Extended Neck Regions Stabilize Tetramers of the Receptors DC-SIGN and DC-SIGNR*

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The human cell surface receptors DC-SIGN (dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin) and DC-SIGNR (DC-SIGN-related) bind to oligosaccharide ligands found on human tissues as well as on pathogens including viruses, bacteria, and parasites. The extracellular portion of each receptor contains a membrane-distal carbohydrate-recognition domain (CRD) and forms tetramers stabilized by an extended neck region consisting of 23 amino acid repeats. Cross-linking analysis of full-length receptors expressed in fibroblasts confirms the tetrameric state of the intact receptors. Hydrodynamic studies on truncated receptors demonstrate that the portion of the neck of each protein adjacent to the CRD is sufficient to mediate the formation of dimers, whereas regions near the N terminus are needed to stabilize the tetramers. Some of the intervening repeats are missing from polymorphic forms of DC-SIGNR. Two different crystal forms of truncated DC-SIGNR comprising two neck repeats and the CRD reveal that the CRDs are flexibly linked to the neck, which contains α-helical segments interspersed with non-helical regions. Differential scanning calorimetry measurements indicate that the neck and CRDs are independently folded domains. Based on the crystal structures and hydrodynamic data, models for the full extracellular domains of the receptors have been generated. The observed flexibility of the CRDs in the tetramer, combined with previous data on the specificity of these receptors, suggests an important role for oligomerization in the recognition of endogenous glycans, in particular those present on the surfaces of enveloped viruses recognized by these proteins.

The dendritic cell receptor DC-SIGN2 and the closely related DC-SIGNR found on endothelial cells have been of considerable interest because of their ability to enhance infection of T cells by the human immunodeficiency virus and because of their interactions with glycoproteins found on the surface of other enveloped viruses (1, 2). The physiological functions of these receptors are not known with certainty, but DC-SIGN has been ascribed roles in binding to intercellular adhesion molecule 3 on T cells and intercellular adhesion molecule 2 on endothelia, as well as in uptake of pathogens (3–7). Although DC-SIGN binds to a broad spectrum of glycans, it has highest affinity for N-linked high mannose oligosaccharides and fucose-containing structures that are found both as blood group antigens in human tissues and on the surfaces of certain parasites (7, 8). DC-SIGNR binds only to the high mannose oligosaccharides, and unlike DC-SIGN, it does not mediate uptake and degradation of glycoconjugates (8).

DC-SIGN and DC-SIGNR share nearly 80% sequence identity and are closely similar in overall architecture. Both receptors are type II transmembrane proteins in which C-terminal C-type carbohydrate-recognition domains (CRDs) are projected from the cell surface by a neck comprising a series of highly conserved 23-amino acid acid repeats. There are seven complete repeats preceded by a partial repeat at the N terminus. The sequence present in all repeats is designated A, and the sequence missing in the first repeat is called B (Fig. 1A). Circular dichroism has been used to demonstrate that the neck has a high content of α helix (9). The neck repeats feature a hydrophobic heptad motif characteristic of coiled-coils of α helices (Fig. 1A). However, each repeat starts with a proline and several hydrophilic residues, features that do not favor formation of coiled coils.

The isolated CRDs of DC-SIGN and DC-SIGNR are monomeric, whereas the extracellular domain is tetrameric (9). In other receptors that contain C-type CRDs, the arrangement of the CRDs in clusters leads to enhanced affinity for multivalent ligands. Although the oligosaccharide ligands recognized by DC-SIGN and DC-SIGNR are found on host cells, they are probably more highly clustered on the surfaces of viruses and parasites. Thus, it is likely that the organization of these receptors enhances the affinity for targets that contain appropriately high densities of specific carbohydrate ligands (10, 11).

The role of the neck region in formation of DC-SIGN and DC-SIGNR oligomers is examined here. Structural analysis of a fragment containing a truncated neck reveals possible modes of tetramer formation and shows that the CRDs are flexibly linked to the neck. A combination of hydrodynamic measurements and model building suggests possible arrangements of the full extracellular tetramer and provides insights into the avidity enhancement provided by oligomer formation.

EXPERIMENTAL PROCEDURES

Cross-linking—For cross-linking in situ, one confluent 25-cm² flask of cells expressing DC-SIGN or DC-SIGNR (8) was rinsed twice with phosphate-buffered saline and harvested by scraping and centrifugation for 2 min at 10,000 × g. The pellet was suspended in 1 ml of phosphate-buffered saline, and 25-μl aliquots were treated with bisul-
fosuccinimidyl)suberate (Pierce). Intact protein was isolated from cells harvested as above and solubilized in 2 ml of loading buffer (150 mM NaCl containing 25 mM Tris-Cl, pH 7.8, and 25 mM CaCl₂) supplemented with 1% Triton X-100. The sample was centrifuged at 18,000 g for 5 min, and the supernatant was loaded onto a 1-ml column of mannose-Sepharose (12). After rinsing with 5 ml of loading buffer supplemented with 0.1% Triton X-100, the column was eluted with 5 aliquots (0.5 ml) of eluting buffer (150 mM NaCl containing 25 mM Tris-Cl, pH 7.8, and 2.5 mM EDTA) supplemented with 0.1% Triton X-100. Protein-containing fractions were identified by SDS-polyacrylamide gel electrophoresis followed by blotting with polyclonal rabbit antibodies to DC-SIGN or DC-SIGNR. Before cross-linking, full-length protein as well as fragments expressed in Escherichia coli were dialyzed against 150 mM NaCl containing 25 mM Na-HEPES, pH 7.8, and 5 mM CaCl₂.

Proteolysis—Proteins purified by affinity chromatography and anion exchange chromatography (9) were adjusted to a final concentration of 25 mM CaCl₂. Aliquots of 25 μl containing ~5 μg of protein were incubated with trypsin (Worthington Biochemicals) or subtilisin (Roche Applied Science), prepared as stock solutions in water immediately before use. For sequencing, gels of the digestion reaction were blotted onto polyvinylidene difluoride membranes (13) and sequenced directly in a Beckman LF-3000 protein sequencer.

Protein Expression—Truncated forms of DC-SIGN and DC-SIGNR were prepared in the expression vector pT5T (14) previously used to express the full extracellular domains of these proteins (9). Synthetic double-stranded oligonucleotides were used to link restriction fragments from the cDNA with the initiator methionine from the plasmid. An alanine codon was inserted after the methionine codon so that the formylmethionine residue would be processed in the bacteria, leaving an exposed N terminus to allow confirmation of the N-terminal sequences of the expressed fragments. Most of the proteins were expressed as previously described (9). However, fragments that do not form oligomers and, hence, do not bind tightly to the affinity columns were concentrated by dialysis against water, lyophilization, and resuspension in one-tenth the original volume of loading buffer before application to a 10-ml column of mannose-Sepharose. The column was washed with 10 ml of loading buffer followed by 20 ml of elution buffer, and fractions of 2 ml were collected throughout. Proteins were further purified by anion exchange chromatography on a Mono Q column (Amersham Biosciences) as previously described for the full extracellular domain (9).

Hydrodynamics—For hydrodynamic analysis, proteins were dialyzed extensively against 100 mM NaCl containing 10 mM Tris-Cl, pH 7.8. Sedimentation equilibrium studies were conducted at three concentrations as previously described (2). Sedimentation velocity was performed in a Beckman XL-A ultracentrifuge using 0.45-ml centerpieces in the AN-60 rotor at 50,000 rpm. Scans at 230 nm were recorded at 4.5-min intervals. The results were analyzed by the second moment method using software provided by the manufacturer. Gel filtration was performed on a 30 × 0.75-cm Spherogel-3000 column (Phenomenex) eluted with 100 mM NaCl containing 10 mM Tris-Cl, pH 7.8, at a flow rate of 0.5 ml/min. Absorbance was monitored at 280 nm. Diffusion coefficients were calculated based on standards of thyroglobulin, bovine serum albumin, and thyroglobulin-globin complexes.
Table 1

Crystallographic statistics for ligand-free DC-SIGN

| Data collection | Space group | P3 21 |
|-----------------|-------------|-------|
| Unit cell parameters (Å) | a = 82.1, c = 110.3 |
| Resolution (Å) | 59.73–2.25 (2.37–2.25) |
| R<sub>free</sub> | 5.4 (37.6) |
| % Complete | 99.9 (99.9) |
| Average multiplicity | 3.4 (3.5) |
| Refinement |
| R<sub>free</sub> | 0.249 |
| R<sub>i</sub> | 0.222 |
| Average B<sub>factor</sub> (Å<sup>2</sup>) | 47.8 |
| Bond length r.m.s.d. (Å) | 0.006 |
| Angle r.m.s.d. (°) | 1.33 |
| Ramachandran plot; % in most favored/allowed/generous/disallowed regions | 86.3/13.7/0.0/0.0 |

a R<sub>free</sub> = Σ(h)(h) - (h)(h)/Σ(h)(h), where (h) is observed intensity, and (h) is mean intensity obtained from multiple measurements.

b R<sub>i</sub> = Σ|F<sub>i</sub>| - |F<sub>i</sub>|/Σ|F<sub>i</sub>|, where |F<sub>i</sub>| is observed structure factor amplitude and |F<sub>i</sub>| is calculated structure factor amplitude for the working and test sets, respectively.

Results

Oligomeric Structure of DC-SIGN and DC-SIGNR—The oligomeric states of intact DC-SIGN and DC-SIGNR were investigated by examining protein expressed in rat fibroblasts. These cells have been described previously and have been shown to express the receptors at the cell surface (8). The intact receptors were extracted from the cells in the presence of Triton X-100 and purified by affinity chromatography on mannos-Sepharose. Treatment with a heterobifunctional cross-linking reagent generated dimers, trimers, and tetramers, with the tetramers predominating at higher reagent concentrations (Fig. 2). Cross-linking to form the tetramer was also observed when intact cells were treated with the reagent. These results confirm that each receptor in its native state forms a tetramer. Similar results for DC-SIGN in dendritic cells have recently been reported (21).
Identification of Regions in DC-SIGN and DC-SIGNR That Stabilize Oligomer Formation—Previous studies on the properties of the extracellular domains of the receptors indicate that interactions within these fragments are sufficient to stabilize the tetramers. An initial indication of the overall arrangement of this portion of the protein was obtained by performing limited proteolytic digestions. Treatment of the full extracellular domains with subtilisin resulted predominantly in cleavage at two sites (Fig. 3). The protease-resistant fragments were identified by N-terminal amino acid sequencing. One site of protease digestion occurs after the first, partial neck repeat. This cleavage removes the unique sequence of 15 amino acids that separates the neck repeats from the transmembrane repeat. This cleavage removes the unique sequence of 15 amino acids that separates the neck repeats from the transmembrane repeat.

Removal of more repeats results in a dramatic loss of overall stability of the protein. Thus, truncated proteins containing 3.5 to 2.5 repeats were difficult to purify intact, and they degraded rapidly during centrifugation, so their oligomeric state could not be characterized. Shorter proteins containing two repeats were substantially more stable but formed partially dissociating dimers rather than tetramers. A dimer-monomer dissociation constant of $35 \pm 3 \mu M$ was measured for truncated DC-SIGN containing two neck repeats. Association of monomers of truncated DC-SIGN with two neck repeats was not evident in the ultracentrifugation experiments, but dimers were detected by cross-linking, probably because of the higher protein concentration used in the cross-linking experiment and because the bifunctional reagent traps the dimer form even if it is present in only a small fraction of the sample. Previous studies have demonstrated that the CRDs, in the absence of any of the neck, are monomeric (9), and the presence of half a repeat does not change this behavior. Thus, the results suggest that repeats close to the CRD mediate the formation of dimers, whereas repeats near the N-terminal membrane anchor are needed for association to form tetramers.

Crystal Structures of a Truncated DC-SIGN with Two Neck Repeats—After an extensive screening of crystallization conditions for several truncated DC-SIGN and DC-SIGNR constructs, two crystal forms were obtained from a DC-SIGN fragment comprising the CRD and two full neck repeats. The first form crystallized in the presence of Ca$^{2+}$ and the Lewis$^x$ trisaccharide (8). The asymmetric unit of this crystal form contains three dimers. The dimer interface is formed by contacts between the neck regions of two protomers. The CRDs of the dimer do not contact one another, and they also adopt different positions with respect to the neck, making the dimer asymmetric (Fig. 4, A and B).

In the DC-SIGN + Lewis$^x$ crystals, the neck region of each protomer consists of two discontinuous regions. Residues 246–264, corresponding to the last 19 residues of the last repeat, form a single $\alpha$ helix that ends at the CRD. Moving toward the N terminus of the protein, residues 235–245 are disordered. Residues 219–234, which are the first 16 residues of the penultimate neck repeat, form another $\alpha$ helix that runs antiparallel to, and packs against, the 246–264 helix. This generates an antiparallel, four-helix bundle stabilized by hydrophobic interactions (Fig. 4C). The missing electron density for the residues that connect the two $\alpha$ helices of each protomer makes it impossible to determine the topology of this assembly, i.e. to which chain the first $\alpha$ helix belongs.

The second crystal form of the truncated DC-SIGNR construct contains a single dimer in the asymmetric unit. This dimer packs against a dimer in a neighboring asymmetric unit that is related by a crystallographic 2-fold rotation axis, which generates a tetramer (Fig. 5A). In this crystal, only a portion of the CRD-proximal segment of the last neck repeat, residues 251–264, is visible. These residues form a single helix, which associates with its partners to form a parallel four-helix bundle (Fig. 5B). The disposition of the CRDs relative to the neck helix is different between the two protomers in the asymmetric unit (Fig. 5C), and their orientations also differ from either of those observed in the first crystal structure (Fig. 6 and Table III). These results, combined with the results showing that the junction between the neck and the CRD is very susceptible to proteolysis (Fig. 3), suggest that this linkage is flexible and that in the membrane CRDs can probably adopt a variety of orientations.

Both crystal forms of the truncated DC-SIGNR show that the last 14 residues of the last neck repeat form an $\alpha$ helix that associates in a parallel orientation with the same sequence from the other protomer of the dimer. The conserved hydrophobic heptad residues at positions 9, 13, 16, 20, and 23 of the...
The asymmetrically disposed CRDs. The position of the monomer B neck helix onto that of monomer heptad residues are shown in parallel four-helix bundle. The side chains of the conserved hydrophobic Ca$^{2+}$ are adjacent to the CRD. This protein was crystallized in the presence of a helical neck can be discerned corresponding to the two repeats that m e rAi si n cated DC-SIGNR comprising the CRD and two full neck repeats. Mono-

Crystallization of a protein in the presence of a crystallization agent, such as calcium ions, can facilitate the formation of a stable four-helix bundle. The side chains of conserved hydrophobic residues, such as the heptad residues, interact with the CRD, forming a stable interface. The stability of this interface is crucial for the protein's function as a receptor for specific carbohydrates, such as the Lewis$^a$ blood group substance.

**Table II**

| Number of neck repeats | $M_r$ Determined | $M_r$ Calculated | Cross-linking result | $M_r$ Determined | $M_r$ Calculated | Cross-linking result |
|------------------------|-----------------|-----------------|---------------------|-----------------|-----------------|---------------------|
| 7.5                    | $149 \times 10^3$ | $157 \times 10^3$ | Tetramer            | $143 \times 10^3$ | $150 \times 10^3$ | Tetramer            |
| 7.5                    | $143 \times 10^3$ | $151 \times 10^3$ | Tetramer            | $145 \times 10^3$ | $144 \times 10^3$ | Tetramer            |
| 7.0                    | $140 \times 10^3$ | $145 \times 10^3$ | Tetramer            | $137 \times 10^3$ | $138 \times 10^3$ | Tetramer            |
| 6.5                    | $132 \times 10^3$ | $140 \times 10^3$ | Tetramer            | 28.3 $\times 10^{3}$ | 42.9 $\times 10^{3}$ | Dimer               |
| 6.0                    | $140 \times 10^3$ | $135 \times 10^3$ | Tetramer            | 18.6 $\times 10^{3}$ | 17.7 $\times 10^{3}$ | Dimer               |
| 5.5                    | $84 \times 10^3$  | $130 \times 10^3$ | Tetramer            | 15.3 $\times 10^{3}$ | 16.2 $\times 10^{3}$ | Monomer             |
| 2.0                    | $22 \times 10^3$  | $23 \times 10^3$  | Monomer             |                 |                 |                     |
| 0.5                    | $17.8 \times 10^3$ | $17.2 \times 10^3$ | Monomer             |                 |                 |                     |

* ND, not determined.

**Fig. 4. Dimer crystal structure.** A, dimer crystal structure for truncated DC-SIGNR comprising the CRD and two full neck repeats. Monomer A is in red, and monomer B in cyan. For each monomer, two segments of a helical neck can be discerned corresponding to the two repeats that are adjacent to the CRD. This protein was crystallized in the presence of Ca$^{2+}$ (cyan spheres) and a trisaccharide ligand representing the Lewis$^a$ blood group substance (yellow bonds). B, superposition of the CRD-proximal neck helix of monomer B onto that of monomer A showing the asymmetrically disposed CRDs. C, close-up view of the antiparallel four-helix bundle. The side chains of the conserved hydrophobic heptad residues are shown in ball-and-stick representation.

**Fig. 5. Tetramer crystal structure.** A, tetramer crystal structure for truncated DC-SIGNR comprising the CRD and two full neck repeats. Application of a crystallographic 2-fold rotation to the two monomers in the asymmetric unit generates the tetramer. The two protomers in an asymmetric unit are shown in red and cyan. B, close-up view of the parallel four-helix bundle. The side chains of the conserved hydrophobic heptad residues are shown in ball-and-stick representation. C, superposition of the monomer B neck helix onto that of monomer A showing the asymmetrically disposed CRDs.

The repeat form the contacts between the helices. It appears, however, that this parallel two-helix arrangement is not a stable unit, as in both crystal forms these helices interact with other helices to form four-helix bundles. In particular, the hydrophobic heptad residues form additional contacts that stabilize the four-helix assembly, which largely buries them from the surrounding solvent. In one case the associations occur with a portion of the preceding neck repeat, which forms a helix that binds in an antiparallel orientation, whereas in the other, the four helices associate in a parallel bundle. The antiparallel arrangement seen in the first crystal form could explain the relatively stable dimer formed by this DC-SIGNR construct in solution. The complete absence of electron density for most of the neck sequence in the second crystal form, combined with the antiparallel orientation of the helices in the first crystal form, indicate that the C-terminal portion of the neck is a relatively unstable and dynamic structure in the absence of the N-terminal repeats. The parallel arrangement of helices in the...
second crystal form probably reflects the arrangement of this portion of the molecule in the full-length tetramer. These crystallization conditions would, thus, compensate for the absence of the N-terminal portion of the molecule, favoring the parallel packing of the last repeat.

Both crystal structures show that the A segment has a high propensity to form α-helix, as would be expected from the presence of a strongly conserved hydrophobic heptad motif at positions 9, 13, 16, 20, and 23 (Fig. 1 A). It is perhaps more surprising that residues 219–234, which includes the first B segment as well as a part of the A segment, form a helix in one of the crystal structures. Although the majority of B segments have a hydrophobic residue at motif position 6, which would be part of a canonical heptad pattern (Fig. 1 A), the last three repeats in DC-SIGN and the last repeat of DC-SIGNR have glutamine in this position. In the truncated molecules the glutamine residues may favor the kinking observed in the first crystal form, whereas in the intact molecule the aliphatic portion of the glutamine side chain could mediate hydrophobic packing interactions in a parallel four-helix bundle. Alternatively, the polar moieties of four glutamines present at a heptad position might interact in the core of a parallel four-helix bundle through a network of hydrogen bonds, as in the complex of syntaxin 1a and SNAP-25N (22). In either case, these observations show that the entire 23-residue neck repeat can adopt a helical structure, suggesting that in the intact receptor, the repeats essentially form a continuous helix. The presence of the conserved proline at the beginning of each repeat, however, could interrupt the helix or at least introduce a kink.

Modeling of Tetramer Structure Based on Hydrodynamic Properties—Information about the overall shape of DC-SIGN and DC-SIGNR was obtained from further hydrodynamic experiments conducted on truncated forms lacking the N-terminal linker domain and the first half repeat of the neck. These regions were omitted because the proteolytic digestion experiments suggested that these N-terminal regions may be partially unfolded in the absence of the membrane anchor. Sedimentation equilibrium experiments indicated that these proteins are stable tetramers (Table II). Sedimentation velocity experiments, gel filtration, and dynamic light scattering were used to derive sedimentation and diffusion coefficients for both molecules (Table IV). The molecular weights calculated from these values using the Svedberg equation are consistent with the values obtained from the equilibrium experiments. However, both the sedimentation and diffusion coefficients are significantly lower than expected for spherical molecules of this mass, indicating that the proteins are relatively elongated.

Modeling studies were used to determine what shapes would be consistent with the observed experimental values. The conformations of the neck and arrangement of CRDs observed in the two crystal structures were used as a basis for the modeling. The models assume that there is little flexibility between repeats, as indicated by the lack of digestion within the seven full neck repeats. The analysis of truncated constructs showed that the three N-terminal neck repeats are required for tetramerization, whereas further truncation results in dimers. In principle, this could arise either from destabilizing a single tetrameric neck region or from a neck in which the more N-terminal portions associate as a tetramer, but the more distal regions are dimeric. Therefore, two major classes of models were constructed; one in which the neck is a single, tetrameric domain and the other in which either the first few repeats associate as a tetramer and dimers flare off to form a Y-shaped structure.

The first class of models assumes that each repeat is a parallel, tetrameric coiled-coil of helices. Each of the four α-helices observed in the tetramer crystal structure were extended N-terminally to create a full 23-amino acid repeat. The six other repeats were then generated by rotation and translation of this repeat unit to make an elongated neck domain (Fig. 7A). The effect of the proline present at the start of each repeat was modeled in different ways. A straight stalk composed of continuous, but kinked, helices was constructed, with the conserved proline producing a kink of 20° between adjacent repeats within a protomer (Fig. 7A).

The alternative models have non-helical interruptions around the proline, which generate a somewhat jagged arrangement of tandem four-helix bundles (Fig. 7A).
b) from DC-SIGN.

The program HYDROPRO (19) was used to calculate sedimentation and diffusion coefficients based on the various models. Variations of the two classes of models were generated to match the experimental S and D values. Models for DC-SIGN were generated based on the DC-SIGNR models using the DC-SIGN CRD structure (Fig. 8) (16). DC-SIGN has a proline-rich 12-amino acid extension at its C terminus relative to DC-SIGNR that is not well defined in the crystal structure of the CRD, so it was added to the models in a conformation containing several turns to try to account for its contributions to the hydrodynamics. Because the crystal structures as well as proteolysis experiments indicate that there is a hinge point between the neck and the CRD, several different models with different angles between these two domains were evaluated. For the Y-shaped models, different angles between the dimeric stalks of the Y-shaped models were also considered. As shown in Figs. 7 and 8, models from both classes can be found that agree with the experimental sedimentation and diffusion coefficients. It is clear that models with completely elongated tetrameric stalks do not fit the data as well as slightly shorter and wider models with either the jagged arrangement of parallel tetramers or those with tetrameric stalks of five repeats followed by compact dimeric arms with an antiparallel arrangement of the last two repeats.

Differential Scanning Calorimetry—The structural analysis suggests that the CRDs of DC-SIGN and DC-SIGNR are flexibly linked to the tetrameric neck. This arrangement contrasts with the close and fixed association of the CRD with trimeric \( \alpha \)-helical neck regions in the collectins (23–25), and it suggests that the CRDs and the necks may be independently folded. Because the crystal structures as well as proteolysis experiments indicate that there is a hinge point between the neck and the CRD, several different models with different angles between these two domains were evaluated. For the Y-shaped models, different angles between the dimeric stalks of the Y-shaped models were also considered. As shown in Figs. 7 and 8, models from both classes can be found that agree with the experimental sedimentation and diffusion coefficients. It is clear that models with completely elongated tetrameric stalks do not fit the data as well as slightly shorter and wider models with either the jagged arrangement of parallel tetramers or those with tetrameric stalks of five repeats followed by compact dimeric arms with an antiparallel arrangement of the last two repeats.

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common lengths. The variant forms have been characterized by examining the length of PCR fragments that cover the entire exon. However, the sequences of the different forms have not been examined in detail. Because distinct roles can now be assigned to the different portions of the neck of DC-SIGNR, it was of interest to determine which repeats are present in each of the variants.

A panel of human genomic DNA was screened to identify examples of the variant forms in different human populations, and alleles corresponding to 4.5, 5.5, 6.5, and 7.5 repeats were sequenced. At least two examples of each form were sequenced, and in each case the same results were obtained. The results show that the length variation corresponds to the absence of specific repeats in the middle of the neck (Fig. 10). In all cases, the C-terminal two repeats adjacent to the CRD are present, consistent with the suggestion that these repeats are important in dimer formation. The N-terminal two repeats are also conserved, probably reflecting the importance of these repeats in stabilization of the tetramer. Thus, the pattern of genetic variation is consistent with the proposed importance of specific repeats in formation of the oligomer of DC-SIGNR.

**DISCUSSION**

Although the full structure of the neck regions of DC-SIGN and DC-SIGNR remain to be established, the results presented here suggest that the necks form extended stalks, stabilized largely by lateral interactions of α-helical regions in the 23-amino acid repeats. These results are consistent with previous data demonstrating that the neck is largely helical in character despite the presence of proline residues in the repeat regions (9). The cross-linking studies on intact receptors in cells provide evidence that the arrangement of subunits in the soluble extracellular domain faithfully represents the arrangement of these proteins at the cell surface. Based on the hydrodynamic data and modeling, the neck regions of DC-SIGN and DC-SIGNR would be expected to project the ligand binding CRDs at least 20 Å above the lipid bilayer. Like the sialic acid binding receptors sialoadhesin and CD22, DC-SIGN and DC-SIGNR have the ability to bind glycans that might be present on the same cells on which they are expressed. An extended neck region in sialoadhesin appears to place the ligand binding domain above the endogenous glycans, whereas the shorter neck in CD22 favors cis ligation (26). The extended necks of DC-SIGN and DC-SIGNR would, thus, be important in directing these receptors away from glycans on glycoproteins in the same cell membrane and toward pathogen surfaces.

DC-SIGN and DC-SIGNR are members of a large group of pathogen binding receptors that appear to have related and perhaps overlapping abilities to bind mannose-, GlcNAc-, and fucose-containing ligands. These sugar-binding proteins have been ascribed multiple roles in the innate and adaptive immune responses. Aside from DC-SIGN and DC-SIGNR, the best-characterized receptor in this group is mannose-binding protein. Whereas DC-SIGN has been proposed to function by binding to pathogens at the surface of dendritic cells, mannose-binding protein is a soluble serum protein that binds to pathogens and initiates complement fixation (27). Combined with recent studies of the DC-SIGN and DC-SIGNR binding sites (8), the present results demonstrate fundamental differences in the way that these receptors and mannose-binding protein bind to pathogens. Mannose-binding protein interacts exclusively with terminal monosaccharide units in oligosaccharides, and therefore, the individual CRDs interact with different glycans with roughly the same low affinity (28). Enhanced affinity and specificity for pathogen surfaces is achieved by formation of rigid trimers in which the binding sites are held in relatively widely spaced, fixed positions (23). This arrangement is ideally suited for binding to repeated terminal monosaccharide units on the surfaces of bacteria and fungi but prevents high affinity binding to endogenous mammalian glycoproteins.

In contrast to mannose-binding protein, the binding sites in DC-SIGN and DC-SIGNR are extended (8, 16); therefore, these CRDs display enhanced affinity for specific oligosaccharides, including endogenous high-mannose structures that are particularly abundant on envelope glycoproteins of human immunodeficiency virus and other viruses (29). Moreover, the CRDs in DC-SIGN and DC-SIGNR are flexibly linked to the neck regions that project them from the cell surface, which allows these receptors to interact with glycans presented with variable spacings on viral surfaces. The flexibility in DC-SIGN and DC-SIGNR can be likened to the flexibility in the hinge region of immunoglobulins, which enhances the ability of antibodies to bind with high avidity to polyclonal target antigens. Thus, both mannose-binding protein and DC-SIGN and DC-SIGNR bind avidly to target surfaces, but the balance in achieving this high affinity is different. Unlike mannose-binding protein, there would be an entropy penalty associated with the CRDs in a particular orientation upon binding of DC-SIGN or DC-SIGNR to a polyclonal ligand. This cost is presumably compensated by the higher affinity of the individual DC-SIGN and DC-SIGNR CRDs for particular oligosaccharides. Thus, although the extended binding sites of DC-SIGN and DC-SIGNR lead to recognition of a relatively restricted set of glycan structures (8), the ability of these receptors to interact multivalently with differently spaced glycans likely broadens their spectrum of high avidity ligands and results in the recognition of a distinct set of pathogens relative to mannose-binding protein.

**Acknowledgments**—We thank Dawn Torgersen for help with protein purification and Russell Wallis for help with the analytical ultracentrifugation experiments. Some of this work is based upon research at the Stanford Synchrotron Radiation Laboratory, a national facility operated by Stanford University for the Department of Energy, Office of Basic Energy Sciences. The Stanford Synchrotron Radiation Laboratory Structural Molecular Biology Program is supported by the Department of Energy, Office of Biological and Environmental Research and by the NIGMS, National Institutes of Health. Some diffraction data were measured at the Advanced Light Source, Lawrence Berkeley National Laboratory. The University of Oxford analytical ultracentrifugation facility is supported by grants from the Biotechnology and Biological Sciences Research Council and the Wellcome Trust.

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