Polymorphisms that affect the length of the extracellular neck region of the endothelial receptor DC-SIGNR (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin-related protein) have been linked to differences in susceptibility to infection by enveloped viruses. We have characterized the effects of these polymorphisms on the ability of DC-SIGNR to form tetramers containing the clusters of sugar-binding sites needed for binding to viral envelope glycoproteins. Chemical cross-linking and analytical ultracentrifugation experiments have been used to show that only the smallest form of DC-SIGNR is defective in homotetramer assembly. A novel affinity-tagging approach has been employed to demonstrate that, contrary to previous speculation, heterotetramers can be assembled efficiently from DC-SIGNR polypeptides of different lengths. The heterotetramers are stable and can be detected in fibroblasts transfected with multiple forms of DC-SIGNR. These results provide a molecular basis for interpreting the way polymorphisms affect interactions with viruses.

All but the Shortest Polymorphic Forms of the Viral Receptor DC-SIGNR Assemble into Stable Homo- and Heterotetramers*

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Polymorphisms that affect the length of the extracellular neck region of the endothelial receptor DC-SIGNR (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin-related protein) have been linked to differences in susceptibility to infection by enveloped viruses. We have characterized the effects of these polymorphisms on the ability of DC-SIGNR to form tetramers containing the clusters of sugar-binding sites needed for binding to viral envelope glycoproteins. Chemical cross-linking and analytical ultracentrifugation experiments have been used to show that only the smallest form of DC-SIGNR is defective in homotetramer assembly. A novel affinity-tagging approach has been employed to demonstrate that, contrary to previous speculation, heterotetramers can be assembled efficiently from DC-SIGNR polypeptides of different lengths. The heterotetramers are stable and can be detected in fibroblasts transfected with multiple forms of DC-SIGNR. These results provide a molecular basis for interpreting the way polymorphisms affect interactions with viruses.

The extracellular portion of DC-SIGNR (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin-related protein, also known as L-SIGN or CD209L) binds to high mannose oligosaccharides that are found on many viral coat proteins and mediates or facilitates infection of target cells. DC-SIGNR serves as a primary receptor for entry of Ebola and West Nile viruses into cells (1, 2), while presentation of hepatitis C virus (HCV) by DC-SIGNR on liver endothelial cells leads to infection of hepatocytes (3). Similarly, DC-SIGNR facilitates transmission of human immunodeficiency virus (HIV) across the placenta (4). For the severe acute respiratory syndrome coronavirus (SARS-CoV), roles in both infection of permissive cells and facilitating viral access to the target cells in lung and intestine have been postulated (5, 6).

The extracellular portion of DC-SIGNR consists of an extended neck region and a C-terminal carbohydrate recognition domain (CRD) that interacts with glycans on viral envelope glycoproteins (7). The neck region of the predominant form of DC-SIGNR is comprised of 7.5 highly conserved repeats containing 23 amino acids. In individual individuals, the number of repeats varies from 4.5 to 9.5 (Fig. 1) (8, 9). The allele frequency for the 7.5-repeat form is over 50%, but the 6.5- and 5.5-repeat forms are also common, with frequencies in the ranges of 12–16 and 26–37%. At the cell surface, the neck region of the 7.5-repeat form is over 50%, but the 6.5- and 5.5-repeat forms are also common, with frequencies in the ranges of 12–16 and 26–37%. At the cell surface, the neck region of the 7.5-repeat form is over 50%, but the 6.5- and 5.5-repeat forms are also common, with frequencies in the ranges of 12–16 and 26–37%. At the cell surface, the neck region of the 7.5-repeat form is over 50%, but the 6.5- and 5.5-repeat forms are also common, with frequencies in the ranges of 12–16 and 26–37%.
and mixed before renaturation. Material remaining soluble after dialysis was purified initially on mannose-Sepharose affinity columns. Following addition of CaCl₂ to a final concentration of 25 mM, the bound and eluted proteins were repurified on 1-ml galactose-Sepharose affinity columns.

Analysis of Doubly Transfected Fibroblasts—Reconstructed cDNAs encoding full-length DC-SIGNR with necks containing 6.5, 5.5, or 4.5 repeats and galactose-binding CRDs were inserted into vector pIREShyg 2 (Clontech), and Lipofectamine reagent (Invitrogen) was used to transfect rat fibroblasts expressing the 7.5-repeat form of full-length mannos-binding DC-SIGNR (14). Cell lines expressing two forms of DC-SIGNR, selected with G418 and hygromycin B, were washed with phosphate-buffered saline, harvested by scraping and centrifugation for 2 min at 1500 rpm, and sonicated briefly in Tris-buffered saline containing 1% Triton X-100. Following centrifugation, the supernatant was incubated with 150 mM glycine in phosphate-buffered saline for 20 min at 25 °C prior to sonication in lysis buffer (Tris-buffered saline containing 1% Triton X-100 before purification on galactose- and mannose-Sepharose. Proteins were detected by Western blotting using rabbit polyclonal antibody to DC-SIGNR (14).

Cell Surface Protein Biotinylation—Transfected cells were treated with 1 mg/ml biotinamidocaproate N-hydroxysulfosuccinimide ester (15) (Perbio) in phosphate-buffered saline for 30 min at 25 °C. Cells were washed and incubated with 100 mM glycine in phosphate-buffered saline for 20 min at 25 °C prior to sonication in lysis buffer (Tris-buffered saline containing 1% Triton X-100). Following centrifugation, the supernatant was incubated with 150 μl of a 50% suspension of avidin-conjugated beads (ImmunoPure immobilized avidin, Perbio) for 1 h at 25 °C. Biotinylated proteins were released by boiling in SDS sample buffer containing 1% β-mercaptoethanol.

RESULTS

Necks with at Least 5.5 Repeats Are Required to Generate DC-SIGNR Tetramers—The extracellular portion of the common 7.5-repeat form of DC-SIGNR was previously found to be a stable tetramer (12). In similar experiments, cross-linked tetratomers could be detected for the 6.5- and 5.5-repeat forms, but only monomers of the 4.5-repeat form were observed (Fig. 2). Analytical ultracentrifugation demonstrated that the 6.5- and 5.5-repeat forms are stable tetratomers and confirmed that the 4.5-repeat form does not self-associate (Table 1).

The shortest polymorphic form of DC-SIGNR could fail to assemble into tetratomers because a minimum number of repeats are needed to stabilize tetratomers or because the second repeat, present in the 5.5-repeat form but absent from the 4.5-repeat form, is critical for oligomerization. The sequence of repeat 2 differs from repeats 3 to 5 at a single amino acid residue (10). N-terminal truncation studies provide evidence to support both interpretations. The importance of repeat 2 is evident from comparison of the 5.5-repeat polymorphic form investigated here, which lacks repeats 5 and 6 and is a stable tetramer, with an artificially created 5.5-repeat version of DC-SIGNR lacking repeats 1 and 2, which was previously found to be partially dissociated (10).

The effect of differences in neck length was further evaluated by analyzing truncated versions of the polymorphic forms from which the first half repeat of the neck has been removed (Fig. 1). Cross-linking experiments showed that the resulting 6- and 5-repeat forms can assemble into tetratomers, while the 4-repeat form remains monomeric (Fig. 2). Analytical ultracentrifugation experiments confirmed that the 6-repeat form is a stable tetramer and the 4-repeat form is a single species corresponding to a monomer, but the 5-repeat form is partially dissociated at low protein concentrations (Table 1). In this case, a tetramer–dimer dissociation constant of 0.14 μM was measured. Thus, at least 5.5 neck repeats are required to stabilize the tetramer even when repeat 2 is present.

Taken together, these experiments show that stable homo-tetramer formation requires a minimum neck length and that repeat 2 plays a significant role in stability of the oligomer. Because the natural 4.5-repeat form of DC-SIGNR is short and lacks repeat 2, it is particularly unstable.

Extracellular Segments of DC-SIGNR with Different Neck Lengths Assemble into Heterotetramers—Because changes to either the N or C terminus of the DC-SIGNR polypeptide might perturb the formation of
oligomers, an alternative strategy was developed to monitor the assembly of hetero-oligomers containing polypeptides with different neck lengths. A short sequence from the galactose-binding CRD of the asialoglycoprotein receptor can confer galactose-binding activity on serum mannose-binding protein (16, 17), so this sequence was introduced into the CRD of the 7.5-repeat form of DC-SIGNR. The resulting protein could be purified on galactose-Sepharose but not mannose-Sepharose. The specificity swap was confirmed in solid phase binding assays, which revealed that the ratio of the $K_I$ values for $\alpha$-methylgalactoside and $\alpha$-methylmannoside changed by more than five orders of magnitude for the galactose-binding version (Table 2). Heterotetramers containing both this modified polypeptide and a smaller form with a normal mannose-binding CRD should have affinity for both mannose- and galactose-Sepharose (Fig. 3A).

Extracellular segments of mannose-binding 6.5-repeat and galactose-binding 7.5-repeat forms of DC-SIGNR were expressed separately in E. coli, and the guanidine-solubilized proteins were mixed and renatured by dialysis. Following initial chromatography on mannose-Sepharose, SDS-polyacrylamide gel electrophoresis revealed the presence of both 6.5- and 7.5-repeat polypeptides (Fig. 3B). Because the 7.5-repeat form does not bind to the mannose-Sepharose column on its own, the 7.5-repeat polypeptide must be associated with the mannose-binding 6.5-repeat polypeptide. Re-chromatography on a galactose-Sepharose column was used to remove homo-oligomers of the 6.5-repeat form, which do not bind this column. Thus the final double affinity-purified material, containing bands corresponding to both 6.5- and 7.5-repeat forms, represents hetero-oligomers of the two polypeptides.

Binding of C-type CRDs to monosaccharide affinity columns generally requires clusters of two or more CRDs interacting with the resin, so it seemed likely that the purified hetero-oligomer consists predominantly of two of each type of polypeptide. The tetrameric structure of the hetero-oligomers was confirmed by gel filtration chromatography (Fig. 4A). The double affinity-purified hetero-oligomer elutes at the same position as the 7.5- and 6.5-repeat homo-tetramers, showing that it also consists of four polypeptides.

**TABLE 2**

Inhibition of neoglycoprotein binding to wild type and modified DC-SIGNR

| Protein            | $K_I$, $\text{Man-}$ | $K_I$, $\text{Me-Gal}$ | $K_I$, $\text{Me-Gal}$/$\text{Me-Man}$ |
|--------------------|----------------------|------------------------|----------------------------------------|
| Wild type DC-SIGNR | 3.4 ± 0.3            | 111 ± 8                | 33 ± 5                                  |
| Modified DC-SIGNR  | 1100 ± 10            | 10.8 ± 2               | 0.010 ± 0.001                          |

**FIGURE 3.** Demonstration of hetero-oligomer formation by extracellular domain fragments of DC-SIGNR. A, scheme showing how double affinity chromatography can be used to detect hetero-oligomers containing DC-SIGN with mannose-binding CRDs and CRDs modified to bind galactose. B and C, isolation of hetero-oligomers using double affinity chromatography. Proteins were mixed in guanidine solution, renatured by dialysis, and loaded onto mannose-Sepharose affinity columns. Elution fraction E3 was further purified on galactose-Sepharose. For comparison, homotetramers of the 6.5- and 5.5-repeat forms were purified on mannose-Sepharose and homo-tetramers of the tagged 7.5-repeat form were purified on galactose-Sepharose. Fractions were analyzed on SDS-polyacrylamide gels that were stained with Coomassie Blue.
Heterotetramers of Viral Receptor DC-SIGNR

FIGURE 4. Gel filtration analysis of purified homo- and heterotetrameric extracellular segments. A, homotetramer of 7.5-repeat form (blue line), homotetramer of 6.5 repeat form (green line), and heterotetramer of 7.5- and 6.5-repeat forms (red line). B, homotetramer of 7.5-repeat form (blue line), homotetramer of 5.5-repeat form (green line), and heterotetramer of 7.5- and 5.5-repeat forms (red line).

FIGURE 5. Double affinity chromatography analysis of DC-SIGNR hetero-oligomers expressed in fibroblasts. The 6.5- and 5.5-repeat forms of DC-SIGNR tagged with galactose-binding CRDs were co-expressed with the mannose-binding 7.5-repeat form. Triton extracts of cells were loaded on galactose-Sepharose affinity columns, and bound material was repurified on mannose-Sepharose affinity columns.

Following incubation at 4°C for 2 weeks, both polypeptides in the hetero-oligomers could be re-bound quantitatively to galactose-Sepharose (data not shown). Thus, the oligomer must be extremely stable, because the 6.5-repeat form that was no longer associated with the galactose-binding 7.5-repeat form as a result of dissociation and random re-association would have passed through the column. Reverse experiments, in which homotetramers of 6.5- and 7.5-repeat forms were mixed and incubated, also failed to show evidence of dissociation and re-association to form hetero-oligomers.

All of the experiments were repeated for the 5.5-repeat form of DC-SIGNR in combination with a galactose-binding 7.5-repeat form, with comparable results (Figs. 3C and 4B). In analogous experiments in which the 4.5-repeat form was combined with the 7.5-repeat form, some hetero-oligomer was detected after the first affinity chromatography step, but it dissociated before the galactose-Sepharose chromatography (data not shown). The inability of the 4.5-repeat form to assemble into hetero-oligomers is consistent with the finding that this form also fails to assemble into stable homo-oligomers.

Hetero-oligomers Are Expressed in Cells—Assembly of hetero-oligomers with transmembrane and cytoplasmic domains was investigated by introducing galactose-binding versions of the full-length 4.5-, 5.5-, and 6.5-repeat forms into fibroblasts expressing the mannose-binding 7.5-repeat form of DC-SIGNR. Following initial purification on galactose-Sepharose, bands corresponding to both forms were observed on Western blots of cell lines expressing 7.5- and 6.5-repeat forms (Fig. 5A) or 7.5- and 5.5-repeat forms (Fig. 5B). Both polypeptides were still present following rechromatography on galactose-Sepharose, demonstrating that heterotetramers were formed. Because both homo- and hetero-oligomeric DC-SIGNR proteins are very stable, heterotetramers could not result from re-assortment of homotetramers following solubilization, and they must have assembled in cells before purification.

The presence of the hetero-oligomers at the cell surface was confirmed by cell-surface biotinylation followed by purification on avidin-conjugated beads (Fig. 6).

Cells transfected with the 4.5-repeat form of DC-SIGNR and selected for co-expression with the 7.5-repeat form were studied using the same protocols. Following initial chromatography of cell extracts on galactose-Sepharose, faint bands were detected only after trichloroacetic acid precipitation so that the entire elution fraction could be run on the gel (data not shown). This result indicates that the 4.5-repeat form is present at ~25-fold lower levels than the 5.5- and 6.5-repeat forms, so it must be inefficiently expressed or unstable.

DISCUSSION

The demonstration that DC-SIGNR with 5.5 or more repeats in the neck region can assemble into stable homo-oligomers as well as into hetero-oligomers with the more common 7.5-repeat form rules out several of the possible ways that the more common DC-SIGNR polymorphisms could be linked to differences in susceptibility to viral infections. These results suggest that functional DC-SIGNR would be expressed in individuals who are homozygous for the 5.5-, 6.5-, or 7.7-repeat forms as well as in individuals who are heterozygotes with at least one of these alleles. However, our results demonstrate that the behavior of the 4.5-repeat form of DC-SIGNR is significantly different from the other forms. Instability of homo- and hetero-oligomers of the extracellular domain, combined with inefficient expression of the full-length protein in cells, suggest that DC-SIGNR function would be severely compromised in individuals who are homozygous for this form, while those who are heterozygous for this form along with a longer form would express largely homo-oligomers of the longer form.

Although oligomers of most of the different forms of DC-SIGNR are stable, there are other ways that the neck length polymorphism could affect interaction with viruses. Shortening of the neck would significantly change the position of the CRDs relative to the cell surface, because hydrodynamic studies indicate that the neck is extended and each repeat projects the binding domains ~25 Å from the surface of the membrane (10). Evidence from other sugar-binding receptors suggests that projection of the binding domains above the glycocalyx may be necessary to prevent blocking of the binding sites with sugars on the
host cell surface (18, 19). Although initial studies indicate that the 5.5-, 6.5-, and 7.5-repeat forms can all bind to HIV at the cell surface (20), longer forms could be more accessible for binding of other viruses.

Given that hetero-oligomers of DC-SIGNR differing by at least two repeat lengths can assemble and remain stable, it is interesting to consider how hetero-oligomers of DC-SIGNR might be arranged. The membrane anchors ensure that the N-terminal ends of the extracellular domains will be in register. Based on structural and hydrodynamic evidence that the DC-SIGNR tetramer might be a dimer of dimers (10), it is possible that the hetero-oligomers might consist of two homodimers that associate at their N termini but diverge further from the membrane. The double affinity chromatography approach favors isolation of such heterotetramers with two of each type of polypeptide. However, for the extracellular domains, the data provide evidence that a portion of each mixed protein binds to the galactose affinity column with reduced affinity and elutes in the wash fractions (Fig. 3, B and C). This is the behavior that would be expected for an oligomer that contains only a single galactose-binding subunit and three mannose-binding subunits, suggesting that a 2:2 stoichiometry is not obligatory.

Although CRDs in a hetero-oligomer may project different distances from the membrane, the structural plasticity of the neck region observed in different crystal forms (10) means that individual repeats might be able to loop-out from the main stalk of the molecule, allowing both the N termini and the CRDs at the C termini to remain in register. Further structural analysis of the neck region may provide a basis for modeling such alternative conformations.

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**References**

1. Simmons, G., Reeves, J. D., Grogan, C. C., Vandenbergh, L. H., Baribaur, F., Whitbeck, J. C., Burke, E., Buchmeier, M. J., Solleux, E. J., Riley, J. L., Doms, R. W., Bates, P., and Pöhlmann, S. (2003) *Virology* **305**, 115–123

2. Davis, C. W., Nguyen, H.-Y., Hanka, S. L., Sánchez, M. D., Doms, R. W., and Pierson, T. C. (2006) *J. Virol.* **80**, 1290–1301

3. Gardner, J. P., Durso, R. J., Arrigale, R. R., Donovan, G. P., Mabbott, P. J., Dragic, T., and Olson, W. C. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 4498–4503

4. Solleux, E. J. (2003) *Clinical. Sci.*, **104**, 437–446

5. Jeffers, S. A., Tussell, S. M., Gillim-Ross, L., Hemmlia, E. M., Achenbach, J. E., Babcock, G. J., Thomas, W. D., Jr., Thackray, L. B., Young, M. D., Mason, R. J., Ambrosino, D. M., Wentworth, D. E., DeMartini, J. C., and Holmes, K. V. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 15748–15753

6. Chan, V. S. F., Chan, K. Y. K., Chen, Y., Poon, L. L. M., Cheung, A., N. Y., Zheng, B., Chan, K.-H., Mak, W., Ngan, H. Y. S., Xu, X., Scretton, G., Tam, P. K. H., Austyn, J. M., Chan, L.-C., Yip, S.-P., Peiris, M., Khoo, U.-S., and Lin, C.-L. S. (2006) *Nat. Genet.* **38**, 38–46

7. Solleux, E. J., Barton, R., and Trowsdale, J. (2000) *J. Immunol.* **165**, 2937–2942

8. Lichterfeld, M., Nischalke, H. D., van Lunzen, J., Söhne, J., Schneisser, J. K., and Spengler, U. (2003) *Clin. Immunol.* **107**, 55–59

9. Liu, H., Carrington, M., Wang, C., Holte, S., Lee, J., Greene, B., Hladik, F., Koele, D. M., Wald, A., Kurosawa, K., Rinaldo, C. R., Celum, C., Detels, R., Corey, L., McElrath, M. J., and Zhu, T. (2006) *J. Infect. Disease* **193**, 698–702

10. Feinberg, H., Guo, Y., Mitchell, D. A., Drickamer, K., and Wei, W. I. (2005) *J. Biol. Chem.* **280**, 1327–1335

11. Nattermann, J., Ahlensetil, G., Berg, T., Feldmann, G., Nischalke, H. D., Müller, T., Rockstroh, J., Woitas, R., Sauerbruch, T., and Spengler, U. (2006) *J. Viral. Hepatitis* **13**, 42–46

12. Mitchell, D. A., Fadden, A. J., and Drickamer, K. (2001) *J. Biol. Chem.* **276**, 28939–28945

13. Forstner, N., and Forath, J. (1975) *FEBS Lett.* **77**, 187–191

14. Guo, Y., Feinberg, H., Conroy, E., Mitchell, D. A., Alvarez, R., Blixt, O., Taylor, M. E., Wei, W. I., and Drickamer, K. (2004) *Nat. Struct. Mol. Biol.* **11**, 591–598

15. Davis, K. E., Straff, D. J., Weinstein, E. A., Bannerman, P. G., Correale, D. M., Rothstein, J. D., and Robinson, M. B. (1998) *J. Neurosci.* **18**, 2475–2485

16. Drickamer, K. (1992) *Nature* **360**, 183–186

17. Jobst, S. T., and Drickamer, K. (1994) *J. Biol. Chem.* **269**, 15512–15519

18. Crocker, P. R., Hartnell, A., Munday, J., and Nath, D. (1997) *Glycoconj. J.* **14**, 601–609

19. Crocker, P. R. (2002) *Curr. Opin. Struct. Biol.* **12**, 609–615

20. Gramberg, T., Zhu, T., Chaipan, C., Marzi, A., Liu, H., Wegele, A., Andrus, T., Hofmann, H., and Pohlman, S. (2006) *Virology* **347**, 354–363