The counterreceptor binding site of human CD2 exhibits an extended surface patch with multiple conformations fluctuating with millisecond to microsecond motions

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Abstract

We have used ¹⁵N NMR relaxation experiments to probe, for the glycosylated human CD2 adhesion domain, the overall molecular motion, as well as very fast nanosecond–picosecond (ns–ps) and slow millisecond–microsecond (ms–μs) internal motions. Using a novel analysis method that considers all residues, we obtained a correlation time for the overall motion of 9.5 ± 0.3 ns. Surprisingly, we found a large contiguous patch of residues in the counterreceptor (CD58) binding site of human CD2 exhibiting slow conformational exchange motions (ms–μs). On the other hand, almost none of the residues of the CD58 binding site display fast (ns–ps) internal motions of amplitudes larger than what is seen for well-ordered regions of the structure. Residues close to the N-glycosylation site, and the first N-acetylglucosamine of the high mannose glycan are as rigid as the protein core. Residues conserved in the immunoglobulin superfamily V-set domain are generally very rigid.

Keywords: glycosylated human CD2 adhesion domain; immunoglobulin superfamily V-set domain; ¹⁵N NMR relaxation; reduced spectral density mapping

CD2 is a transmembrane glycoprotein receptor expressed on the cell surface of T lymphocytes and natural killer cells and is important in mediating both cellular adhesion and signal transduction (reviewed in Moingeon et al., 1989a; Hahn et al., 1993). CD2 mediates adhesion by binding to its counterreceptor CD58 in humans (Selvaraj et al., 1987). CD2-mediated adhesion facilitates T cell recognition of antigens by the T cell receptor (Moingeon et al., 1989b; Koyasu et al., 1990). In addition, CD2 can transduce acti-

vation signals that synergize with those mediated by the TCR (Meuer et al., 1984; Bierer et al., 1988), and in vivo studies in rodents have shown that administration of anti-CD2 monoclonal antibodies effectively prolong allograft survival (Chavin et al., 1993; Qin et al., 1994). Moreover, the CD2–CD58 pathway plays a critical role in the maintenance and reversal of anergy (Boussiotis et al., 1994), and anti-CD2 and anti-CD58 mAbs can specifically inhibit interleukin-12-induced proliferation and interferon γ production by activated T cells (Gollob et al., 1995).

Structural studies of both rat (Driscoll et al., 1991; Jones et al., 1992) and human CD2 (Withka et al., 1993; Wyss et al., 1993; Bodian et al., 1994) have shown that the extracellular region of CD2 consists of a nine-stranded NH₂-terminal immunoglobulin superfamily V-set domain and a seven-stranded membrane-proximal IgSF C2-set domain. The CD58 binding site of human CD2 is a highly charged surface area located on the GFCC'C" face of the NH₂-terminal adhesion domain (Arulanandam et al., 1993; Sommoza et al., 1993). The affinity of the interaction between human CD2 and human CD58 is only in the micromolar range (Sayre et al., 1989; Recny et al., 1990; van der Merwe et al., 1994), and models for the interaction of human CD2 with human CD58 have been proposed (Arulanandam et al., 1994). The adhesion domain in human CD2 contains a single N-glycosylation site at Asn 65 in the center of the DE loop that is conserved in equine and mouse,

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Abbreviations: TCR, T cell receptor; mAbs, monoclonal antibodies; IgSF, immunoglobulin superfamily; hCD2d1, adhesion domain of human CD2 including its heterogeneous high-mannose N-glycan [-(N-acetylglucosamine)₂-(mannose)_{5–8}]; $J(\omega)$, spectral density functions; $R_N(N_z)$, nitrogen longitudinal relaxation rate; $R_N(N_x)$, nitrogen transverse relaxation rate; $R_{HN}(H_z \rightarrow N_z)$, proton-nitrogen heteronuclear cross relaxation rate; $J(0)$, $J(\omega_N)$, and $J(\omega_H)$, spectral density function derived at zero, nitrogen, and proton frequencies, respectively; $J_{eff}(0)$, in the absence of exchange its value equals $J(0)$; $J_{avg}(\omega_{HH})$, the average of the spectral density function at the proton frequency as well as the sum and difference of the proton and nitrogen frequencies; τ_c , overall tumbling time; GlcNAc-1, N-acetylglucosamine.

but not in rat CD2 (Tavernor et al., 1994). In contrast to earlier data, which suggested that glycosylation is critical for human CD2 adhesion domain structure and function (Recny et al., 1992; Arulanandam et al., 1993), Davis et al. (1995) proposed that ligand binding by human CD2 is glycosylation-independent. Using NMR, we recently determined the three-dimensional structure of a glycosylated soluble version of the adhesion domain of human CD2 (hsCD2d1), including its heterogeneous high-mannose N-glycan (Wyss et al., 1995a, 1995b). These studies revealed that this N-glycan is located opposite to the CD58 binding site and thus, is not directly involved in ligand binding (Wyss et al., 1995a). However, studies on both enzymatically treated hsCD2d1 and a series of mutants of full-length human CD2 suggested that the N-glycan in the adhesion domain is crucial for human CD2 adhesion function by stabilizing the protein fold and counterbalancing an unfavorable clustering of five positive charges centered on Lys 61 of human CD2 (Wyss et al., 1995a). The ensemble of calculated NMR structures exhibited varying degrees of disorder that were in good agreement with qualitative mobility data obtained by measuring the carbon line widths of C-H groups in the sugar and the polypeptide units of hsCD2d1 in a highly resolved 2D ¹H-¹³C spectrum (Wyss et al., 1995a). These data provided valuable, but limited information about the relative flexibility of the carbohydrate and the polypeptide moieties, and indicated that the proximal core residues of the N-glycan are well ordered and their mobility is restricted through specific interactions with side chains of the polypeptide.

In this study, we have used ¹⁵N NMR relaxation data in combination with the "reduced spectral density mapping" approach (Farrow et al., 1995; Ishima & Nagayama, 1995; Dayie et al., 1996a; Lefèvre et al., 1996) to derive a detailed map of the distribution of picosecond to millisecond molecular motions at almost every N-H group of hsCD2d1. The discussion focuses on the relative flexibility of carbohydrate and polypeptide groups, and the motions experienced by human CD2 residues that are part of the CD58 binding site, conserved within species, or highly conserved between members of the IgSF. Understanding such motions is crucial for establishing a correlation between structure, dynamics, and function of macromolecules.

Results and discussion

Frequency spectrum of motions from NMR relaxation

Internal motions in proteins have been studied extensively with a variety of techniques, notably NMR relaxation, or molecular dynamics simulations (see Dayie et al., 1996b for a recent review). Nuclear relaxation rates can be used to map the distribution of the frequencies of rotational diffusive motions of N-H bond vectors contained in the spectral density functions, $J(\omega)$. Relaxation processes are sensitive to the values of the $J(\omega)$ at discrete frequencies and, thus, represent frequency filters of the large timescale range of motions exhibited by macromolecules in solution. Using ¹⁵N relaxation experiments, we measured three rates [the longitudinal relaxation rate $R_N(N_z)$, the transverse relaxation rate $R_N(N_x)$, and the heteronuclear cross relaxation rate $R_{HN}(H_z \rightarrow N_z)$]. These can be used to calculate $J(\omega)$ at discrete resonance frequencies of both proton and nitrogen (see Materials and methods).

The relaxation rates and the values of the spectral density function derived at zero [$J(0)$], nitrogen [$J(\omega_N)$], and proton [$J(\omega_H)$] frequencies can yield information on three aspects of protein mo-

tion: (1) the overall rotational motion of the protein; (2) internal rotational diffusive motions that are faster than the overall motion; and (3) slow conformational exchange motions on the millisecond-microsecond (ms-μs) time scale. $J(0)$ is related directly to the timescale on which the N-H bonds fluctuate and, as such, is a very useful parameter for characterizing the whole range of motions found along the protein backbone. Due to slow conformational exchange processes, we measured $J_{eff}(0)$ instead of $J(0)$, which is the value of $J_{eff}(0)$ in the absence of exchange. Using the reduced mapping approach (see Materials and methods), we calculated $J_{avg}(\omega_{Hi})$, the average of the spectral density function at the proton frequency, as well as the sum and difference of the proton and nitrogen frequencies. It has been shown previously that $J_{avg}(\omega_{Hi})$ is identical to $J(\omega_H)$, and both are sensitive to motions on the picosecond timescale (Lefèvre et al., 1996).

Regions of the molecule that are highly constrained are characterized by high $J_{eff}(0)$ and $J(\omega_N)$ values, and corresponding small $J_{avg}(\omega_{Hi})$ values. In contrast, regions displaying unrestricted motions have low values of $J_{eff}(0)$ and $J(\omega_N)$ and high values of $J_{avg}(\omega_{Hi})$. This compensatory behavior is revealed by the anticorrelation of $J_{eff}(0)$ and $J_{avg}(\omega_{Hi})$ (Fig. 1A). In addition, unusually small values of $J_{eff}(0)$ and large values of $J_{avg}(\omega_{Hi})$ indicate very rapid internal motions, on the nanosecond-picosecond (ns-ps) time scale.

Unusually large values of $J_{eff}(0)$ suggest exchange contributions to the motion that are believed to be important for biological function (Peng & Wagner, 1995; Lefèvre et al., 1996). The timescale of these slow motions (ms-μs range) can be estimated from the value of the spectral density function at zero frequency, $J_{eff}(0)$,

Fig. 1. Plots of (A) $J_{avg}(\omega_{Hi})$ and (B) $J(\omega_N)$ as a function of $J_{eff}(0)$. The correlation coefficients, R , for the linear least-squares fits of the data are also indicated. From the linear fit of plot B, a value of $\tau_c = 9.5 \pm 0.3$ ns was obtained for hsCD2d1 (see Materials and methods).

because such contributions increase the transverse relaxation rate (Peng & Wagner, 1995).

Frequency spectrum of motions in human CD2

We have measured the values of the $J(\omega)$ for most NH groups of hsCD2d1 and the results are shown in Figure 2. The values are color coded according to motional behavior, and the code is maintained in the structure displayed in Figure 3A. The measured values of $J(\omega)$ are also compared with those expected for a rigid body motion of the protein. The latter values were obtained with a procedure developed by Lefèvre et al. (1996), who have shown that a linear fit of $J_{eff}(0)$ versus $J(\omega_N)$ affords the overall tumbling time, τ_c (see Materials and methods). For hsCD2d1, this yields an overall tumbling time (τ_c) of 9.5 ± 0.3 ns. Assuming the protein tumbles isotropically and an individual N-H vector does not undergo any additional internal motions, its $J_{eff}(0)$ value equals 3.8 ± 0.12 ns [$J_{eff}(0) = 2/5\tau_c$]. In Figure 2A, this value is indicated as a blue horizontal line to indicate the limit of the rigid body motional diffusive motion. Rapid internal motions on the ns-ps timescale reduce this value, whereas slow motions on the ms-μs

timescale increase this value. Therefore, residues with values of $J_{eff}(0) > 3.8$ ns are probably affected by such slow motions and are color coded green in Figures 2 and 3A. In contrast, residues color coded blue, purple, and red have decreasing values of $J_{eff}(0)$, suggesting that they exhibit increasing mobility on the ns-ps timescale (in that order). In particular, residues in red represent very flexible regions. The overall trend in $J_{eff}(0)$, $J(\omega_N)$, and $J_{avg}(\omega_{H1})$ is complementary (Fig. 2), and the distribution of all three values of $J(\omega)$ point to the large variation of mobility along the backbone of hsCD2d1.

The high-mannose N-glycan in our samples of hsCD2d1 is composed of heterogenous isomeric structures (glycomers) [-(GlcNAc)₂-(Man)₅₋₈] containing predominantly Man₅ (~20%), Man₆ (~34%), Man₇ (~40%), and Man₈ (~6%) glycoforms (Wyss et al., 1995b). The molecular weight of the Man₈ glycomer is ~14 kDa, which in the Man₅ glycomer is reduced by only ~3.5%. Therefore, it is expected that this limited heterogeneity of the N-glycan causes only a narrow distribution of overall correlation times and the individual glycomers will exhibit very similar values of τ_c . Due to the high-mannose N-glycan, the overall shape of hsCD2d1 is very spherical and hence, the assumption that the protein tumbles iso-

Fig. 2. Reduced spectral density values of hsCD2d1. Color coding of the bars in each plot is taken from A (see below). The errors displayed are based on Monte Carlo simulations. β -Strands are labeled according to a IgSF V-set domain and depicted with lines above the bar graphs; their location and extents are taken from Wyss et al. (1995a). Values of some side-chain nitrogens are also included in the right-hand corner and represent, from left to right, those of Asn 65 at the glycosylation site, GlcNAc-1, Arg 48, Arg 105, Trp 10, and Trp 35. **A:** $J_{eff}(0)$. Blue, purple, and red horizontal lines are drawn at values of $J_{eff}(0) = [2/5\tau_c]$, $[\text{mean}]$ and $[\text{mean} - (2/5)\tau_c - \text{mean}]$, respectively. Green bars indicate residues with values of $J_{eff}(0) > 2/5\tau_c$ and hence, are implicated in slow conformational exchange processes on the ms-μs timescale. Blue, purple, and red bars highlight residues with increasing mobility on the ns-ps timescale with values of $J_{eff}(0)$ that fall into the ranges between the blue and purple horizontal lines, between the purple and red horizontal lines, and below the red horizontal line, respectively. Residues implicated in CD58 binding are marked with (●) at the top (Arulanandam et al., 1993). **B:** $J(\omega_N)$. Blue, purple, and red horizontal lines are drawn at values of $J(\omega_N) = [\text{mean} + 1 \text{ standard deviation } (\sigma)]$, $[\text{mean}]$, and $[\text{mean} - \sigma]$, respectively. **C:** $J_{avg}(\omega_{H1})$. Blue, purple, and red horizontal lines are drawn at values of $J_{avg}(\omega_{H1}) = [\text{mean} + \sigma]$, $[\text{mean}]$, and $[\text{mean} - \sigma]$, respectively. **D:** B -factors. Crystallographic B -factors were taken from Bodian et al. (1994).

Fig. 3. A: Stereo view of a representative NMR solution structure of hsCD2d1 (Wyss et al., 1995a). Heavy atoms of the backbone and side chains of Asn 65, GlcNAc-1, Trp 10, Trp 35, and Arg 105 are shown and color coded according to Figure 2A (green, slow ms- μ s conformational exchange; blue, rigid; purple and red, increasingly mobile on the ns-ps time scale). In addition, backbone heavy atoms of residues for which no relaxation data were available are highlighted in white. Heavy atoms of side chains implicated in CD58 binding (Arulanandam et al., 1993) are shown in yellow. Unlike Figure 2A, the side chain of Arg 48 is consequently colored yellow instead of red. Sequence numbers of residues implicated in slow conformational exchange processes on the ms- μ s timescale are indicated in green. **B:** Ribbon diagram of hsCD2d1 in the same orientation as Figure 3A with red residue numbers indicating the start and end of each β -strand [figure prepared with the MOLSCRIPT (Kraulis, 1991)]. β -Strands are labeled in black letters according to a IgSF V-set domain and their location and extents are taken from Wyss et al. (1995a). The view is with the GFCC'C'' β -sheet (yellow) in the front.

tropically is justified. The overall tumbling time of 9.5 ± 0.3 ns of hsCD2d1 agrees well with the range of τ_c values (9.3–10.5 ns) obtained with the model-free approach for other proteins of similar size, such as the 14-kDa protein, villin 14T (Markus et al., 1996).

Fast (ns-ps) motions

The N-terminus is highly flexible and exhibits very rapid internal motions. A stiffening of the motion occurs as the residues approach the center of strand A, which then becomes flexible again at the end of strand A and the beginning of the AB loop. Residues in strand B become progressively more rigid until the beginning of the BC loop, where at Ser 23 a sudden drop to very low values of

$J_{eff}(0)$ and $J(\omega_N)$ occurs. These low values coincide with correspondingly high values of $J_{avg}(\omega_{Hi})$, indicating that the first part of the BC loop is highly mobile. Starting at Asp 28, residues toward the end of the BC loop become more rigid and the C strand generally shows very restricted motions on this timescale, as indicated by high values of $J_{eff}(0)$ and $J(\omega_N)$ and low values of $J_{avg}(\omega_{Hi})$. Notably, starting at Asp 32, several residues show unusually large values of $J_{eff}(0)$ (color coded green in Figs. 2A and 3A), suggesting that they may undergo extensive slow conformational exchange processes on the ms- μ s timescale besides the motions discussed here (see below). The CC' loop and the C' strand are also relatively constrained in their motions, whereas the C'C'' loop and the C'' strand are more flexible, as indicated by their decreased

values of $J_{eff}(0)$ and $J(\omega_N)$ and increased values of $J_{avg}(\omega_{Hi})$. The end of the C'D loop, as well as the start and the end of the D strand are relatively rigid, but the center of strand D is more flexible. Most residues between Phe 63 in strand D and Ser 84 in strand F undergo very restricted motions on this timescale. Residues from Ile 85 to the C-terminus alternate between rigid and flexible regions, and Arg 105 undergoes very rapid internal motions.

In summary, the central strands C, E, and F seem to be the most rigid parts of hsCD2d1. They appear to be more restricted in their mobility on the ns-ps timescale when compared to other strands, such as strands A, C'', and G. In contrast, the terminal residues exhibit very rapid internal motions, and parts of the G strand and the BC, C'C'', and FG loops appear to be significantly more mobile than other regions of the glycoprotein.

Figure 2 also depicts how the values of $J(\omega)$ vary for the side-chain NH groups of Asn 65 at the glycosylation site, the first N-acetylglucosamine (GlcNAc-1), Arg 48, Arg 105, Trp 10, and Trp 35. In agreement with previous carbon line width measurements (Wyss et al., 1995a), the two nitrogens at the glycosylation site show restricted mobility similar to the side chain nitrogen of the highly conserved and buried Trp 35. In contrast, the other side chains bearing N-H groups are highly mobile.

Slow (ms-μs) motions

Besides this general trend of ns-ps motion discussed above, $J_{eff}(0)$ in combination with results from measuring $R_N(N_x)$ at various spin lock fields allows one to identify residues that might be involved in slow motions on the ms-μs timescale. Analysis of the primary relaxation rates and $J_{eff}(0)$ values implicate Asp 32, Ile 33, Lys 34, Ser 39, Ile 44, Gln 46, Phe 47, Glu 50, Thr 53, Phe 54, Thr 59, Phe 63, Lys 64, Tyr 81, and Val 93 in slow exchange (green residues in Figs. 2A and 3A). Many of these residues map to the region between Thr 38 and Lys 57, which was difficult to assign due to exchange broadening of the corresponding NMR resonances and, hence, the NH resonances of Arg 48, Glu 56, and Lys 57 were not observed in the NMR spectra (Wyss et al., 1993).

Comparison with X-ray crystallographic temperature factors

Crystallographic B -factors also provide information on internal mobility. However, the B -factor depends also on static crystal disorder. If one assumes that the atoms undergo simple isotropic motion in a harmonic potential, the B -factors are related to the mean-square atomic displacement, and a comparison with mobility measurements from NMR relaxation experiments appears to be meaningful (Brüschweiler & Wright, 1994).

Figure 2D shows the B -factors reported for the crystal structure of a two-domain construct of human CD2. In this case, the crystal structure was obtained on an endo-H treated two-domain fragment where only single GlcNAc-1 residues were retained at the three N-glycosylation sites (Bodian et al., 1994). The nitrogens at the glycosylation site (side chain NH of Asn 65 and GlcNAc-1) have high B -factors. This is inconsistent with the NMR data, which do not indicate higher internal mobility for these sites [high $J_{eff}(0)$, $J(\omega_N)$, and low $J_{avg}(\omega_{Hi})$ values]. This discrepancy may be due to the fact that the carbohydrate was truncated for the X-ray studies, but not for the NMR experiments, which were performed on a glycosylated adhesion domain fragment containing a single heterogeneous high-mannose N-glycan (Wyss et al., 1993; Withka et al., 1993; Wyss et al., 1995a, 1995b). Increased mobility is

observed for the N-terminus, and the C'C'' loops, as evidenced by higher B -factors. These residues have correspondingly low $J_{eff}(0)$ and high $J_{avg}(\omega_{Hi})$ values. However, only part of the BC loop shows clear mobility from the NMR data. In contrast, all the BC loop has high B -factors. Overall, the value of the linear correlation coefficient between the B -factors and $J_{avg}(\omega_{Hi})$ has a low value of 0.38. The corresponding values for $J_{eff}(0)$ and $J(\omega_N)$ are even lower at -0.07 and -0.26, respectively. These correlation coefficients are dominated by relatively few residues, with very high $J_{avg}(\omega_{Hi})$ and B -factors at the N-terminus and the C'C'' loop, but, for most residues, there is very little correlation. More generally, the striking difference between the NMR relaxation data and the X-ray B -factors lies in the alternation in high and low B -factors for loops and strands respectively; this is clearly absent from the NMR data.

These contradictory results are perhaps not surprising for a number of reasons. First, static disorder, absent in NMR, contributes to the spreading of the electron density. Second, the averaging of the collected data over the duration of the experiment (days or weeks) removes any information about the time scales of fluctuations; the dynamic information is preserved in an NMR experiment. Third, as discussed by Petsko and Ringe (1986), absolute values of individual mean-square displacements may be unreliable due to errors introduced by application of restraints in the refinement process, poor resolution (>2 Å), poorly determined coordinates (R -factor $> 20\%$), and radiation damage of the crystal. Fourth, if the B -factors are dominated by translational motions, these motions would not be visible in the NMR experiments, because NMR relaxation is sensitive primarily to rotational diffusive motions in macromolecules, whereas translational motions are rather ineffective for relaxation. Finally, B -factors reflect only the amplitudes of the motions, whereas the NMR relaxation parameters presented here depend on both the amplitude and the rate of the motions; thus, both methods provide different but useful information.

Location of mobile sites in hsCD2d1 and relation to biological function

What does the relaxation data reveal about the kind of motions experienced by residues near the sugar moiety, the CD58 binding site, and residues that are conserved in the IgSF?

CD58 binding site undergoes ms-μs motions

Figures 2A and 3A indicate that most of the residues that are implicated in slow conformational exchange processes, as suggested by high $J_{eff}(0)$ values, map either to the CD58 binding site directly (Asp 32, Lys 34, Gln 46, Val 93) or its vicinity (Ile 33, Ser 39, Ile 44, Phe 47, Glu 50, Thr 53, Phe 54, and Tyr 81). This suggests that large parts of the CD58 binding site of human CD2 are involved in a slow motional process that may play an important role in CD58 recognition and thus, may affect CD58 binding. Besides these motions on the ms-μs timescale, the majority of the CD2 residues involved in CD58 binding show restricted mobility (Asp 32, Lys 34, Lys 41, Lys 43, Gln 46, Lys 82, Tyr 86, and Lys 91) on the ns-ps timescale and only Lys 51, Gly 90, and Val 93 seem to be slightly more mobile than the average residue, suggesting that CD58 binding will not significantly change the conformational entropy along the backbone (Akke et al., 1993; Yang & Kay, 1996).

Using site-directed mutagenesis in conjunction with NMR structural data, we showed previously that the CD58 binding site of

human CD2 is a flat and positively charged ($\approx 54\%$) surface area covering $\approx 770 \text{ \AA}^2$ on the GFCC'C" face of this IgSF V-set domain (Arulanandam et al., 1993). Theoretical studies suggest that the desolvation costs associated with the removal of charged and polar groups from water upon formation of a protein-protein interface in a complex appear in general to be larger than the Coulombic attraction energy between surface groups in the interface (Honig & Nicholls, 1995). Thus, the presence of polar and charged groups in a protein-ligand interface may have an overall destabilizing effect on complex formation. On the other hand, these groups may contribute to the specificity of the protein-ligand interaction, because they must arrange to form ion pairs and hydrogen bonds to minimize the free energetic cost of their removal from water (Honig & Nicholls, 1995). The nature of the CD58 binding surface of human CD2, which contains a large number of polar and charged residues ($\approx 77\%$), may account for the low binding affinity ($K_d \approx 10^{-6} \text{ M}$ – 10^{-5} M) seen for the CD2-CD58 interaction (Sayre et al., 1989; Recny et al., 1990; van der Merwe et al., 1994) and suggests that electrostatic complementarity at the CD2-CD58 interface may contribute significantly to the binding specificity. Given the highly charged and polar CD58 binding surface of human CD2, a large loss in entropy upon binding, which would also favor the unfolded state, is unlikely to be tolerated. Our dynamics data indeed suggest a contiguous surface area fluctuating on the ms- μ s timescale that shows restricted internal motions on the ns-ps timescale. The association rate constant (k_{on}) for the CD2-CD58 interaction has been estimated to be $\geq 400,000 \text{ M}^{-1} \text{ s}^{-1}$ using surface plasmon resonance technology (van der Merwe et al., 1994). Interestingly, the rate of CD2-CD58 encounters becomes comparable to the slow motions exhibited by the CD58 binding site if we assume a CD58 concentration of about 1 mM. Whether this is biologically relevant or not remains to be seen.

Residues in the vicinity of the N-glycan show restricted mobility

Previous structural studies revealed that the first two N-acetylglucosamine residues (GlcNAc-1-GlcNAc-2) interact with the polypeptide residues Asn 18, Asp 20, Lys 61, Phe 63, Asn 65, Thr 67, and Lys 69 located on the DEB β -sheet surface opposite to the CD58 binding site of human CD2 (Wyss et al., 1995b). Biochemical and mutational studies showed that the glycan crucially stabilizes the folded protein structure, and demonstrated that GlcNAc-1 is sufficient to achieve this (Davis et al., 1995; Wyss et al., 1995a). Based on point mutations of all side chains contacting the glycan, however, it has not been possible to identify a particular stabilizing interaction between glycan and protein in the folded state. On the other hand, the presence of GlcNAc-1 could decrease the entropy of the unfolded state and thus, favor the folded form.

Our relaxation data report directly on the motions of regions near the N-glycosylation site. The amide nitrogen of the glycosylation site shows a very restricted mobility on the ns-ps timescale. The motion of this site is similar to many backbone nitrogens in the more rigid β -strands C, E and F, and the side-chain nitrogen of the highly conserved Trp 35, which is completely buried and at the center of the hydrophobic core of the protein. The amide nitrogen of the N-acetyl group of GlcNAc-1 shows a slightly higher, but still restricted mobility, similar to many nitrogens of the polypeptide backbone. These findings are in good agreement with both the degree of disorder observed in calculated NMR structures, and the qualitative measurements of mobility obtained earlier (Wyss et al.,

1995a). These previous studies revealed that, through the interaction with several solvent-exposed amino acid residues within the DEB β -sheet of hsCD2d1, the N-glycosylation site and the trisaccharide core of the N-glycan are very well defined and rigid (Wyss et al., 1995a). On the other hand, the sugar units become more mobile with increasing distance from the N-glycosylation site such that the terminal sugar residues are highly flexible. This variation in mobility of the monosaccharides of the N-glycan agrees with a motional model derived from NMR and MD studies of glycans being part of a glycoprotein. In this model, the mobility of the GlcNAc-1 residue relative to the polypeptide chain is largely determined by its interactions with the polypeptide. The other monosaccharides gradually increase their mobility with increasing distance from the glycosylation site due to the additive effects of torsional oscillations and conformational transitions about the glycosidic linkages (Rutherford et al., 1993; Woods et al., 1994; Lommerse et al., 1995). A consequence of this model is that the mobilities of the other monosaccharides of the N-glycan are correlated with the mobility of GlcNAc-1.

Most backbone nitrogens of residues in both the DE loop and the vicinity of the N-glycan show very restricted mobilities on the ns-ps timescale, similar to the N-glycosylation site. These findings agree with the notion that the N-glycan of hsCD2d1 is essential for human CD2 adhesion function by increasing the rigidity of the surrounding polypeptide region and thus, crucially stabilizes the glycoprotein. Several recent studies on glycoproteins have shown that glycosylation globally decreases the dynamic fluctuations of the molecule and increases its stability (Wyss & Wagner, 1996). In two cases, an increase in stability of about 1.2 kcal/mol was measured and, in two examples, this stabilizing function was achieved by a single sugar unit, as in hsCD2d1.

Residues conserved in the IgSF V-set domain are rigid

The IgSF is a large group of related proteins that function, mainly, in the immune system in cell-cell recognition, or in the structural organization and regulation of muscle (Harpaz & Chothia, 1994). The evolutionary and structural basis of the IgSF rests on gene duplication and divergence of homologous domains of approximately 100 residues. These residues show a characteristic folding pattern of two β -sheets stabilized by a hydrophobic core and a disulfide bond between the sheets (Williams & Barclay, 1988). This bond is absent in domain 1 of CD2, however. The evolutionary success of IgSF domains is believed to derive from their ability to form a stable platform upon which a diversity of recognition determinants can be displayed by varying the amino acids that are exposed on the external faces of the β -sheets or on the loop regions connecting the β -strands (Williams & Barclay, 1988). Individual members of the IgSF can differ in the number and size of strands in the two β -sheets and in the size and conformation of the links between the strands. Conserved patterns in sequence and structures are observed that allow the different members to be clustered into different sets (Williams & Barclay, 1988). Recently, Harpaz and Chothia (1994) constructed a so-called "V-frame" sequence profile for searching for I set domains that contain 20 residues (key sites), mainly responsible for the V frame fold. Interestingly, in hsCD2d1 13 (65%) (Ile 17, Leu 19, Ile 21, Trp 35, Tyr 60, Leu 68, Ile 70, Leu 73, Asp 77, Tyr 81, Val 83, Phe 98, and Leu 100) of these 20 residues are less mobile than the average residue and 5 (25%) residues (Gln 15, Ile 33, Ile 44, Thr 59, Asp 79) showed an average restricted mobility on the ns-ps timescale as judged by their values

of $J_{\text{eff}}(0)$, $J(\omega_N)$, and $J_{\text{avg}}(\omega_{\text{Hi}})$ (Fig. 2). Only Ser 23 and Ile 102 at the key sites "B7" and "G10," respectively, are more mobile than the average residue. However, the increased mobility seen for Ile 102 may be due to the absence of domain 2 in hsCD2d1. In the crystal structure of hsCD2d1d2, residues 103–105 form part of the interdomain interface and, hence, it is likely that the C-terminal end of hsCD2d1 is stabilized by these contacts in full-length human CD2. Interestingly, the key site "B7" is not conserved in human CD2, in which the end of the B strand is disrupted and the D and E strands are shortened. Also, the salt bridge between the key sites "D1" and "EF6" is not conserved in human CD2, and the corresponding residues Thr 59 and Asp 77, respectively, show an average mobility. Therefore, almost all human CD2 residues that are conserved in the "V-frame" sequence profile are very rigid.

Conclusion

Our ^{15}N relaxation studies have revealed that hsCD2d1, as an example of an IgSF V-set domain, undergoes large variations of motions on the ms–ps timescale that may have structural and functional significance. The densely packed interior of hsCD2d1, as exemplified by residues conserved in IgSF V-set domains, exhibits very restricted motions. This may underscore their evolutionary and biological roles as rigid stabilizing scaffolds. In contrast, the CDR loops (BC, C'C", and FG) show enhanced fluctuations on the ns–ps timescale. These fluctuations may arise from lack of close packing with the rest of the polypeptide chain, and may be important for their role in providing specificity for antigen binding. Interestingly, a contiguous patch of residues in the CD58 binding site not only have rigid ns–ps motions imposed by the overall tumbling, but also experience slow conformational fluctuations on the ms– μs timescale. The natures of both the motions present in this site and the highly charged and extended surface area may account for the low binding affinity of the CD2–CD58 interaction. Notably, the N-glycosylation site and residues near the N-glycan of hsCD2d1 are rigid, consistent with previous data, which showed that the N-glycan is crucial for CD58 binding by stabilizing the polypeptide fold.

Materials and methods

Sample preparation

A ~ 1.0 mM [^{15}N]-labeled hsCD2₁₀₅ NMR sample in 95% (v/v) $\text{H}_2\text{O}/\text{D}_2\text{O}$ and 20 mM deuterated acetate buffer, pH 4.5, was prepared as described previously (Wyss et al., 1993), by providing all amino acids [^{15}N]-labeled, except asparagines and cysteines.

NMR spectroscopy

NMR experiments were performed at 25 °C on a Varian UNITY-plus 500 spectrometer with a ^1H operating frequency of 500.0 MHz and equipped with a triple-resonance, pulsed-field gradient probe with an actively shielded z gradient and a gradient amplifier unit. All ^{15}N correlation experiments were performed using "gradient-enhanced" pulse sequences to suppress unwanted water magnetization, select for both orthogonal components of the in-phase ^{15}N - ^1H magnetization, and minimally perturb the water (Kay et al., 1992; Cavanagh & Rance, 1993; Grzesiek & Bax, 1993). The rates $R_N(N_z)$ and $R_N(N_x)$ were measured using similar pulse sequences as described in Daye and Wagner (1994), and the heteronuclear NOE en-

hancements were measured using the pulse sequence described in Farrow et al. (1994). Delays and gradient strengths and durations were optimized to minimize losses due to relaxation.

Spectral widths in the ^1H dimension(s) and the ^{15}N dimension were 8,000 Hz and 1,500 Hz, respectively. The ^1H and the ^{15}N carrier positions were set on the residual water resonance and at 120.1 ppm relative to liquid ammonia, respectively. The 2D data sets comprised 150 and 1,024 complex points, which were zero filled to 512 and 2,048 points and multiplied with a $\pi/3$ shifted squared sine bell apodization function in both dimensions prior to Fourier transformation. Sixteen scans were acquired for each t_1 value. T_1 values were measured from the spectra recorded with 14 different relaxation delays, $T = 30.1, 60.2, 90.2, 120.3, 150.4, 210.5, 300.8, 451.2, 601.6, 802.1, 1,052.7, 1,503.9, 2,506.5, 4,992.9$ ms, and 11 different relaxation delays, $T = 5.5, 11.0, 22.0, 33.0, 44.1, 66.1, 88.2, 99.2, 110.2, 137.8, 165.3$ ms, were used to determine T_2 values. To permit the estimation of noise levels, duplicate spectra were recorded for $T = 30.1$ ms (T_1 spectra) and $T = 5.5$ and 22.0 ms (T_2 spectra). Relaxation delays of 2 s were employed in the measurement of ^{15}N T_1 and T_2 values. The $R_N(N_x)$ rates were measured at an ^{15}N spin-locking field strength of 2.9 kHz. Heteronuclear NOE values were determined from spectra recorded in the presence and absence of a proton presaturation period of 3 s preceded by an additional relaxation delay of 3 s. Both experiments were repeated for error estimates.

Analysis of NMR relaxation data

Monoexponential relaxation rate determinations and error estimations were done as described previously (Peng & Wagner, 1992). The fitting to monoexponential decay was performed using the Levenberg–Marquardt algorithm as described previously (Peng & Wagner, 1992). The error estimation was done using established Monte Carlo procedures (Kamath & Shriver, 1989), with the difference between the repeated time points as the standard deviation of the Gaussians. An ensemble of 500 synthetic data sets was generated for each NH vector. For each of the 500 synthetic data sets, relaxation rates were obtained using the same fitting procedures. The estimated errors in the relaxation rates were taken to be the standard deviations derived from the ensemble of simulated data.

Extracting spectral density functions and the overall correlation time

Heteronuclear NMR relaxation experiments are sensitive to the dynamics of singly protonated nuclei such as ^{15}N - ^1H or ^{13}C - ^1H generously distributed along the backbone and side chains of macromolecules. The three rates measured [the longitudinal relaxation rate, $R_N(N_z)$, the transverse relaxation rate, $R_N(N_x)$, and the heteronuclear cross relaxation rate, $R_{\text{HN}}(\text{H}_z \rightarrow \text{N}_z)$] relate directly to the relative distribution of the values of the spectral density functions as follows:

$$J_{\text{eff}}(0) = \frac{3}{2E} \left[-\frac{1}{2} R_N(N_z) + R_N(N_x) - \frac{3}{5} R_{\text{HN}}(\text{H}_z \rightarrow \text{N}_z) \right]$$

$$J(\omega_N) = \frac{1}{E} \left[R_N(N_z) - \frac{7}{5} R_{\text{HN}}(\text{H}_z \rightarrow \text{N}_z) \right] \quad (1)$$

$$J_{\text{avg}}(\omega_{\text{Hi}}) = \frac{1}{5A} R_{\text{HN}}(\text{H}_z \rightarrow \text{N}_z)$$

where $A = \gamma_{\text{HN}}^2 \gamma_{\text{N}}^2 \hbar^2 / 4r_{\text{HN}}^6$, $B = \Delta^2 \omega_{\text{N}}^2 / 3$, and $E = 3A + B$. For a CSA value of $\Delta = -160$ ppm (Hiyama et al., 1988) and an internuclear ^{15}N - ^1H bond distance of $r_{\text{HN}} = 1.02$ Å (Keiter, 1986), the constants A and B become $\approx 1.3 \times 10^9$ (rad/s)² and 0.9×10^9 (rad/s)², respectively, at 500 MHz. Linear combinations of the three rates therefore provide a direct measure of $J(\omega)$, without any assumptions about the shape of the spectral density function.

It has been shown recently that the $J_{\text{eff}}(0)$ and $J(\omega_{\text{N}})$ are correlated linearly for nearly all proteins for which such studies were conducted (Dayie et al., 1996a, 1996b; Lefèvre et al., 1996); thus:

$$J(\omega_{\text{N}}) = \alpha J_{\text{eff}}(0) + \beta. \quad (2)$$

α and β are the slope and intercept of the linear fit. A Lorentzian spectral density function $J(\omega) = 2\tau/5(1 + \omega^2\tau^2)$ leads to a third degree equation in τ :

$$\tau^3(2\alpha\omega_{\text{N}}^2) + \tau^2(5\beta\omega_{\text{N}}^2) + \tau\{2(\alpha - 1)\} + 5\beta = 0. \quad (3)$$

The positive roots (τ) of this equation give the correlation times of the various motions contributing to the low frequency part of the spectral density function. From the linear least-square fit of the CD2 data, the second root yields the overall tumbling rate of the molecule of 9.5 ± 0.3 ns.

Supplementary material in Electronic Appendix

For each residue of hsCD2d1, the three rates $R_{\text{N}}(\text{N}_z)$, $R_{\text{N}}(\text{N}_x)$, and $R_{\text{HN}}(\text{H}_z \rightarrow \text{N}_z)$ are listed in the file CD2_Rates±errors. The Table was prepared on a Macintosh using KaleidaGraph Version 3.0.2.

Supplementary information

Auto relaxation rates $R_{\text{N}}(\text{N}_z)$, $R_{\text{N}}(\text{N}_x)$, and cross relaxation rates $R_{\text{HN}}(\text{H}_z \rightarrow \text{N}_z)$ with error estimates. The cross relaxation rates are calculated from the steady state NOEs and auto relaxation rate $R_{\text{N}}(\text{N}_z)$ according to:

$$R_{\text{HN}}(\text{H}_z \rightarrow \text{N}_z) = \frac{H_{\text{sat}} - H_{\text{eq}}}{H_{\text{eq}}} \cdot \frac{\gamma_{\text{N}}}{\gamma_{\text{H}}} \cdot R_{\text{N}}(\text{N}_z).$$

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