DC-SIGN Binds to HIV-1 Glycoprotein 120 in a Distinct but Overlapping Fashion Compared with ICAM-2 and ICAM-3*

Received for publication, January 8, 2004, and in revised form, February 7, 2004 Published, JBC Papers in Press, February 16, 2004, DOI 10.1074/jbc.M400184200

Stephen V. Su‡, Patrick Hong‡, Sarah Baik‡, Oscar A. Negrete‡, Kevin B. Gurney‡, and Benhur Lee‡§∥

From the §Department of Microbiology, Immunology, and Molecular Genetics, the §Department of Pathology and Laboratory Medicine, ¶UCLA AIDS Institute, David Geffen School of Medicine, UCLA, Los Angeles, California 90095

DC-SIGN is a C-type lectin that binds to endogenous adhesion molecules ICAM-2 and ICAM-3 as well as the viral envelope glycoprotein human immunodeficiency virus, type 1, glycoprotein (gp) 120. We wished to determine whether DC-SIGN binds differently to its endogenous ligands ICAM-2 and ICAM-3 versus HIV-1 gp120. We found that recombinant soluble DC-SIGN bound to gp120-Fc more than 100- and 50-fold better than ICAM-2-Fc and ICAM-3-Fc, respectively. This relative difference was maintained using DC-SIGN expressed on three different CD4-negative cell lines. Although the cell surface affinity for gp120 varied by up to 4-fold on the cell lines examined, the affinity for gp120 was not a correlate of the ability of the cell line to transfer virus. Monosaccharides with equatorial 4-OH groups competed as well as D-mannose for the affinity for gp120 was not a correlate of the ability of the cell line to transfer virus. Monosaccharides with equatorial 4-OH groups competed as well as D-mannose for the cell line to transfer virus. Monosaccharides with equatorial 4-OH groups competed as well as D-mannose for the cell line to transfer virus. Monosaccharides with equatorial 4-OH groups competed as well as D-mannose for the cell line to transfer virus. Monosaccharides with equatorial 4-OH groups competed as well as D-mannose for the cell line to transfer virus. Monosaccharides with equatorial 4-OH groups competed as well as D-mannose for the cell line to transfer virus.

*This work was supported in part by the UCLA AIDS Institute, flow cytometry core UCLA CFAR Grant AI-28697 from the National Institutes of Health, and the James B. Pendleton Charitable Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Charles E. Culpepper Medical Scholar supported by the Rockefeller Brothers Fund, a recipient of the Burroughs Wellcome Fund career development award, and National Institutes of Health Grants RO1-AI55291 and R21-AI055305. To whom correspondence should be addressed: Dept. of Microbiology, Immunology, and Molecular Genetics, 3821 Molecular Sciences Bldg., 609 Charles E. Young Dr. East, Los Angeles, CA 90095. Tel: 310-794-2132; Fax: 310-267-2580; E-mail: benhurst@microbio.ucla.edu.

§The abbreviations used are: DCs, dendritic cells; HIV-1, human immunodeficiency virus, type 1; gp, glycoprotein; CRDs, carbohydrate recognition domains; HRF, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody.

This paper is available on line at http://www.jbc.org
some CRDs are able to bind to both protein and saccharide ligands (37, 38). Biochemical and structural studies using synthetic substrates demonstrate that DC-SIGN preferentially binds to oligomannosides such as those found on N-linked high mannose type glycoproteins (39, 39–41). Indeed, binding studies with envelope glycoproteins gp120 of feline immunodeficient virus (59) and gp120 of HIV-1 (2, 40) along with ICAM-2 (28) and ICAM-3 (30) confirm that DC-SIGN binds high mannose glycans on these glycoproteins. Additional carbohydrate profiling has led to the identification of Lewis blood group Ags (Lewis x, Lewis y, Lewis a, and Lewis b) (22) as additional oligosaccharide structures that are specifically bound by DC-SIGN. Furthermore, recent reports (25, 27) also identified the highly mannosylated surface lipoglycan liporabinomannan of M. tuberculosis as a ligand that specifically binds DC-SIGN.

Recently, a mucosually transmitted pathogenic SHIV variant (SHIV 162P) was shown to bind to DC-SIGN 3-fold better than its parental derivative, which was non-pathogenic and poorly transmissible. This gain in DC-SIGN binding function was mapped to an addition of an N-linked glycosylation site in the V2 loop (42). In addition, highly purified DC-SIGN+ DCs from human gut biopsies can bind and transfer HIV 1-100-fold more efficiently than DC-SIGN− DCs from the same tissue (43). These data suggest that DC-SIGN may play a role in the pathogenesis of mucosal HIV transmission, and the DC-SIGN/gp120 interface may be a legitimate target for antimicrobial therapeutics. However, because DC-SIGN also binds to endogenous ICAM-2 and ICAM-3, we wished to determine whether there were any differences that distinguish DC-SIGN binding to gp120 from binding to the two endogenous ligands ICAM-2 and ICAM-3.

In this study, we showed that recombinant gp120 binds with much greater affinity to DC-SIGN than ICAM-2 and ICAM-3 in in vitro and cell surface binding assays. The cell surface affinity of DC-SIGN for gp120 varied by up to 4-fold among the cell lines examined, but the cell surface affinity for gp120 did not correlate with the ability of DC-SIGN to transfer virus suggesting that cell-specific cofactors may be involved. Competition experiments using gp120, ICAM-2, and ICAM-3 revealed a preference for saccharide ligands with an equatorial 4-hydroxyl group. More extensive glycan array profiling also indicated that the presence of an α-anomeric glycosidic linkage contributed to the high affinity binding of cognate oligosaccharide ligands. Finally, targeted alanine-scanning mutagenesis of the CRD of DC-SIGN identified critical residues that differentiate DC-SIGN binding to each of the three ligands.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA restriction enzymes were obtained from New England Biolabs (Beverly, MA). High fidelity Ffu Turbo DNA polymerase was obtained from Invitrogen, and oligonucleotides were supplied from MWG Biotech (High Point, NC). All chemicals and monosaccharides were purchased from Sigma. Disaccharides and high binding certified 96-well plates were purchased from Fisher. Isopropyl-β-n-thiogalactoside was obtained from BioVectra (Oxford, CT). Anti-human Fc monoclonal antibodies conjugated with horseradish peroxidase (HRP) and the 1-step Ultra TMB substrate for ELISA were purchased from Pierce. Clonal antibodies conjugated with horseradish peroxidase (HRP) and the 1-step Ultra TMB substrate for ELISA were purchased from Pierce. The cell surface affinity for gp120 did not correlate with the ability of DC-SIGN to transfer virus suggesting that cell-specific cofactors may be involved. Competition experiments using gp120, ICAM-2, and ICAM-3 revealed a preference for saccharide ligands with an equatorial 4-hydroxyl group. More extensive glycan array profiling also indicated that the presence of an α-anomeric glycosidic linkage contributed to the high affinity binding of cognate oligosaccharide ligands. Finally, targeted alanine-scanning mutagenesis of the CRD of DC-SIGN identified critical residues that differentiate DC-SIGN binding to each of the three ligands.

**Differential Binding of DC-SIGN to gp120, ICAM-2, and ICAM-3**

A summary of this study is carried out in 1 liter of Luria broth supplemented with 100 μg/ml ampicillin. The culture was incubated further for at least 3 h before being harvested by centrifuging at 4000 × g for 10 min at 4 °C. The resulting pellet was resuspended in 10 ml of 100 mM NaH₂PO₄, 10 mM Tris·HCl, and 6 μg guanidine HCl, pH 8, and lysed by repeated sonication (5 bursts of 1-min duration at 4°C). The lysate was then supplemented with 0.01% β-mercaptoethanol and incubated at 4°C for 2 h. The lysate was then centrifuged at 150,000 × g for 30 min at 4°C in a Beckman 55.2Ti rotor, and the supernatant was incubated with 800 μl of nickel-nitritrolactic acid-agarose resin (Qiagen, Valencia, CA) (pre-equilibrated with denaturing buffer) at 4°C overnight. The resin was loaded onto a 30-cm chromatography column, and all subsequent washes were done with a 30-fold resin-volume excess of wash buffer starting with 1 M NaCl pH 8, 1 M CaCl₂, 6 μm urea, and 10 mM imidazole. The column was then washed again with same buffer except 15 mM imidazole was used. Successive washes of 30 mM Tris·HCl, pH 8, 0.5 mM NaCl in decreasing concentrations of urea starting with 5 mM urea were performed to renature the protein. The protein was eluted with 30 mM Tris·HCl, pH 8, 0.5 mM NaCl, 1 mM CaCl₂, and 1 mM imidazole.

**Saccharide Competition Assays**—20 μl of a 200 mM sDC-SIGN concentrate were coated onto high binding 96-well plates in the presence of 30 mM Tris·HCl, 30 mM NaH₂PO₄, and 1 mM CaCl₂ overnight at 4°C (20 μl per well). The plates were then blocked in the same buffer but supplemented with 5% bovine serum albumin (50 μl per well) at 37°C for 3 h. The plates were washed five times with wash buffer (Tris-buffered saline supplemented with 1 mM CaCl₂ and 0.1% Tween). 2.4, 300, and 150 mM of recombinant gp120-Fc, ICAM-2-Fc, and ICAM-3-Fc, respectively, were incubated in parallel with the indicated amounts of the various saccharides at room temperature for 2 h. The plates were then washed five times with wash buffer and incubated with anti-human Fc monoclonal antibody conjugated with HRP (Pierce) for 1 h at room temperature. The plates were then washed five times, and the amount of bound ligand was assessed with 1-step Ultra TMB substrate (Pierce). The colorimetric reading was performed on a spectrophotometer (Dynex Technologies, Chantilly, VA). For each saccharide, each experiment was performed at least three times, each time in duplicate.

**Direct Binding Assays**—A 200 nm solution of sDC-SIGN concentrate was coated onto high binding 96-well plates as described above. Increasing amounts of purified recombinant ligand were added and incubated at room temperature for 1 h. The plates were washed five times with wash buffer and incubated with anti-human Fc monoclonal antibody conjugated with HRP as above for 1 h at room temperature. The plates were washed five more times, and the binding was assessed by colorimetry as described above. For each ligand (gp120-Fc, ICAM-2-Fc, or ICAM-3-Fc), each experiment was performed at least three times in triplicate. Dissociation constants (K₅) were calculated using GraphPad Prism software (San Diego, CA). For the differential lectin binding experiments, 0.2 μg/ml of the indicated lectins were coated onto streptavidin plates (Pierce) for 2 h. Plates were washed three times with wash buffer (phosphate-buffered saline supplemented with 0.5% Tween and 2% bovine serum albumin). Ligands were added and bound at room temperature for 1 h, followed by three additional washes. HRP-conjugated goat anti-human Fc antibodies were added for 30 min to detect the amount of bound ligand. The activity of HRP was quantified colorimetrically as described above.

**Cell Surface Binding Assays**—The B-cell line HS Sultan and the monocytic cell line THP-1 stably expressing DC-SIGN were derived from a stable integration of a retroviral vector MIG/HOGF/-vector containing DC-SIGN (6). 293T cells expressing DC-SIGN were produced by transient CaPO₄ transfections with pCDNA3-DCSIGN construct according to standard protocols (6). The cell surface binding reaction was done as described previously (40). The cell surface K₅ values were calculated using the GraphPad Prism software and were obtained by titrating increasing amounts of the indicated ligand and binding each to the highest mean fluorescent intensity value obtained to 100%.

**Saccharide Chip Array**—96-Well format of monosaccharides was purchased from Glycominds (Israel). Binding assays with sDC-SIGN were performed according to manufacturer’s instructions. Briefly, the 96-well plate was washed five times with Tris-buffered saline supplemented with 0.5% Tween and 2% bovine serum albumin. The plate was washed five times with wash buffer and incubated with a mouse anti-DC-SIGN monoclonal antibody (DC028) for 1 h at room temperature. DC028 recognizes the repeat
Differential Binding of DC-SIGN to gp120, ICAM-2, and ICAM-3

RESULTS

Recombinant Soluble DC-SIGN Oligomerizes Similarly to DC-SIGN on Dendritic Cells—To delineate the differences in the binding affinities of gp120, ICAM-2, and ICAM-3 to DC-SIGN, we first measured the dissociation constant ($K_d$) of the three ligands in an immobilized solid-phase ELISA format. To this end, we expressed the extracellular domain of DC-SIGN (sDC-SIGN) as an N-terminal His$_6$-tagged fusion protein. To determine whether sDC-SIGN multimerizes into tetramers as has been demonstrated previously (39), we fractionated the recombinant protein on a 5–20% sucrose gradient. As can be seen in Fig. 1, purified recombinant sDC-SIGN readily formed trimers and tetramers in agreement with previous biochemical and physical studies (39). Most important, the higher ordered oligomers of purified sDC-SIGN corresponded approximately to the oligomeric state of full-length DC-SIGN proteins isolated from immature monocyte-derived dendritic cells (Fig. 1). To our knowledge, this is the first demonstration that full-length DC-SIGN on primary dendritic cells can also exist in the oligomeric state found for recombinant sDC-SIGN.

gp120 Binds to DC-SIGN with Greater Affinity Than ICAM-2 and ICAM-3—In order to facilitate our binding studies, HIV-1 gp120 was produced as an IgG1-Fc fusion protein (40). By using purified sDC-SIGN, we found that gp120-Fc bound to sDC-SIGN with 100- and 50-fold higher affinity than ICAM-2-Fc and ICAM-3-Fc, respectively (Fig. 2A). We then asked if the differential affinity of DC-SIGN for the three ligands was maintained when DC-SIGN was expressed on the cell surface. We chose three cell lines (THP-1, HS-Sultan, and 293T HEK cells) with no appreciable binding to gp120, ICAM-2, and ICAM-3 in the absence of DC-SIGN (Ref. 2 and data not shown). THP-1 and HS-Sultan cells were retrovirally transduced, and clones were isolated that stably expressed DC-SIGN at similar levels. HS Sultan is a mature B-cell line, and the functionality of DC-SIGN on B-cells has not been established previously. 293T HEK cells were transiently transfected with DC-SIGN. In all three cell lines, much like the in vitro binding studies, gp120-Fc bound to DC-SIGN with much greater affinity than ICAM-2-Fc and ICAM-3-Fc (Fig. 2B). However, the relative differences in binding affinity between ICAM-2-Fc and ICAM-3-Fc to DC-SIGN were cell-type dependent. For example, although ICAM-2 ($K_d = 6.66 \pm 0.88$ nM) bound better than ICAM-3 ($K_d = 17.0 \pm 1.74$ nM) on THP-1 DC-SIGN$^+$ cells, it bound less avidly than ICAM-3 on HS Sultan DC-SIGN$^+$ cells ($K_d = 57.9 \pm 4.09$ versus $13.92 \pm 1.38$ nM, respectively). Whereas gp120 showed the highest affinity binding to all three cell lines, gp120 bound to HS Sultan DC-SIGN$^+$ cells ($K_d = 0.017$ nM) cells 4-fold better than to 293T DC-SIGN$^+$ cells ($K_d = 0.078$ nM) transfectants and the THP-1 DC-SIGN$^+$ cells ($K_d = 0.068$ nM) cells (Fig. 2B). These data suggest that the ligand binding behavior of DC-SIGN may be modulated in a cell type-specific manner, although we cannot for-
with gp120. Although gp120 has a larger number of potential N-linked glycosylation sites than ICAM-2 and ICAM-3, the type of glycans (complex/hybrid versus high mannose) on each site can only be determined empirically. Therefore, we used different lectins of various specificities to determine the relative amounts of the major types of glycans on these three ligands. In an equilibrium binding assay, we found that Galanthus nivalis agglutinin bound to gp120 \( (K_d/H_{11005} = 0.15 \text{ mM}) \) with about 150-fold higher affinity compared with ICAM-2 \( (K_d/H_{11005} = 22.7 \text{ mM}) \) and ICAM-3 \( (K_d/H_{11005} = 20.4 \text{ mM}) \), respectively (Fig. 4).

Because \( G. \) nivalis agglutinin is a lectin that reacts most strongly with multiple terminal \( \alpha(1,3) \text{mannose} \) residues \((45)\), the data showed that gp120 contained more high mannose-type carbohydrates than ICAM-2 and ICAM-3. We also found that Datura stramonium agglutinin bound to gp120 \( (K_d/H_{11005} = 0.74 \text{ mM}) \) with a higher affinity than either ICAM-2 \( (K_d/H_{11005} = 76.3 \text{ mM}) \) or ICAM-3 \( (K_d/H_{11005} = 58.7 \text{ mM}) \) (Fig. 4). On mammalian cells, \( D. \) stramonium agglutinin binds to terminal GlcNAc moieties if they are not masked by galactose or sialic acid. Thus, gp120 may contain more terminal GlcNAc residues compared with ICAM-2 or ICAM-3.

In toto, the lectin binding profiles suggest that gp120 is glycosylated differently from ICAM-2 and ICAM-3 but that the similar \( K_d \) values of ICAM-2 and ICAM-3 for \( G. \) nivalis agglutinin and \( D. \) stramonium agglutinin, respectively, indicate that ICAM-2 and ICAM-3 are glycosylated similarly.

Glycan Specificities of DC-SIGN-mediated Binding—To assess further whether there are any differences in the type of glycans on gp120, ICAM-2, and ICAM-3 that mediate DC-SIGN binding, we used various monosaccharides and disaccharides to compete for ligand binding to immobilized sDC-SIGN. Monosaccharides with equatorial 4-hydroxyl groups (e.g. mannose, mannose derivatives, glucose, and fucose) preferentially com-

![Fig. 2.](http://www.jbc.org/figures/)

**Fig. 2.** gp120 binds with much higher affinity to DC-SIGN than ICAM2 and ICAM3. A, binding affinity of gp120-Fc, ICAM2-Fc, and ICAM3-Fc to recombinant sDC-SIGN. 200 nM of sDC-SIGN was coated on each well, and increasing concentration of the indicated ligand was added. The amount of ligand bound was detected colorimetrically using a human Fc-specific antibody conjugated to HRP. B, binding of gp120, ICAM-2, and ICAM-3 to cell surface DC-SIGN. Binding of the three ligands to cell surface DC-SIGN expressed on THP-1, HS-Sultan, and 293T cells was assessed by flow cytometry using R-phycoerythrin-conjugated anti-Fc antibodies (see "Experimental Procedures"). \( K_d \) values were obtained by titrating in the amount of ligand added and normalizing the maximal binding seen (in geometric mean fluorescence intensities) to 100%. Binding curves and \( K_d \) values were generated via Graphpad Prism™.

![Fig. 3.](http://www.jbc.org/figures/)

**Fig. 3.** HS Sultan DC-SIGN+ cells do not facilitate virus infection in trans despite having a higher affinity for HIV-1 gp120. 250-µg p24 equivalents of JR-CSF (an R5 HIV isolate) were added to \( 2.5 \times 10^4 \) of the indicated cells for 2 h at 37°C. Excess virus was washed away (4x) by media, and \( 2.5 \times 10^4 \) T-cell blasts were subsequently added to each well with the respective cells. Supernatants were half-exchanged with fresh media on days 0, 3, 5, and 7, and p24 levels in the supernatant were determined by a commercial p24 ELISA kit. Increased p24 levels over the course of 7 days is indicative of viral transfer and replication in the T-cell blasts as the parental HS Sultan and THP-1 cells are not permissive for viral replication. With gp120, although gp120 has a larger number of potential N-linked glycosylation sites than ICAM-2 and ICAM-3, the type of glycans (complex/hybrid versus high mannose) on each site can only be determined empirically. Therefore, we used different lectins of various specificities to determine the relative amounts of the major types of glycans on these three ligands. In an equilibrium binding assay, we found that Galanthus nivalis agglutinin bound to gp120 \( (K_d/H_{11005} = 0.15 \text{ mM}) \) with about 150-fold higher affinity compared with ICAM-2 \( (K_d/H_{11005} = 22.7 \text{ mM}) \) and ICAM-3 \( (K_d/H_{11005} = 20.4 \text{ mM}) \), respectively (Fig. 4). Because G. nivalis agglutinin is a lectin that reacts most strongly with multiple terminal \( \alpha(1,3) \text{mannose} \) residues (45), the data showed that gp120 contained more high mannose-type carbohydrates than ICAM-2 and ICAM-3. We also found that Datura stramonium agglutinin bound to gp120 \( (K_d/H_{11005} = 0.74 \text{ mM}) \) with a higher affinity than either ICAM-2 \( (K_d/H_{11005} = 76.3 \text{ mM}) \) or ICAM-3 \( (K_d/H_{11005} = 58.7 \text{ mM}) \) (Fig. 4). On mammalian cells, D. stramonium agglutinin binds to terminal GlcNAc moieties if they are not masked by galactose or sialic acid. Thus, gp120 may contain more terminal GlcNAc residues compared with ICAM-2 or ICAM-3. In toto, the lectin binding profiles suggest that gp120 is glycosylated differently from ICAM-2 and ICAM-3 but that the similar \( K_d \) values of ICAM-2 and ICAM-3 for G. nivalis agglutinin and D. stramonium agglutinin, respectively, indicate that ICAM-2 and ICAM-3 are glycosylated similarly.
Differential Binding of DC-SIGN to gp120, ICAM-2, and ICAM-3

Inhibition constants ($K_i$) for each monosaccharide and disaccharide were determined by a solid-phase competition assay. At least three independent experiments were done for each condition stated, and each experiment was done in duplicate. 95% confidence intervals (95% C.I.) are indicated in parentheses. ND, not done. The $K_i$ of each saccharide tested relative to the $K_i$ of d-mannose is shown by the ratio of $K_i$ saccharide/$K_i$ mannose.

| Saccharide       | gp 120-Fc | ICAM-2-Fc | ICAM-3-Fc |
|------------------|-----------|-----------|-----------|
|                  | $K_i$ (95% C.I.) | $K_i$ saccharide/$K_i$ mannose | $K_i$ (95% C.I.) | $K_i$ saccharide/$K_i$ mannose | $K_i$ (95% C.I.) | $K_i$ saccharide/$K_i$ mannose |
| Monosaccharides  | mM        |          | mM        |          | mM        |          |
| d-mannose        | 5.6 (3.7–8.7) | 1.0       | 3.5 (2.5–5.5) | 1.0       | 1.6 (0.6–3.8) | 1.0       |
| l-mannose        | 350.0 (105.3–1164) | 61.6      | ND        | ND        | 110.9 (57.2–214.9) | 68.8      |
| l-fucose         | 3.3 (2.0–5.4) | 0.5       | ND        | ND        | ND        | ND        |
| CH3-mannoside    | 3.0 (1.0–8.9) | 1.5       | 19.3 (8.1–46.5) | 5.5       | 0.9 (0.5–1.9) | 0.6       |
| d-galactose      | 63.5 (32.3–124.8) | 11.1      | 16.8 (4.5–63.3) | 4.7       | 68.8 (22.3–211.6) | 42.7      |
| CH3-galactose    | 163.0 (75.3–339.0) | 28.7      | 196.3 (94.5–457.1) | 55.7      | 117.9 (56.5–248.0) | 73.2      |
| l-glucose        | 8.0 (4.5–14.4) | 1.4       | 423.3 (149.5–1198) | 120.2     | 2.3 (0.6–8.3) | 1.4       |
| Disaccharides    |           |          |           |          |           |          |
| Maltose          | 0.2 (<0.1–0.7) | 0.1       | 5.5 (3.2–9.4) | 1.5       | 13.0 (4.2–40.1) | 8.1       |
| Celllobiose      | 1.4 (0.6–3.0) | 0.2       | ND        | ND        | ND        | ND        |
| Lactose          | 22.5 (11.5–43.9) | 3.9       | 21.3 (10.0–45.3) | 6.0       | 39.0 (8.5–178.7) | 19.8      |
| Sucrose          | 11.2 (5.6–22.6) | 1.9       | 76.8 (33.7–175) | 21.8      | 6.8 (3.1–15.2) | 4.2       |
played in a 96-well ELISA plate format. The carbohydrates were covalently coupled via a flexible linker to the surface, and this technology has been used to profile the saccharide binding specificities of carbohydrate-binding proteins such as lectins and antibodies (47–49). Here we used sDC-SIGN to bind to the immobilized saccharides in an attempt to characterize the glycan binding specificities of DC-SIGN. These direct binding studies are consistent with the competition data presented in Fig. 5 and Table I. Specifically, sDC-SIGN does not bind to any galactose, galactosides, or any various galactose linked to another sugar (Table II and data not shown). Rather, sDC-SIGN exhibited the greatest binding to glucose and mannose moieties, showing a strict specificity for the 4-OH group in an equatorial position (data not shown and see Ref. 39). In this assay, sDC-SIGN also tends to prefer glycosidic bonds linked in the α-anomeric configuration (Table II), consistent with the competition data shown in Table I.

**Mutational Analysis of DC-SIGN**—The competition and binding data suggest that DC-SIGN may bind differentially to gp120, ICAM-2, and ICAM-3. Therefore, we next sought to determine whether specific residues on DC-SIGN differentially contribute to gp120, ICAM-2, and ICAM-3 binding. To this end, we generated alanine scan mutants that correspond to most of the solvent-exposed amino acids implicated in the maintenance of calcium coordination or carbohydrate contacts in the crystal structure of the CRD of DC-SIGN (Fig. 6A). The 16 different mutations created represent various degrees of conservation among C-type mannose-specific lectins. All alleles were expressed in the HEK 293T cell line, and ligand binding was done similarly to that noted in Fig. 2B. A panel of conformation-independent and conformation-dependent anti-DC-SIGN monoclonal antibodies was used to assess the cell surface expression level and gross structural integrity of each DC-SIGN mutant. mAb 507 is a conformation-dependent antibody directed against the CRD and has the ability to block gp120

### Table II

**Oligosaccharide array analysis of DC-SIGN binding specificity**

| Saccharide                          | Relative light units |
|-------------------------------------|----------------------|
| Gal(β1-3) GalNac(β)                | <100                 |
| Gal(β1-4) Glc(β)                   | <100                 |
| Gal(β1-4) GlcNac(β)                | <100                 |
| Gal(β1-6) Gal(β)                   | <100                 |
| Glc(α1-4) Glc(α)                   | 2.87 × 10⁶           |
| Glc(α1-4) Glc(β)                   | 2.51 × 10⁶           |
| Glc(β)                             | 1.40 × 10⁵           |
| Glc(β1-3) Glc(β)                   | 5.29 × 10⁵           |
| Glc(β1-4) Glc(β)                   | 5.61 × 10⁵           |
binding to DC-SIGN (50), although its exact epitope is not known. mAb DC028 (10) is a conformation-independent antibody directed against the repeat domain of DC-SIGN and should not be directly affected by mutations in the CRD. As seen in Fig. 6B, the expression of wild-type DC-SIGN and its alleles was compared by normalizing the mean channel fluo-
rescence obtained for each allele (and for each antibody) to the wild-type expression, which was set at 100%. mAb DC028 will necessarily measure the absolute amounts of cell surface DC-SIGN, whereas mAb 507, whose epitope is unknown, could potentially be affected by a particular mutation. Therefore, the gp120, ICAM-2, and ICAM-3 binding to DC-SIGN and its alleles were normalized to the expression level as determined by mAb DC028 staining (Fig. 6C). In addition, because all the alleles except for P348A were recognized by mAb 507 at 50% or more of the wild-type level, we believe that none of these mutants exhibit gross perturbation of the structure of DC-SIGN. We believe the decreased expression of P348A is because of the absolute decrease in cell surface DC-SIGN as both DC028 and 507 staining revealed the same percent decrease in expression (Fig. 6B).

The observed phenotypes of the 16 mutants fall into several classes. First, as expected, mutations in residues highly conserved in all C-type lectins, Glu-354, Asp-355, and Asp-366, severely compromised binding to all three ligands. Glu-354 coordinates with calcium and interacts with 4-OH of mannose in the co-crystal structures of an oligosaccharide with both DC-SIGN and DC-SIGNR (41). The amino acid Asp-366 of DC-SIGN coordinates extensively with the calcium ion only. Residues Glu-347, Pro-348, and Asn-349 represent the tripeptide sequence that is a highly conserved signature motif for mannose-specific lectins. Both Glu-347 and Asn-349 residues make extensive contacts with the calcium ion and 3-OH of mannose as observed in the DC-SIGN CRD/oligosaccharide co-crystal structure. Most interesting, mutating Glu-347, Pro-348, and Asn-349 to alanine reduced gp120 binding much more substantially than ICAM-2 and ICAM-3 binding. Notably, the P348A mutant appeared to exhibit enhanced binding to the two adhesion molecules (Fig. 6C), although this could be an artificial effect of normalization as P348A was the only mutant that was expressed at significantly lower levels than all the other alleles (Fig. 6B). In sum, mutagenesis of these conserved residues suggests that the primary binding determinants of all three ligands to DC-SIGN were calcium- and mannose-dependent to a certain degree but also that DC-SIGN binds to gp120 in a similar but distinct fashion from ICAM-2 and ICAM-3.

Mutations of amino acids less conserved in C-type mannose lectins show a wide range of informative phenotypes. Mutating residues Asn-311, Arg-345, Val-351, Ser-360, Gly-361, and Asn-362 to alamines showed only a minimal effect on binding to all three ligands (Fig. 6C). Some of these results were somewhat surprising. For example, Asn-311 was postulated in the co-crystal structure, alongside Phe-313, to form a fitting groove for high mannose recognition that specifically discriminates binding against the inner branch point mannose (41) (Fig. 7D), and Ser-360 maintains extensive contacts with Man3 and Man4 in the pentasaccharide co-crystallized with the CRD of DC-SIGN. On the other hand, the mutants G346A and E353A diminished binding to gp120 by at least 50%, while not having a negative effect on ICAM-2 and ICAM-3 binding. Indeed, the G346A mutation appeared to enhance ICAM-2 and ICAM-3 binding by up to 2-fold. Surprisingly, Gly-346 and Glu-353 did not have direct interactions with the pentasaccharide moiety in the co-crystal structure with the CRD of DC-SIGN. Once again, these data suggest that DC-SIGN binding to the two endogenous ligands (ICAM-2 and ICAM-3) may involve unique elements from that required for gp120 binding, and that our mutagenic analysis using the “natural” ligands of DC-SIGN.

---

**C. DC-SIGN Mutant Binding to gp120, ICAM-2 and ICAM-3**

![Graph showing the % of wild-type binding for gp120, ICAM2Fe, and ICAM3Fe.]

**Legend:**
- Low (< 40%)
- Medium (40-80%)
- High (80-100%)

**Fig. 6—continued**

---
can provide information about the binding behavior of DC-SIGN that is not immediately obvious even from structural data. Finally, the mutant D367A diminished ICAM-2 and ICAM-3 binding by about 50% while enhancing gp120 binding by up to 2-fold, suggesting yet again that DC-SIGN binding to gp120 is qualitatively different from its binding to ICAM-2 and ICAM-3.

**DISCUSSION**

The present study provides evidence for the molecular determinants of DC-SIGN binding to a viral ligand, gp120, and its two endogenous ligands, ICAM-2 and ICAM-3. First, using purified components in an *in vitro* equilibrium binding assay, we showed that gp120 binds to sDC-SIGN with nanomolar affinity, 100- and 50-fold better than ICAM-2 and ICAM-3, respectively. When DC-SIGN is expressed on the cell surface, gp120 still bound with relatively much greater affinity than ICAM-2 and ICAM-3 on all three cell types examined. It is interesting to note that the affinity is much higher when binding is done on cell surface-expressed DC-SIGN. This difference in affinity may be due to cellular factor(s) affecting the binding behavior of DC-SIGN and/or the presence of other cognate binding partners that contribute to the total cell surface affinity of examined ligands. We note, however, that all three cell lines were CD4-negative and lack appreciable gp120, ICAM-2, and ICAM-3 binding activity in the absence of DC-SIGN expression.

We also note that the cell surface affinity of DC-SIGN for gp120 does not correlate with its ability to transfer HIV-1 to permissive T-cells. It has been reported that the capacity of DC-SIGN to transmit HIV to permissive T-cells is greater when it is expressed in THP-1 versus 293 cells (44), suggesting the presence of contributory cellular factors to the ability of DC-SIGN to facilitate infection in *trans*. Here we show that the difference in transmission between these cell lines was not due to the cell surface affinity for gp120 because the measured $K_d$ values for the two cell types were similar. Intriguingly, we also show that even on an antigen-presenting cell line (HS-Sultan) with a 4-fold higher affinity for gp120, DC-SIGN was still not able to facilitate virus infection in *trans*. For the moment, it appears that the ability of DC-SIGN to transfer virus is restricted to a cellular factor(s) common to dendritic cells and THP-1 cells.

Our competition data provide further insights into the molecular specificities of DC-SIGN glycan binding. Unlike the rat mannose-binding protein (51, 52) and the macrophage mannose receptor (46), structural and functional data all support the notion that DC-SIGN prefers to bind mannose- or glucose-derived oligosaccharides linked in an $\alpha$-anomeric configuration. Because our data indicate that gp120 contains more high mannose type sugars than ICAM-2 and ICAM-3, and it has been shown previously that most of the high mannose moieties in gp120 are linked in the $\alpha$-anomeric configuration (53-55), it is therefore not surprising that DC-SIGN binds with so much higher affinity to gp120 than to ICAM-2 or ICAM-3 (Fig. 2). Our competition data also indicate that DC-SIGN has a relatively high affinity for 1-fucose. This is consistent with published reports of the ability of DC-SIGN to bind to Lewis blood group antigens (22), which are composed of glycosphingolipids, largely defined by a difference in the position of the linkage following a difference in the number of fucose sugars present (56). Indeed, DC-SIGN specifically binds the bacterium *H. py*-
Differential Binding of DC-SIGN to gp120, ICAM-2, and ICAM-3

lori (22) and the parasitic worm S. mansonii (24) both of which express the Lewis x antigen.

However, it has become increasingly evident that discerning the glycan binding specificities of DC-SIGN is a bit more complex with respect to what glycans it is predicted to bind versus the actual glycan moieties it does bind on its natural ligands. Although DC-SIGN seems to bind to the vector-borne parasite L. pifanoi, the interaction is not inhibitable by mannan (23), which to date has inhibited DC-SIGN binding to all known ligands. It is possible that DC-SIGN binds via another high affinity glycan found on L. pifanoi that is yet to be characterized or that protein/protein interactions are actually involved in binding. Also, our data showed that maltose is a highly effective inhibitor of gp120 binding to DC-SIGN suggesting that dimannosides would be as good or better ligands for DC-SIGN. Yet synthetic glycolusters harboring dimannoside substrates failed to bind to cells expressing DC-SIGN (57). Nonetheless, convincing evidence shows that the binding of DC-SIGN to M. tuberculosis is due to the dimannoside, Manα1,2Man, cap of the lipoglycan lipoparabinomannan (ManLam) (25, 27). The binding of this native substrate ManLam is specific to the dimannoside cap because AraLam (ManLam devoid of the mannosic cap) fails to bind to DC-SIGN, and enzymatic cleavage of the dimannosides from ManLam abrogated its ability to compete for DC-SIGN binding to M. tuberculosis. Thus, it prudent to bear in mind the difference between what ligands DC-SIGN can or cannot bind when presented as neo-glycoconjugates versus what ligands it does bind when presented on a biologically relevant molecule.

Our alanine-scanning mutagenesis of most of the solvent-exposed amino acids of DC-SIGN CRD reveals several insights into DC-SIGN function. By using the schematic presented in Feinberg et al. (41) (Fig. 7A), we have mutations at the highly conserved end of the “oligosaccharide binding valley” as exemplified by residues Glu-347, Asn-349, Glu-354, Glu-355, and Asp-366 that significantly diminished binding to all three ligands presumably by destroying calcium coordination and hydrogen bonding to the 4-OH of Man2 (Fig. 7B). It is most likely that this one end of the valley contributes the greatest amount of binding energy to the DC-SIGN/ligand interaction because the consensus motif for all mannose-specific and C-type lectins involves protein/protein in addition to protein/carbohydrate interactions. It is not uncommon that lectins bind to both saccharides while enhancing ICAM-2 and ICAM-3 binding, suggesting that Phe-313 may partially obscure more important interactions with gp120 (as changing this residue to alanine enhances gp120 binding). Conversely, the G346A mutant almost abrogated gp120 binding while enhancing ICAM-2 and ICAM-3 binding. In the crystal structure, Asp-367 resides in the highly conserved end of the pocket and makes extensive water-mediated bonding to the 6th carbon hydroxyl group of Man2. The mutant suggests that Asp-367 is important for ICAM-2 and ICAM-3 because substituting alanine at this position reduced binding by more than 50% and also suggests that Asp-367 may partially obscure more important interactions with gp120 (as changing this residue to alanine enhances gp120 binding). Conversely, the G346A mutant almost abrogated gp120 binding while enhancing ICAM-2 and ICAM-3 binding, suggesting that ICAM-2 and ICAM-3 binding to DC-SIGN may be qualitatively different from gp120. This same residue in DC-SIGNR, a homologue of DC-SIGN, makes water-mediated contacts with the 6th carbon hydroxyl group and may be important for saccharide binding. This observation, coupled with the fact that D-glucose competes more than 100-fold less efficiently than D-mannose for ICAM-2-Fc binding to DC-SIGN, argues once again that ICAM-2 and ICAM-3 binding to DC-SIGN may involve protein/protein in addition to protein/carbohydrate interactions. It is not uncommon that lectins bind to both saccharide and protein determinants concurrently (37, 38). Further experimentation is warranted to determine the validity of this hypothesis.

In this study, we show that DC-SIGN binds to HIV-1 gp120, ICAM-2, and ICAM-3 in a distinct but overlapping fashion. We also provide biochemical and genetic evidence that DC-SIGN binding to ICAM-2 is qualitatively different from DC-SIGN binding to gp120 and ICAM-3. Most important, we have identified residues that differentially contribute to binding all three ligands. Detection of such differences may be useful for future therapeutic intervention targeting the gp120-DC-SIGN interface.

Acknowledgments—We thank Linda Baum for critical review of this manuscript. We thank Kurt Drickamer for the initial gift of recombinant soluble DC-SIGN and Sophia Young for technical assistance in making recombinant soluble DC-SIGN in the Lee Lab.
