measured by the approach

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used. As the affinity increases, the frequency of the exchange reaction of the bound and free ligand decreases and may become substantially less than the differences in the resonant frequencies of the sugar protons in the bound and free states. Thus the conditions for intermediate exchange, resulting in line broadening and shifting, are no longer met. Under these conditions of slow exchange, the bound form of the ligand would still show changes in the positions of the proton resonances, but it is not possible to identify the resonances corresponding to the bound sugar because they are present only at a level equivalent to the protons on the protein. A 10-fold increase in affinity would be sufficient to change from intermediate to slow exchange. An alternative explanation may lie in the disposition of aromatic amino acids in the sialic acid-binding sites of the different siglecs. In particular, the nature and precise position of the residue at position 2 in the different siglecs may result in different interactions with the N-acetyl group of sialic acid.

DISCUSSION

Millimolar affinity constants for neutral monosaccharide ligands are typical for animal lectins in several structural groups, including, in particular, the Ca^{2+}-dependent C-type lectins [26]. Although the siglecs represent a structurally distinct family of lectins, it appears that they resemble the C-type lectins in terms of the relatively weak interactions with terminal monosaccharides. High-affinity cell adhesion is achieved from the multivalent interactions between multiple lectins and sugars on apposing cell surfaces.

The essentially absolute discrimination for α- over β-linked sialic acid demonstrated in these studies can be explained by the structure of the sialic acid-binding site [16]. The carboxy group in the β-anomer would project away from the surface of the protein and would not be able to make the key stabilizing interactions with Arg^{97}. The titrations described here suggest that the affinities for α2,3- and α2,6-linked sialic acid differ only by roughly 2-fold. As in the case of the absolute affinities, the relatively weak selectivity can be amplified in the case of multivalent cell-surface interactions. Thus the observed selectivity can account for the preferential agglutination of erythrocytes bearing α2,3- rather than α2,6-linked sialic acid [6]. Similar amplification from very small solution-phase differences in binding to major differences in haemagglutination activity has also been observed for influenza-virus haemagglutinin [17].

The fact that the affinity for both isomers of sialyl-lactose is higher than the affinity for the simple methyl sialoside suggests that differential binding of these ligands results from enhancing contacts rather than differential exclusion of one of the ligands. The relatively weak additional binding energies for these ligands reflects a net balance between the enthalpy gain from additional contacts and the entropy loss from immobilization of the sialic acid moiety relative to the galactose. These linkages are known to populate several different, shallow energy minima. The potentially greater flexibility of the α2,6 linkage would result in a higher entropy penalty for the bound form, which may at least in part explain the weaker binding of this ligand.

In summary, these studies provide strong evidence that the binding interaction between sialoadhesin and terminal sialic residues observed in crystals is closely similar to the interaction in solution and they provide confirmation of the importance of charge and hydrophobic interactions in the binding site. The data also provide direct evidence that strong cell–cell adhesion mediated by sialoadhesin results from an accumulation of weak interactions and that selectivity for cell-surface sialic acid in particular linkages is based on very modest differences in affinity for individual sialic acids. Finally, the results suggest that there may be subtle, but important, differences in the sialic acid-docking region of different members of the siglec family.

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REFERENCES

1 Drickamer, K. (1995) Curr. Opin. Struct. Biol. **5**, 612–616
2 Crocker, P., Clark, E. A., Filbin, M., Gordon, S., Jones, Y., Kehrl, J. H., Kelm, S., Le Douarin, N., Powell, L., Roder, J. et al. (1998) Glycobiology **8**, v
3 Crocker, P. R., Mucklow, S., Bouckson, V., McWilliam, A., Willis, A. C., Gordon, S., Milon, G., Kelm, S. and Bradfield, P. (1994) EMBO J. **13**, 4490–4503
4 Powell, L. D., Sgroi, D., Sjoberg, E. R., Stamenkovic, I. and Varki, A. (1993) J. Biol. Chem. **268**, 7019–7027
5 Freeman, S. D., Kelm, S., Barber, E. K. and Crocker, P. R. (1995) Blood **85**, 2005–2012
6 Kelm, S., Pelz, A., Schauer, R., Filbin, M. T., Tang, S., de Bellard, M.-E., Schnaar, R. L., Mahoney, J. A., Hartnell, A., Bradfield, P. and Crocker, P. R. (1994) Curr. Biol. **4**, 965–972
7 Cornish, A. L., Freeman, S., Forbes, G., Ni, J., Zhang, M., Cepeda, M., Gentz, R., Augustus, M., Carter, K. C. and Crocker, P. R. (1998) Blood **92**, 2123–2132
8 O'Keefe, T. L., Williams, G. T., Davies, S. L. and Neuberger, M. S. (1996) Science **274**, 798–801
9 Crocker, P., Freeman, S., Gordon, S. and Kelm, S. (1995) J. Clin. Invest. **95**, 635–643
10 Fruttiger, M., Montag, D., Schachner, M. and Martini, R. (1995) Eur. J. Neurosci. **7**, 511–515
11 Klein, A., Krishna, M., Varki, N. M. and Varki, A. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 7782–7786
12 Kelm, S., Schauer, R., Manuguerra, J. C., Gross, H. J. and Crocker, P. R. (1994) Glycoconjugate J. **11**, 576–585
13 Crocker, P. R., Kelm, S., Hartnell, A., Freeman, S., Nath, D., Vinson, M. and Mucklow (1996) Biochem. Soc. Trans. **24**, 150–156
14 Nath, D., van der Merwe, P. A., Kelm, S., Bradfield, P. and Crocker, P. R. (1995) J. Biol. Chem. **270**, 26184–26191
15 Vinson, M., van der Merwe, P. A., Kelm, S., May, A., Jones, E. Y. and Crocker, P. R. (1996) J. Biol. Chem. **271**, 9267–9272
16 May, A. P., Robinson, R. C., Vinson, M., Crocker, P. R. and Jones, E. Y. (1998) Mol. Cell **1**, 719–728
17 Sauter, N. K., Bednarski, M. D., Wurzburg, B. A., Hanson, J. E., Whitesides, G. M., Skehel, J. J. and Wiley, D. C. (1989) Biochemistry **28**, 8388–8396
18 Kronis, K. A. and Carver, J. P. (1982) Biochemistry **21**, 3050–3057
19 May, A. P., Robinson, R. C., Aplin, R. T., Bradfield, P., Crocker, P. R. and Jones, E. Y. (1997) Protein Sci. **6**, 717–721
20 Bebbington, C. (1991) Methods **2**, 136–145
21 Reuter, G. and Schaurer, R. (1994) Methods Enzymol. **230**, 168–199
22 Kelm, S., Brossmer, R., Isecke, R., Gross, H.-J., Strenge, K. and Schauer, R. (1998) Eur. J. Biochem. **255**, 663–672
23 Strenge, K., Schauer, R., Bovin, N., Hasegawa, A., Ishida, H., Kiso, M. and Kelm, S. (1998) Eur. J. Biochem. **258**, 677–685
24 Perkins, S. J. and Dwek, R. A. (1980) Biochemistry **19**, 245–258
25 Kraulis, P. J. (1991) J. Appl. Crystallogr. **24**, 946–950
26 Weis, W. I. and Drickamer, K. (1996) Annu. Rev. Biochem. **65**, 441–473

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