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Epitope mapping of sialyl Lewis^x bound to E-selectin using saturation transfer difference NMR experiments

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A complex between sialyl Lewis^x (α-D-Neu5Ac-[2 → 3]-β-D-Gal-[1 → 4]-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc-O-[CH_2]$_8$ COOMe) and E-selectin was studied using saturation transfer difference (STD) nuclear magnetic resonance (NMR) experiments. These experiments allow the identification of the binding epitope of a ligand at atomic resolution. A semi-quantitative analysis of STD total correlation spectroscopy spectra provides clear evidence that the galactose residue receives the largest saturation transfer. The protons H4 and H6 of the galactose residue are in especially close contact to the amino acids of the E-selectin binding pocket. The fucose residue also receives a significant saturation transfer. The GlcNAc and Neu5Ac residues, with the exception of H3 and H3′ of Neu5Ac, were found to interact weakly with the protein surface. These findings are in excellent agreement with a recently published X-ray structure and with the earlier findings from syntheses and activity assays. To further characterize the binding pocket of E-selectin, an inhibitory peptide, Ac-TWDQLWDLMK-$CONH_2$, was synthesized and the binding to E-selectin studied utilizing transfer nuclear Overhauser effect spectroscopy (trNOESY) experiments. Finally, competitive trNOESY experiments were performed, showing that the synthetic peptide is a competitive inhibitor of sialyl Lewis^x.

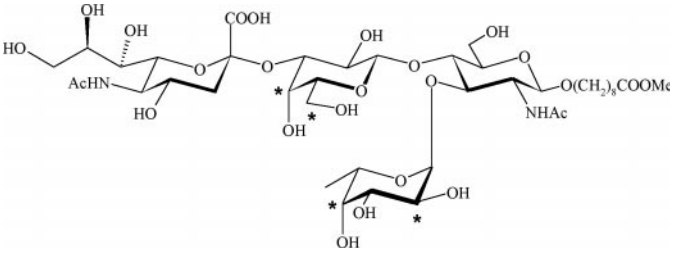
Key words: epitope mapping/E-selectin/sialyl Lewis^x/STD NMR/transfer NOESY

Introduction

E-selectin is a glycoprotein that selectively recognizes the sialyl Lewis^x epitope (see Scheme 1) and belongs to a family of cell adhesion molecules that mediate initial binding of leucocytes to endothelial cells (Phillips *et al.*, 1990; Walz *et al.*, 1990). This binding process plays a key role in a variety of pathological states (Tedder *et al.*, 1995; McEver, 1997) and therefore has been the target for numerous studies (Varki, 1992; Vestweber, 1996). The binding activity of E-selectin is due to the *N*-terminal carbohydrate recognition domain (CRD) that displays a significant homology with CRD domains in other calcium-dependent animal lectins (Drickamer, 1994; Bouyain *et al.*, 2001). The three-dimensional structure of the CRD and the adjacent epidermal growth factor-like domain had been determined by X-ray crystallography (Graves *et al.*, 1994).

Nuclear magnetic resonance (NMR) studies utilizing transfer nuclear Overhauser effect (NOE) techniques complemented the crystallographic studies and identified the conformation of sialyl Lewis^x bound to E-selectin (Scheffler *et al.*, 1995; Poppe *et al.*, 1997; Harris *et al.*, 1999). These studies were extended recently to compare the binding of sialyl Lewis^x and fragments thereof to E-selectin and fucose-recognizing lectins (Haselhorst *et al.*, 2001). Models for the sialyl Lewis^x/E-selectin complex have been constructed based on other C-type lectins for example the mannose-binding protein (MBP) (Weis, 1994). In one study with a mutant of MBP that recognizes fucose instead of mannose, it was found that the 2-OH and 3-OH groups of the fucose residue formed coordinative bonds to Ca^{2+} (Ng and Weis, 1997). Finally, a recent crystallographic study succeeded in delivering 3D data for the E-selectin/sialyl Lewis^x complex (Somers *et al.*, 2000). The results are in good agreement with the earlier NMR studies (Table I). The conformation of the Lewis^x core is almost identical to the conformation that had been derived originally from transfer (tr) NOE data (Scheffler *et al.*, 1995). Compared to the MBP mutant protein, significant differences were observed for the orientation and conformation of sialyl Lewis^x in the binding pocket. For instance, the new crystallographic data unambiguously show that the 3-OH and the 4-OH instead of the 2-OH and 3-OH of the fucose residue complex the Ca^{2+} in E-selectin.

Here, experimental data are presented that complement the structural data collected to date. Saturation transfer



Scheme 1. Sialyl Lewis^x. Asterisks designate positions of protons that are in close contact with protons of E-selectin.

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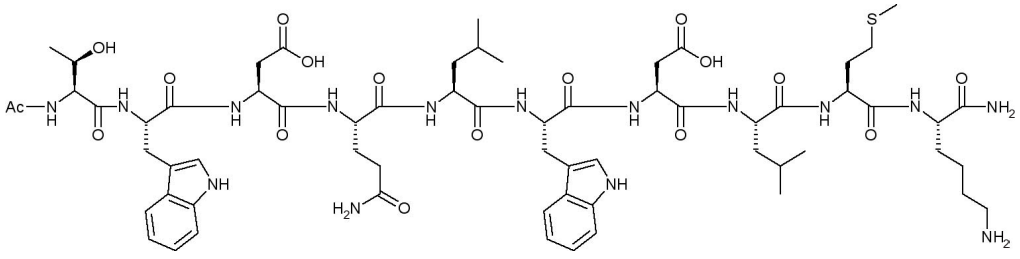
Table I. Torsion angles at the glycosidic linkages of sLe^x bound to E-selectin from NMR and X-ray data

Reference	ϕ/ψ Neu5Ac(2 → 3)Gal	ϕ/ψ Gal(1 → 4)GlcNAc	ϕ/ψ Fuc(1 → 3)GlcNAc
NMR (Scheffler *et al.*, 1995)	−76°/+6°	+39°/+12°	+38°/+26°
NMR (Poppe *et al.*, 1997)	−58°/−22°	+24°/+34°	+71°/+14°
NMR (Harris *et al.*, 1999)	−43°/−12°	+45°/+19°	+29°/+41°
X-ray (Somers *et al.*, 2000)	−65°/−12°	+34°/+16°	+41°/+22°

At the Neu5Ac(2 → 3)Gal linkage ϕ and ψ are defined as follows: ϕ, $C1^N$–$C2^N$–$O2^N$–$C3^G$; ψ, $C2^N$–$O2^N$–$C3^G$–$H3^G$. For the other two linkages the definitions of ϕ and ψ are: ϕ, H1–C1–O1–Cx; ψ, C1–O1–Cx–Hx; with x the glycosidic linkage site.



Scheme 2. Ac-TWDQLWDLMK-$CONH_2$.

difference (STD) NMR has been established as an important technique to identify binding activities in mixtures and to characterize binding epitopes of ligands with atomic resolution (Klein *et al.*, 1999; Biet and Peters, 2001; Mayer and Meyer, 2001). Utilizing STD NMR techniques, a model for the binding epitope of sialyl $Lewis^x$ bound to E-selectin was derived under physiological conditions and compared to the crystallographic results. Preliminary results on mapping the binding epitope of sialyl $Lewis^x$ had been previously published (Poppe *et al.*, 1997). Here, we show that with the use of 2D STD–total correlation spectroscopy (TOCSY) experiments it is possible to overcome restrictions that result from severe signal overlap in 1D STD spectra. Finally, a comparison of sialyl $Lewis^x$ to an inhibitory peptide (see Scheme 2) complements our understanding of the respective binding reactions.

Results and discussion

STD NMR experiments of sialyl $Lewis^x$ bound to E-selectin

The STD NMR protocol is based on the transfer of saturation from the protein to bound ligands, which in turn by exchange is moved into solution where it is detected (Mayer and Meyer, 1999). Subtracting a spectrum, where the protein is saturated from one without protein saturation produces a spectrum where only signals of the ligand(s) remain in the difference spectrum. The irradiation frequency is set to a value where only protein resonances and no ligand resonances are located. Therefore, in the on-resonance experiment selective saturation of the protein is achieved. For the on-resonance irradiation frequency values around −1 ppm are practical because no ligand resonances are found in this spectral region, whereas the significant line width of protein signals still allows selective saturation. If the ligands show no resonances in the aromatic spectral region the saturation frequency may also be placed here or even further downfield. To achieve the desired selectivity and to avoid side band irradiation, shaped pulses are employed for the saturation of the protein signals. One of the major advantages of this technique is that it may be combined with any NMR pulse sequence generating a whole suite of STD NMR experiments, such as STD TOCSY or STD heteronuclear single quantum coherence (Mayer and Meyer, 1999; Vogtherr and Peters, 2000).

The protocol for STD NMR experiments is as follows. Ligands are added to a solution of the receptor protein, in this case E-selectin, and one ^1H NMR experiment is performed where the protein is selectively irradiated at a frequency at least 700 Hz away from the closest ligand signal (on-resonance experiment). For a carbohydrate ligand, such as sialyl $Lewis^x$, the choice is not difficult because of the limited range of chemical shift values, and a value of 7.5 ppm was used. Even though the irradiation is highly selective and has usually only a band width of a few Hz, irradiation at such frequencies still yields full saturation of the protein via efficient spin diffusion within about 50–200 ms.

A ligand that binds to the protein, for instance, sialyl $Lewis^x$ binding to E-selectin, will also be saturated. The degree of ligand saturation obviously depends on the residence time of the ligand in the protein binding pocket. The dissociation of the ligand will then transfer this saturation into solution, where the free ligand has again narrow line

widths. For those ligand protons that interact with protein protons through an intermolecular NOE a decrease in intensity is observed. However, in the presence of other molecules, such as impurities and other nonbinding components, it is not usually possible to identify such attenuated signals. Therefore, in a second experiment the irradiation frequency is set at a value that is far from any signal, ligand or protein. Here, a value of 40 ppm was used (off-resonance spectrum). Subtraction of the on-resonance from the off-resonance spectrum leads to a difference spectrum in which only signals of protons are visible that were attenuated via saturation transfer.

This protocol enables binding and nonbinding ligands to be distinguished and the binding epitope to be characterized as the extent of saturation transfer to the ligand strongly depends on its proximity to the protein surface. Therefore, the binding epitope of a ligand, in this case sialyl Lewis^x, can be determined at atomic resolution.

Some characteristic features of the sialyl Lewis^x binding epitope are revealed by the inspection of 1D STD NMR spectra. An inspection of the STD spectra demonstrates that it is possible to discriminate between the two *N*-acetyl groups attached to the *N*-acetyl glucosamine and to the neuramic acid. The *N*-acetyl group of neuramic acid is clearly receiving less saturation transfer, suggesting that this part of the neuramic acid residue only weakly interacts with the protein surface (Figure 1). From the crystallographic studies it is seen that both *N*-acetyl groups only should have few contacts with the protein. It is concluded that the GlcNAc residue may have a slightly different orientation in the binding E-selectin pocket in solution. The STD spectra also reveal that the fucose C6 methyl group is not in very close proximity to the amino acids of the binding pocket of E-selectin.

To obtain information also on ligand signals that are heavily overlapped with other signals, STD TOCSY experiments were performed and the cross-peaks integrated. The resulting intensities were compared to a normal TOCSY spectrum acquired under the same conditions. The corresponding spectra are shown in Figure 2. The analysis of STD TOCSY spectra differs from the analysis of 1D STD spectra because the changes in the intensity of the cross-peaks reflect the saturation transfer to all protons participating in the magnetization transfer that the cross-peak identifies. For instance, a cross-peak between $H1^G$ and $H4^G$ reflects the saturation transfer to all protons involved in the TOCSY transfer $H1^G$–$H2^G$–$H3^G$–$H4^G$. Therefore, the analysis is more complex but potentially more powerful than the analysis of 1D STD spectra.

A simple epitope mapping on the basis of STD TOCSY spectra is easily achieved for isolated spin systems, such as the ones found for pyranose rings in oligosaccharides. Spin diffusion across glycosidic linkages can be neglected, and therefore a qualitative comparison of the corresponding cross-peaks readily identifies pyranose units in intimate contact with the protein binding pocket. The STD TOCSY and TOCSY spectra are shown in Figure 2. From these spectra it is immediately obvious that the spacer attached to the reducing end of the sialyl Lewis^x tetrasaccharide has no contacts to the protein surface because all corresponding cross-peaks have vanished (compare Figure 2A and B). It is

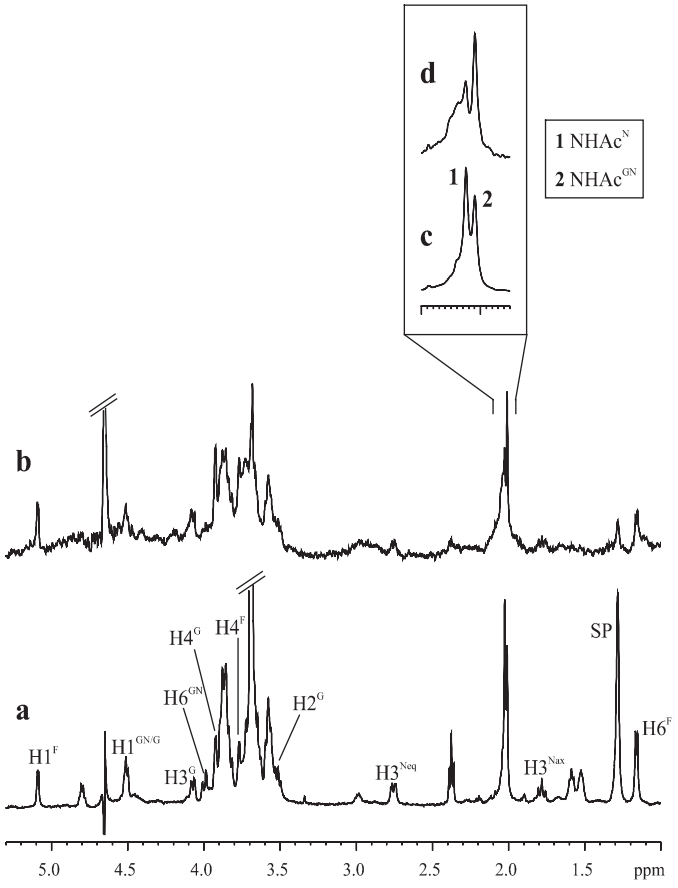


Fig. 1. 1D NMR spectra of the complex E-selectin/sialyl Lewis^x (molar ratio 1:15) at 310 K and 500 MHz. (**a**) Normal 1D spectrum and (**b**) STD spectrum recorded with on resonance irradiation at 7.2 ppm and a saturation time of 2 s. **c** and **d** show expansions of the region containing the *N*-acetyl resonance for the normal and STD spectra, respectively.

also obvious that mainly the cross-peaks of the fucose and the galactose pyranose rings display the largest overall intensities, indicating that the protons of these two pyranose rings are in close contact to protons of the E-selectin binding pocket (compare Figure 2C and D).

For a more detailed analysis of saturation transfer within a particular pyranose ring, the comparison of different TOCSY transfers leads to a relative ranking of the amount of saturation that is transferred to the individual spins. As the cross-peaks from the Fuc, Gal, and GlcNAc anomeric protons, the C6 methyl group protons of the Fuc residue, and the protons of C3 of the Neu5Ac, are well separated from other peaks; they are simple to integrate. This analysis leads to the histogram in Figure 3. A comparison of the intensities of the cross-peaks $H1^G$–$H2^G$, $H1^G$–$H3^G$, and $H1^G$–$H4^G$ shows that $H4^G$ receives the largest fraction of saturation transfer, because the cross-peaks $H1^G$–$H4^G$ have the greatest intensity. The cross-peaks $H1^G$–$H2^G$ and $H1^G$–$H3^G$ reflect parts of the complete TOCSY transfer from $H1^G$ to $H4^G$. Therefore, the greater intensity of $H1^G$–$H4^G$ can only be due to a larger amount of saturation transfer to $H4^G$. This is in accordance with the large intensity of the $H3^G$–$H4^G$ cross-peak, which for the same reason must be

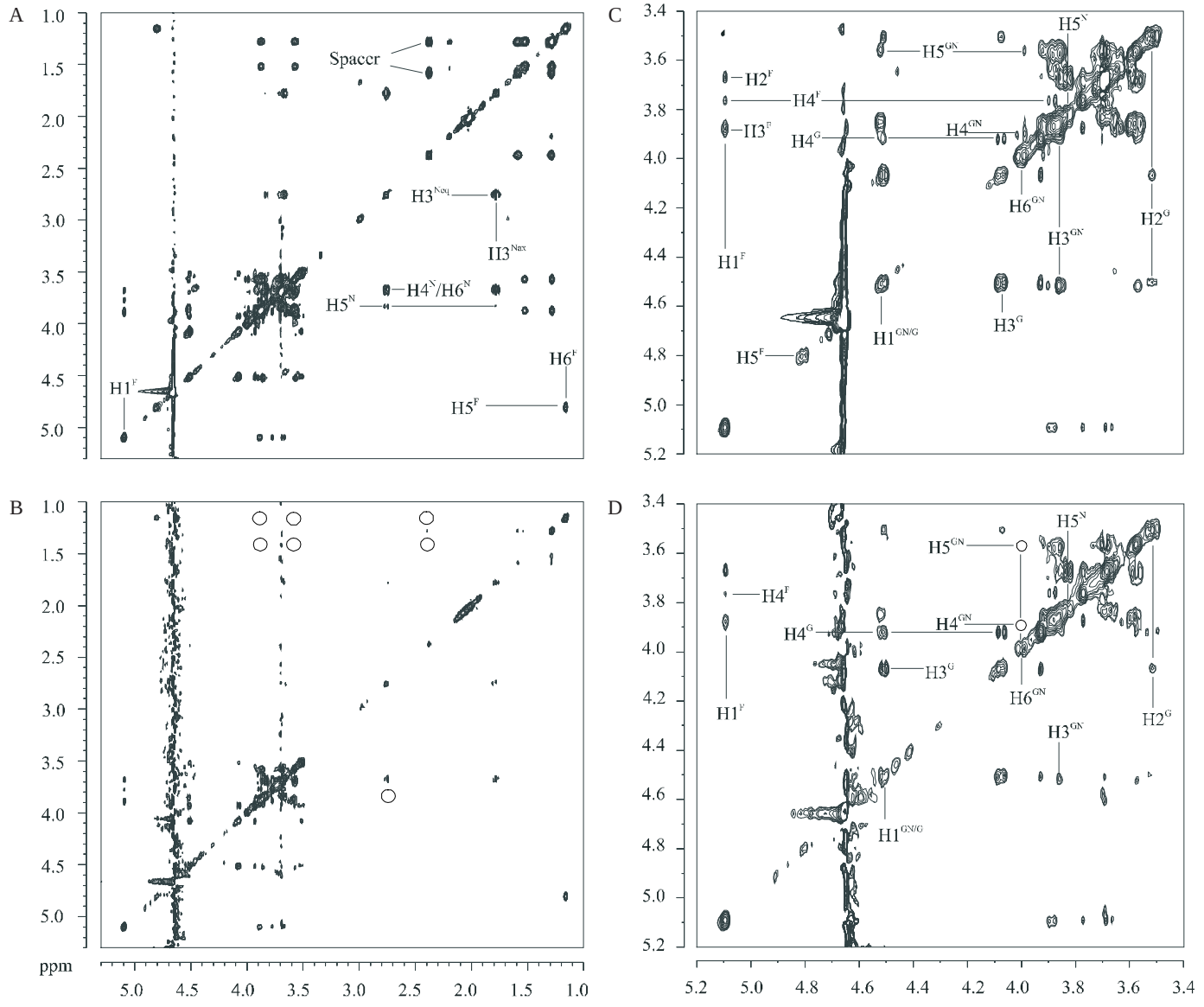


Fig. 2. Comparison of the 2D TOCSY spectrum (**A**) and the STD TOCSY spectrum (**B**) of sialyl Lewis^x in the presence of E-selectin at 310 K. The STD experiment was recorded with on resonance irradiation at 7.2 ppm and 2 s saturation time. Signals that receive no saturation transfer disappear (○). Spectra **C** and **D** show the ring proton regions of the spectra in **A** and **B**, respectively.

due to the large amount of saturation transfer to H4^G. Following a similar line of reasoning, the larger intensity of the cross-peak for H6^G–H5^G (the two protons H6^G have almost identical chemical shifts and are therefore a system of higher order) (Scheffler *et al.*, 1995), compared to the cross-peak H4^G–H6^G, is attributed to a difference in saturation transfer to H4^G. Saturation transfer to H5^G or H6^G has to be more efficient. Whether it is the saturation transfer to H5^G or to H6^G cannot be deduced from the current data, but a comparison to the crystal structure strongly suggests that it is H6^G and not H5^G (see later discussion).

It can be also seen that the fucose residue receives a considerable saturation transfer, whereas almost no saturation transfer is observed to the GlcNAc residue or to the Neu5Ac residue, with the exception of the protons attached to the C3 of Neu5Ac (Figure 1). For the fucose it is observed that the intensities of the TOCSY cross-peaks H1^F–H2^F and H3^F–H4^F experience the largest change on saturation transfer. Therefore, these protons, or at least one of each pair, are in rather close contact to the protein binding site.

These results were compared to the recently published X-ray data (Somers *et al.*, 2000).

- The X-ray data show that the H4 proton of the Gal residue is in van der Waals contacts to side chain protons of Y94 and Y48, and the H6 proton of Gal makes van der Waals contacts with Y94, E92, and N105. These structural details are in accordance with the STD NMR data that identify these protons as being in intimate contact with the E-selectin binding pocket.
- The data from Figure 3 suggest that the H2 and H4 protons of fucose should also interact with the side

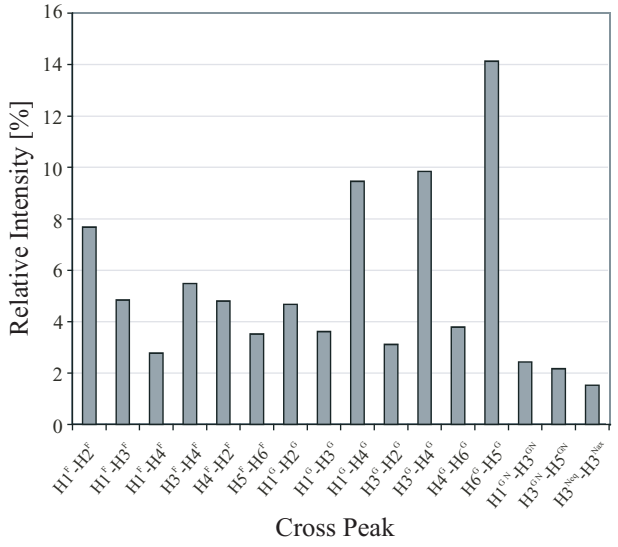


Fig. 3. Relative intensities of STD TOCSY cross-peaks. The intensity is normalized to the intensity of the corresponding cross-peak in the reference TOCSY spectrum. It is obvious that galactose protons receive the largest amount of saturation transfer.

chains of amino acids in the binding pocket. This is confirmed by the X-ray data, which show that the H2 and H4 protons are close to the side chains of N82 and N105. The distances between the other protons of the fucose and the protein are larger. In particular, the C6 methyl group is pointing away from the surface of the binding pocket in accordance with the small observed STD effect (compare Figures 1 and 3).

- The interpretation of the signals of the neuramic acid is complicated by severe overlap even in the STD TOCSY spectra. The only exception is observed for the protons of the C3 carbon and the *N*-acetyl moiety; both show small STD effects. The remaining seven protons of the neuramic acid residue contribute to the broad STD signal that is visible in Figure 1 between 3.4 and 3.9 ppm. The protons of the other monosaccharide residues also contribute to this broad signal. It is observed, however, that the cross-peaks from the protons attached to C3 of Neu5Ac to protons H4 and H5 have a significantly reduced intensity in the STD TOCSY (Figure 4). From the spectrum it is also obvious that the overall STD response for GlcNAc and for Neu5Ac is significantly smaller than for the Gal and Fuc residues. This is also reflected in the graph in Figure 3 for those signals that were integrated.
- There is no straightforward explanation for the observed differences between the STD effects for the *N*-acetyl groups of the Neu5Ac and the GlcNAc residues. Both groups in the crystal structure display relatively large distances to the protein surfaces; a possible explanation for the difference observed in the STD spectra is that amino acid side chain mobility in solution may lead the ligand to adopt at a slightly different binding epitope in solution.

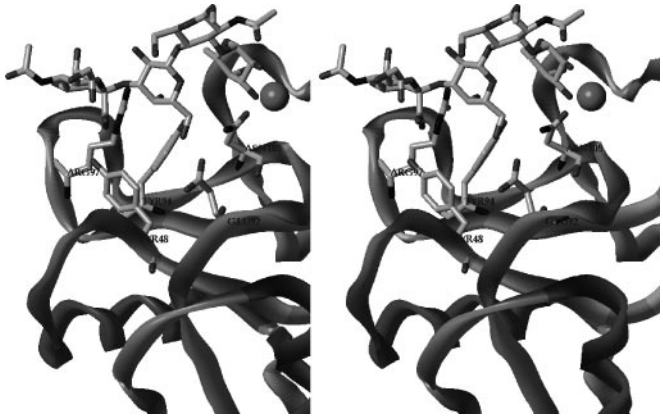


Fig. 4. Part of the crystal structure of the sialyl Lewis^x/E-selectin complex, showing the binding pocket with the carbohydrate ligand. Amino acids involved in contacts with the ligand are labeled. Relaxed eye stereo view.

In general, our experimental data are in very good agreement with the X-ray data. A stereo picture of the carbohydrate ligand in the binding pocket displaying the amino acids that have been mentioned can be seen in Figure 4. It should also be stated that the conformation of sialyl Lewis^x cocrystallized with E-selectin is very similar to the bound conformation that we had derived from the analysis of trNOE experiments (Scheffler *et al.*, 1995) (see the legend to Figure 4).

NMR experiments to study the binding of an inhibitory peptide and sialyl Lewis^x to E-selectin

In the past, peptide libraries had been used for the identification of novel E-selectin inhibitors. Following this strategy a dodecapeptide DITWDQLWDLMK with an IC_{50} in the low nanomolar range was identified (Martens *et al.*, 1995). In contrast to sialyl Lewis^x, it has been reported that the binding of the peptide was independent of calcium and that the binding is noncompetitive with that of sialyl Lewis^x. Therefore, it was concluded that the peptide bound to E-selectin in a different fashion than sialyl Lewis^x. In the same study, the truncated decapeptide TWDQLWDLMK was also found to inhibit E-selectin with an IC_{50} of 2.5 μM. The binding affinity of this peptide (as estimated from the IC_{50}) makes it an ideal target for trNOE and transfer rotating frame Overhauser effect studies. For the NMR studies this peptide was synthesized as Ac-TWDQLWDLMK-$CONH_2$.

The assignment of ^{1}H NMR signals of Ac-TWDQLWDLMK-$CONH_2$ was straightforward, utilizing correlation spectroscopy (COSY), TOCSY, and NOE spectroscopy (NOESY) experiments (Table II). An analysis of the conformation of the peptide bound to E-selectin was performed at 310 K, as at this temperature no NOEs for the free peptide were observed. Consequently no contributions by the free ligand molecules to the trNOE are expected. A portion of the trNOESY spectrum is shown in Figure 5.

Usually, long-range NH-NH NOEs yield information about the secondary structure of a protein (Wüthrich, 1989). For the peptide Ac-TWDQLWDLMK-$CONH_2$ no such NH-NH trNOEs are observed, and therefore no

Table II. ^{1}H NMR chemical shifts (ppm) for the decapeptide TWDQLWDLMK at 310 K and 500 MHz in deuterated acetic acid/acetat-H_2O-buffer (pH 5.5)

Amino acid	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	Other protons
Thr-1	7.90	4.24	4.14	1.10		
Trp-2	8.15	4.58	3.28/3.22		7.21	7.59 (C4H); 7.12 (C5H); 7.20 (C6H); 7.44 (C7H)
Asp-3	7.96	4.39	2.43			
Gln-4	7.90	4.11	1.90	2.25		
Leu-5	7.98	4.17	1.54	1.41	0.83/0.78	
Trp-6	7.85	4.51	3.25/3.13		7.18	7.56 (C4H); 7.12 (C5H); 7.21 (C6H); 7.45 (C7H)
Asp-7	7.95	4.44	2.57			
Leu-8	7.81	4.15	1.64	1.64	0.90/0.84	
Met-9	8.05	4.35	2.05	2.61/2.51		
Lys-10	7.83	4.08	1.72	1.34	1.58	2.93 ($C^{\varepsilon}H$)

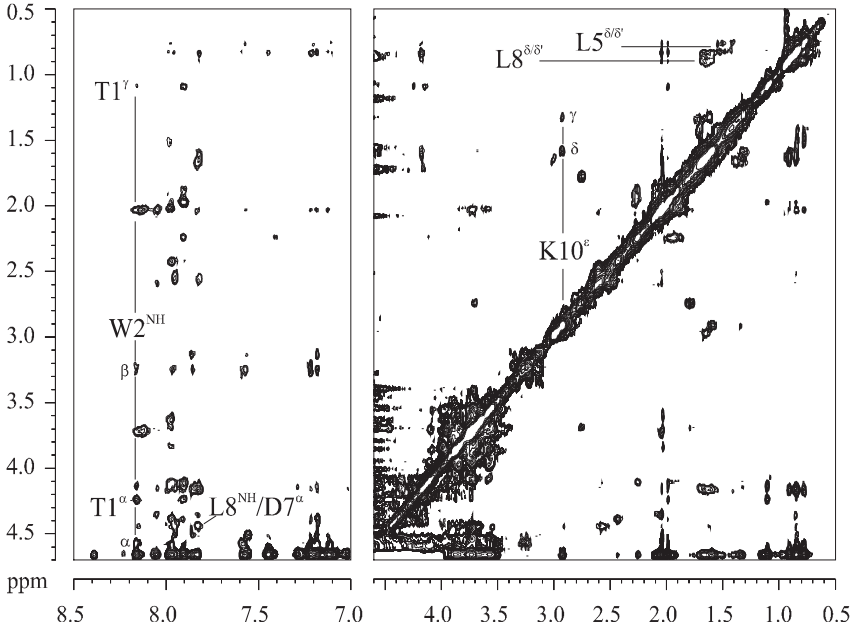


Fig. 5. Transfer NOESY spectrum of the peptide Ac-TWDQLWDLMK-$CONH_2$ complexed with E-selectin at 500 MHz and 310 K. The mixing time was 150 ms.

attempt was made to assign the bound conformation. Presumably, the peptide is bound to the E-selectin in a stretched conformation. A trNOESY spectrum of the peptide in the presence of E-selectin is shown in Figure 5.

The NMR sample was then titrated with a solution of sialyl Lewis^x. Molar ratios ranging from 1:27.5:5 to 1:27.5:20 (E-selectin:peptide:sialyl Lewis^x) resulted. Along the titration a gradual decrease in the size of the peptide trNOEs was observed. A trNOESY spectrum obtained at the final ratio of 1:27.5:20 is shown in Figure 6 and clearly indicates that the peptide ligand has been substituted by the carbohydrate ligand. The observed trNOE signals are almost exclusively those originating from sialyl Lewis^x.

In contrast to the previously published data, our results suggest that the peptide and sialyl Lewis^x bind to E-selectin in a competitive manner. This is in excellent agreement with our finding in the cell free ligand binding assay (Ramphal *et al.*, 1994), in which a competitive binding of inhibitory peptide and sialyl Lewis^x was observed. Because the binding of the inhibitory peptides had been shown to be independent of Ca^{2+}, it may be hypothesized that the peptide and sialyl Lewis^x share the same binding site in an overlapping manner.

Conclusion

The NMR experiments performed allow the definition of the binding epitope of sialyl Lewis^x bound to E-selectin at atomic resolution. This binding epitope comprises mainly

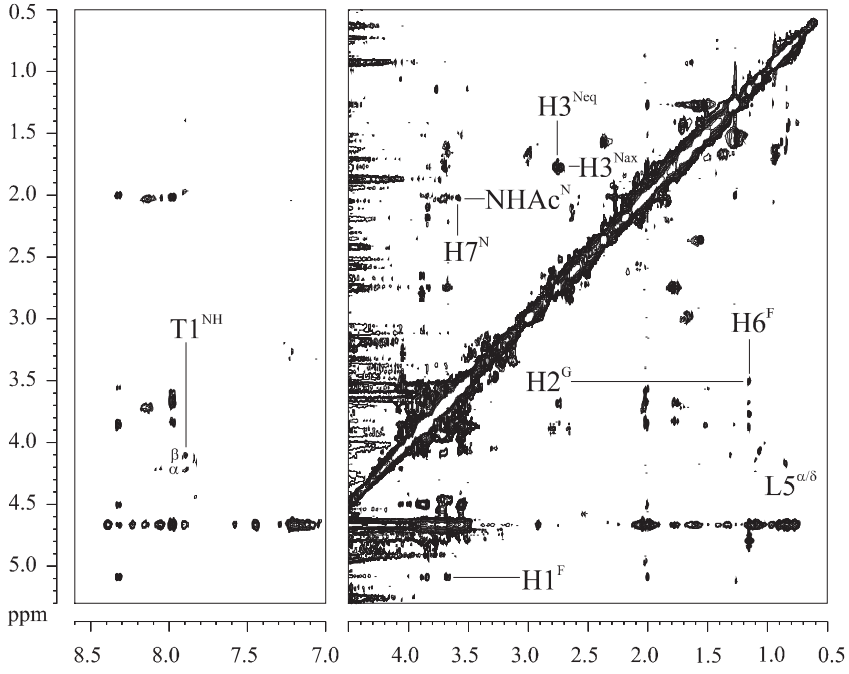


Fig. 6. Transfer NOESY spectrum of the peptide Ac-TWDQLWDLMK-$CONH_2$ and E-selectin in the presence of sialyl Lewis^x at 500 MHz and 310 K. The molar ratio of peptide to sialyl Lewis^x to E-selectin was 1:27.5:20. The mixing time was 150 ms.

the galactose and the fucose residues and is in very good agreement with crystallographic data for the complex (Somers *et al.*, 2000) and the SAR studies (Brandley *et al.*, 1993; Ramphal *et al.*, 1994; Stahl *et al.*, 1994; Bänteli and Ernst, 2001). In addition, the competitive trNOE experiments with a synthetic decapeptide clearly show that the two ligands bind to E-selectin in a competitive fashion. It is therefore likely that sialyl Lewis^x and the synthetic peptide share parts of the same binding pocket. This has implications for the design of new E-selectin inhibitors.

Materials and methods

Peptide synthesis

The synthetic peptide Ac-TWDQLWDLMK-$CONH_2$ was prepared by a standard solid phase peptide synthesizer from Perseptive Biosystems (Wiesbaden, Germany) using the Fmoc-strategy. The Nα-Fmoc-amino acids were purchased from Novabiochem (Heidelberg, Germany). The side chains of functionalized amino acids were protected by the use of ether or ester derivatives that are cleaved rapidly using normal cleavage procedures. Peptide (0.06 mmol) was synthesized on a Fmoc-protected 5-(4-Aminomethyl-3,5-dimethoxyphenoxy) valeric acid linker, Polysterol-(1%-divinylbenzene) copolymer resin (substitution level 0.38 mEq/g) in dimethylformamide (DMF) with a fourfold excess of the amino acids. The carboxyl group of each amino acid was activated with 0.5 M *O*-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate and 1 M *N*,*N*-Diisopropylamine (DIPEA) in DMF. After 30 min of coupling, reaction was deemed to be complete, and the protecting Fmoc-group was removed by 20% piperidine in DMF and the nonreactive amino groups capped using acetic acid anhydride. After the coupling of the last amino acid, the growing peptide on the resin was acylated using a mixture of the corresponding acid anhydride and DIPEA. Subsequently, the peptide was cleaved from the resin with concomitant removal of all side chain protecting groups by treatment with trifluoroacetic acid (TFA) at room temperature for 90 min in the presence of 2.5% H_2O and 2.5% Triisopropylsilane. TFA was evaporated in vacuum, and the residue was triturated with ether. The crude peptide was purified by reverse-phase high-performance liquid chromatography on a Eurospher C18 column (5 mm, 100 Å, 8×250 mm) with a gradient of increasing concentration of acetonitrile in water containing 0.1% TFA. Absorbance was monitored at 280 nm. The amino acid composition of the purified peptide was verified by matrix-assisted laser desorption ionization and time-of-flight and NMR analyses.

NMR sample preparation

Sialyl Lewis^x with a C8 spacer was synthesized according to literature. The E-selectin used throughout this study consists of a recombinant chimera of E-selectin and human IgG and has two carbohydrate-binding sites and a molecular weight of 220 kDa. The E-selectin was obtained as described previously. For the complex a 0.018 mM solution of E-selectin, that is, a 0.036 mM concentration of E-selectin binding sites, in 20 mM deuterated acetic acid/acetate buffer (pH 5.5) in D_2O with 1 mM $CaCl_2$ was prepared. The concentration of sialyl Lewis^x was 0.841 mM, corresponding to the molar ratio of E-selectin binding sites to sialyl Lewis^x of 1:15.

NMR experiments

All NMR spectra were recorded on a Bruker DRX 500 NMR spectrometer using XWINNMR software (Bruker,

Rheinstetten, Germany). Phase-sensitive NOESY experiments were performed using the TPPI methodology (Marion and Wüthrich, 1983) for quadrature detection and with the WATERGATE sequence for suppression the residual water signal (Piotto *et al.*, 1992). After zero filling in t1, 2 K × 1 K data matrices were obtained. For all 2D NOESY spectra, a π/2-shifted squared sine bell window function was applied in both dimensions prior to the Fourier transformation. STD spectra were recorded with on resonance irradiation at 7.2 ppm and off resonance irradiation at 40 ppm. A pulse train of Gaussian-shaped selective pulses each with a 1% truncation and comprising 1000 points and 50 ms length separated by a 1-ms delay were used to saturate the protein. The 1D STD spectra for the E-selectin/sialyl Lewis^x complex were performed at 310 K with saturation times of 0.2, 0.5, 0.7, 1.0, 1.5, 2.0, 2.5, and 3.0 s and a relaxation delay of 1.5 s. A 15-ms spinlock pulse with a strength of 3 kHz was applied to remove residual protein resonances. One hundred twenty-eight scans were recorded for each spectrum with 16 K data points. The 1D STD spectra were obtained by internal subtraction by phase cycling.

The TOCSY experiments were recorded at 310 K with a MLEV17-sequence and 75 ms spinlock time and with a relaxation delay of 1.5 s. A pulse train of 40 Gaussian bell-shaped selective pulses of 50 ms length separated by a 1-ms delay corresponding to a total saturation time of 2 s was used to saturate the protein. The experiment was performed with approximate 20 h measurement time. Sixteen scans were recorded, and 512 experiments were performed in t1 with 2 K data points in t2 that are stored separately for on resonance and off resonance in two data sets. For the two spectra, a π/2-shifted squared sine bell window function was applied in both dimensions prior to the Fourier transformation. After zero filling in t1, 2 K × 1 K data matrices were obtained. The two spectra were phased identically. The 2D STD TOCSY spectrum was obtained by subtraction of the spectrum recorded with on resonance from the spectrum recorded with off resonance.

The extent of saturation transfer in the STD TOCSY spectrum was determined by the comparison of the cross-peak volumes in the STD TOCSY and TOCSY spectra. The integration of these cross-peaks was performed using the Aurelia program (Bruker).

The TOCSY experiment for the free peptide was recorded at 310 K with a MLEV17-sequence and 75-ms spinlock time and a relaxation delay of 1.5 s. Thirty-two scans were recorded, and 512 experiments were performed in t1 with 2 K data points in t2. The experiment was performed with approximate 6 h measurement time. The gradient selected COSY experiment was measured at 310 K and a relaxation delay of 1.5 s. Twelve scans were recorded, and 512 experiments were performed in t1 with 2 K data points in t2. A squared sine bell window function was applied in both dimensions prior to the Fourier transformation. The experiment was performed with approximate 4 h measurement time.

NOESY spectra for the free peptide were recorded at 290 K with mixing times of 75, 150, 350, 500, 800, and 1200 ms and a relaxation delay of 1.5 s. Each experiment was performed with approximate 4 h measurement time. Sixteen scans were recorded for each, and 512 experiments were performed in t1 with 2 K data points in t2. The resulting NOEs were negative.

trNOESY spectra for the complex E-selectin/peptide were recorded at 310 K with mixing times of 75, 150, 250, 350, and 500 ms, and a relaxation delay of 1.5 s. The protein resonances were suppressed by a 12-ms spinlock filter with a field strength of 2.9 kHz. Each experiment was performed with approximate 4 h measurement time. Sixteen scans were recorded for each, and 512 experiments were performed in t1 with 2 K data points in t2. The resulting trNOEs were negative. The integration of the cross-peak volumes was performed with the Aurelia program.

trNOESY spectra for the complex E-selectin:peptide: sialyl Lewis^x were recorded at 310 K with 150-ms mixing times and a relaxation delay of 1.5 s. The protein resonances were suppressed by a 12-ms spinlock filter with a field strength of 2.9 kHz. Each experiment was performed with approximate 4 h measurement time. Sixteen scans were recorded for each, and 512 experiments were performed in t1 with 2 K data points in t2.

Abbreviations

CRD, carbohydrate recognition domain; DMF, dimethylformamide; MBP, mannose-binding protein; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; NMR, nuclear magnetic resonance; DIPEA, *N*,*N*-Diisopropylamine; STD, saturation transfer difference; TFA, trifluoracetic acid; TOCSY, total correlation spectroscopy.

References

Bänteli, R. and Ernst, B. (2001) Synthesis of sialyl Lewis^x mimics. Modifications of the 6-position of galactose. *Bioorg. Med. Chem. Lett.*, **11**, 459–462.

Biet, T. and Peters, T. (2001) Molecular recognition of UDP-Gal by β-1,4-galactosyltransferase T1. *Angew. Chem. Int. Ed. Engl.*, **40**, 4189–4192.

Bouyain, S., Rushton, S., and Drickamer, K. (2001) Minimal requirements for the binding of selectin ligands to a C-type carbohydrate-recognition domain. *Glycobiology*, **11**, 989–996.

Brandley, B.K., Kiso, M., Abbas, S., Nikrad, P., Srivasatava, O., Foxall, C., Oda, Y., and Hasegawa, A. (1993) Structure-function studies on selectin carbohydrate ligands. Modifications to fucose, sialic acid and sulphate as a sialic acid replacement. *Glycobiology*, **3**, 633–641.

Drickamer, K. (1994) Three-dimensional view of a selectin cell adhesion molecule. *Glycobiology*, **4**, 245–248.

Graves, B.J., Crowther, R.L., Chandran, C., Rumberger, J.M., Li, S., Huang, K.S., Presky, D.H., Familletti, P.C., Wolitzky, B.A., and Burns, D.K. (1994) Insight into E-selectin/ligand interaction from the crystal structure and mutagenesis of the lec/EGF domains. *Nature*, **367**, 532–538.

Harris, R., Kiddle, G.R., Field, R.A., Milton, M.J., Ernst, B., Magnani, J.L., and Homans, S.W. (1999) Stable-isotope-assisted NMR studies on ^{13}C-enriched sialyl Lewis^X in solution and bound to E-selectin. *J. Am. Chem. Soc.*, **121**, 2546–2551.

Haselhorst, T., Weimar, T., and Peters, T. (2001) Molecular recognition of sialyl Lewis^X and related saccharides by two lectins. *J. Am. Chem. Soc.*, **123**, 10705–10714.

Klein, J., Meinecke, R., Mayer, M., and Meyer, B. (1999) Detecting binding affinity to immobilized receptor proteins in compound libraries by HR-MAS STD NMR. *J. Am. Chem. Soc.*, **121**, 5336–5337.

Marion, D. and Wüthrich, K. (1983) Application of phase sensitive two-dimensional correlated spectroscopy for measurement of ^{1}H–^{1}H-coupling constants in proteins. *Biochem. Biophys. Res. Commun.*, **113**, 967–974.

Martens, C.L., Cwirla, S.E., Lee, R.Y., Whitehorn, E., Chen, E.Y., Bakker, A., Martin, E.L., Wagstrom, C., Gopalan, P., and Smith, C.W. (1995) Peptides which bind to E-selectin and block neutrophil adhesion. *J. Biol. Chem.*, **270**, 21129–21136.

Mayer, M. and Meyer, B. (1999) Characterization of ligand binding by saturation transfer difference spectroscopy. *Angew. Chem. Int. Ed. Engl.*, **38**, 1784–1788.

Mayer, M. and Meyer, B. (2001) Group epitope mapping by saturation transfer difference NMR to identify segments of a ligand in direct contact with a protein receptor. *J. Am. Chem. Soc.*, **123**, 6108–6117.

McEver, R.P. (1997) Selectin-carbohydrate interactions during inflammation and metastasis. *Glycoconj. J.*, **14**, 585–591.

Ng, K.K. and Weis, W.I. (1997) Structure of a selectin-like mutant of mannose-binding protein complexed with sialylated and sulfated Lewis^x oligosaccharides. *Biochemistry*, **36**, 979–988.

Phillips, M.L., Nudelman, E., Gaeta, F.C., Perez, M., Singhal, A.K., Hakomori, S., and Paulson, J.C. (1990) ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand, sialyl-Le^x. *Science*, **250**, 1130–1132.

Piotto, M., Saudek, V., and Sklenar, V. (1992) Gradient tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *J. Biomol. NMR*, **2**, 661–665.

Poppe, L., Brown, G.S., Philo, J.S., Nikrad, P.V., and Shah, B.H. (1997) Conformation of sLe^x tetrasaccaride, free and bound to E-, P-, and L-selectin. *J. Am. Chem. Soc.*, **119**, 1727–1736.

Ramphal, J.Y., Zheng, Z.L., Perez, C., Walker, L.E., DeFrees, S.A., and Gaeta, F.C. (1994) Structure–activity relationships of sialyl Lewis x-containing oligosaccharides. 1. Effect of modifications of the fucose moiety. *J. Med. Chem.*, **37**, 3459–3463.

Scheffler, K., Ernst, B., Katopodis, A., Magnani, J.L., Wang, W.T., Weisemann, R., and Peters, T. (1995) Determination of the bioactive conformation of the carbohydrate ligand in the E-selectin/sialyl Lewis^x complex. *Angew. Chem. Int. Ed. Engl.*, **34**, 1841–1844.

Somers, W.S., Tang, J., Shaw, G.D., and Camphausen, R.T. (2000) Insights into the molecular basis of leukocyte tethering and rolling revealed by structures of P- and E-selectin bound to SLe^X and PSGL-1. *Cell*, **103**, 467–479.

Stahl, W., Sprengard, U., Kretschmar, G., and Kunz, H. (1994) Synthesis of an RGD-sialyl-Lewis^x glycoconjugate: a new highly active ligand for P-selectin. *Angew. Chem. Int. Ed. Engl.*, **33**, 2096–2098.

Tedder, T.F., Steeber, D.A., Chen, A., and Engel, P. (1995) The selectins: vascular adhesion molecules. *FASEB J.*, **9**, 866–873.

Varki, A. (1992) Selectins and other mammalian sialic acid-binding lectins. *Curr. Opin. Cell Biol.*, **4**, 257–266.

Vestweber, D. (1996) Ligand-specificity of the selectins. *J. Cell. Biochem.*, **61**, 585–591.

Vogtherr, M. and Peters, T. (2000) Application of NMR based binding assays to identify key hydroxy groups for intermolecular recognition. *J. Am. Chem. Soc.*, **122**, 6093–6099.

Walz, G., Aruffo, A., Kolanus, W., Bevilacqua, M., and Seed, B. (1990) Recognition by ELAM-1 of the sialyl-Le^X determinant on myeolid and tumor cells. *Science*, **250**, 1132–1135.

Weis, W.I. (1994) Lectins on a roll: the structure of E-selectin. *Structure*, **2**, 147–150.

Wüthrich, K. (1989) Determination of three-dimensional protein structures in solution by nuclear magnetic resonance. *Methods Enzymol.*, **177**, 125–131.