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Identification of Different Binding Sites in the Dendritic Cell-specific Receptor DC-SIGN for Intercellular Adhesion Molecule 3 and HIV-1*

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The novel dendritic cell (DC)-specific human immunodeficiency virus type 1 (HIV-1) receptor DC-SIGN plays a key role in the dissemination of HIV-1 by DC. DC-SIGN is thought to capture HIV-1 at mucosal sites of entry, facilitating transport to lymphoid tissues, where DC-SIGN efficiently transmits HIV-1 to T cells. DC-SIGN is also important in the initiation of immune responses by regulating DC-T cell interactions through intercellular adhesion molecule 3 (ICAM-3). We have characterized the mechanism of ligand binding by DC-SIGN and identified the crucial amino acids involved in this process. Strikingly, the HIV-1 gp120 binding site in DC-SIGN is different from that of ICAM-3, consistent with the observation that glycosylation of gp120, in contrast to ICAM-3, is not crucial to the interaction with DC-SIGN. A specific mutation in DC-SIGN abrogated ICAM-3 binding, whereas the HIV-1 gp120 interaction was unaffected. This DC-SIGN mutant captured HIV-1 and infected T cells in *trans* as efficiently as wild-type DC-SIGN, demonstrating that ICAM-3 binding is not necessary for HIV-1 transmission. This study provides a basis for the design of drugs that inhibit or alter interactions of DC-SIGN with gp120 but not with ICAM-3 or vice versa and that have a therapeutic value in immunological diseases and/or HIV-1 infections.

Transmission of human immunodeficiency virus type 1 (HIV-1)[1] infection in humans requires the dissemination of virus from sites of infection at mucosal surfaces to T cell zones in secondary lymphoid organs, where extensive viral replication occurs in $CD4^+$ T-helper cells. HIV-1 enters these cells through the interaction of the viral envelope glycoprotein gp120 with its primary receptor CD4 and members of the chemokine receptor family, primarily CCR5 and CXCR4 (1, 2). Mechanisms of early viral dissemination remain uncertain, but anatomical distribution and *in vitro* infectivity studies infer that immature dendritic cells (DC) residing in the skin and at mucosal surfaces are the first cells to be targeted by HIV-1 (3–5).

Upon pathogen infiltration, immature DC migrate specifically to sites of inflammation to capture pathogens. Captured pathogens are processed into antigenic peptides and presented on major histocompatibility complex class II molecules at the surface of DC. DC mature and migrate to the secondary lymphoid organs, where they interact with T cells to initiate an immune response. HIV-1 is thought to subvert the trafficking capacity of DC to gain access to the $CD4^+$ T cell compartment of lymphoid tissues where DC enhance the infection of $CD4^+$ T cells by HIV-1 (3, 4, 6). The molecular basis behind this process remained unclear until we recently identified DC-SIGN (DC-specific ICAM-3-grabbing nonintegrin) through its high affinity interaction with the intercellular adhesion molecule 3 (ICAM-3) (7, 8).

DC-SIGN binds with high affinity to the HIV-1 envelope glycoprotein gp120 (K_d = 1.7 nM (9)), and we demonstrated that DC-SIGN binds both M- and T-tropic HIV-1 and enhances infection of T cells in *trans* (8). Furthermore, DC-SIGN is expressed by immature DC localized in those mucosal tissues involved in sexual transmission of HIV-1. Thus, DC-SIGN endows DC with the ability to efficiently capture HIV-1, even when the virus is present in minute amounts. HIV-1 subsequently exploits the migratory capacity of DC to gain access to the T cell areas of lymphoid tissues, where DC-SIGN enhances the infection of residing $CD4^+$ T cells by HIV-1.

The cellular ligand of DC-SIGN, ICAM-3, is highly expressed on naive T cells, and we demonstrated that DC-SIGN binding to ICAM-3 initiates the DC-T cell interaction necessary for T cell activation (7). Blocking antibodies against DC-SIGN inhibit the DC-T cell clustering and the subsequent T cell activation, demonstrating the importance of this first step in DC-T cell contact (7).

Here, we have investigated the mechanism of both ligand and Ca^{2+} binding by DC-SIGN. Strikingly, ICAM-3 recognition by DC-SIGN is dependent on *N*-glycosylation of the ligand, whereas HIV-1 gp120 binding is independent of both *N*- and *O*-linked glycosylations. Differences in ligand binding were further confirmed by site-directed mutagenesis and three-dimensional modeling. We generated a DC-SIGN mutant that specifically bound HIV-1 gp120 but not ICAM-3 and that efficiently mediated HIV-1 infection of T cells in *trans*. Thus, DC-SIGN has different binding sites for HIV-1 gp120 and ICAM-3. This information will be essential in the successful development of preventative and therapeutic strategies in the treatment of HIV-1 and opens up the possibility of designing drugs specifically inhibiting interactions of DC-SIGN with one ligand without affecting that of the other ligand.

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus type 1; DC, dendritic cell(s); ICAM, intercellular adhesion molecule; ELISA, enzyme-linked immunosorbent assay; CRD, carbohydrate recognition domain; RHL-1, rat hepatic lectin-1; MBP, mannose-binding protein.

EXPERIMENTAL PROCEDURES

Antibodies and Proteins—The monoclonal antibodies against DC-SIGN are AZN-D1 and AZN-D2 (7). Glycosylated and nonglycosylated HIV-1_{SF2} gp120 was provided by M. Quiroga through the NIH AIDS Research and Reference Reagent Program. Nonglycosylated HIV-1 gp120 was produced in yeast using an intracellular expression vector lacking the signal sequence, thereby preventing passage through the secretory pathway and thus the addition of carbohydrates (10). Peptide-*N*-glycosidase F and endo-*N*-acetylgalactosaminidase were obtained from Oxford Glycosciences (Wakefield, MA).

Mutagenesis—Mutations in the cDNA encoding DC-SIGN were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). K562 transfectants either expressing wild-type or mutant DC-SIGN were generated by transfection of K562 cells with 10 μg of plasmid by electroporation as described previously (8). Positive cells were sorted several times to obtain stable transfectants with similar expression levels of DC-SIGN. DC-SIGN-Fc consists of the extracellular portion of DC-SIGN (amino acid residues 64–404) fused at the C terminus to a human IgG1-Fc fragment into the Sig-pIgG1-Fc vector (11). DC-SIGN-Fc was produced in Chinese hamster ovary K1 cells by co-transfection of DC-SIGN-Sig-pIgG1 Fc (20 μg) and pEE14 (5 μg) vector. DC-SIGN-Fc concentrations in the supernatant were determined by an anti-IgG1 ELISA.

Adhesion—The fluorescent bead adhesion assay was performed as described earlier (7). Adhesion was determined in the presence of either 5 mM mannan, 5 mM EGTA, or 20 μg/ml antibodies against DC-SIGN. The soluble DC-SIGN adhesion assay was performed as follows. Soluble ligands were coated in ELISA plates (1 μg/well) for 1 h at 4 °C, followed by blocking with 1% bovine serum albumin for 30 min at 4 °C. Soluble DC-SIGN-Fc supernatant was added, and the adhesion was performed for 30 min at 37 °C. Unbound DC-SIGN-Fc was washed away, and binding was determined by an anti-IgG1 ELISA. Specificity was determined in the presence of either 20 μg/ml blocking antibodies or 5 mM EGTA.

HIV-1 Infection Assays—The infection assays were performed as described previously (8). Pseudotyped viral stocks were generated by calcium phosphate transfections of 293T cells with the proviral plasmid pNL-Luc-E^-R^-, containing a luciferase reporter gene and an expression plasmid for the ADA gp160 envelope. Briefly, DC-SIGN transfectants were incubated for 2 h with identical pseudotyped HIV-1_{ADA} virus concentrations, unbound virus was washed away, and phytohemagglutinin-activated T cells were added. Cell lysates were obtained after 2 days and analyzed for luciferase activity.

Modeling—We have built the model of the C-type lectin domain of DC-SIGN by exploiting the sequence similarity to the carbohydrate recognition domain (CRD) of the asialoglycoprotein receptor, which was solved using x-ray crystallography (12) (Protein Data Bank entry 1DV8). The modeling was done with WHAT IF (13), following the protocol as previously described (14). Three insertions (Arg^{312}-Phe^{313}, Leu^{321}-Asn^{322}, and Tyr^{342}) were added to the model of the asialoglycoprotein receptor by copying the respective loop conformations from two other homologous templates (Protein Data Bank entries 1ESL and 1HLJ) to provide a structure with high homology to the CRD of DC-SIGN. The model was minimized with the YASARA NOVA force field, which was parameterized for the refinement of homology models and was shown to routinely reduce the C<α> root mean square deviation.[2] With 50% sequence identity between DC-SIGN and the template 1DV8, homology modeling is commonly considered straightforward (14). Validation of the model with WHAT_CHECK (15) revealed no large problems in the model structure. The model coordinates are available on the World Wide Web at www.cmbi.kun.nl/gv/service/dcsign/.

RESULTS

Glycosylation Is Required for ICAM-3 but Not for HIV-1 gp120 Binding—DC-SIGN, a type II transmembrane protein with a C-terminal C-type lectin domain, binds with high affinity to both the immunoglobulin superfamily member ICAM-3 (7) and the HIV-1 envelope glycoprotein gp120 (8). We used K562 transfectants stably expressing DC-SIGN to specifically investigate the binding characteristics of DC-SIGN in the absence of any other ICAM-3- or HIV-1-receptors, since this erythroleukemic cell line does not express leukocyte function-associated molecule 1 and CD4. DC-SIGN expressed by these

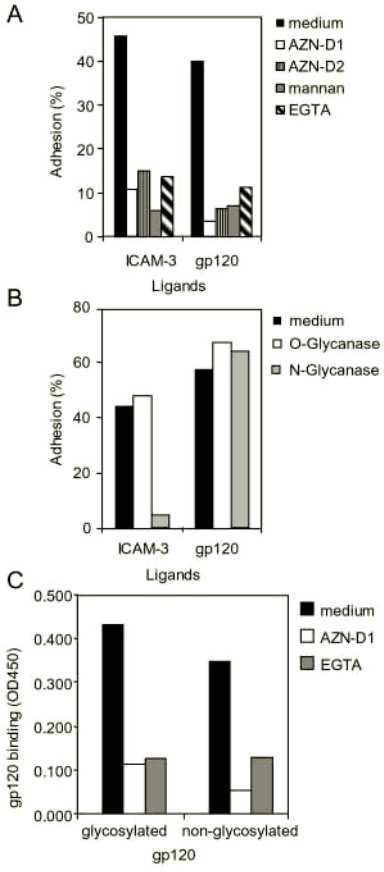

FIG. 1. **The interaction of DC-SIGN with HIV-1 gp120, in contrast to ICAM-3, is glycosylation-independent.** *A*, DC-SIGN, expressed by K562 transfectants, binds strongly to both ICAM-3 and HIV-1 gp120. The adhesion to both HIV-1 gp120 and ICAM-3 was determined using the fluorescent bead adhesion assay. One representative experiment out of three is shown. *B*, enzymatic removal of both *O*- and *N*-linked glycosylations from HIV-1 gp120 does not affect binding to DC-SIGN in contrast to removal of *N*-linked glycosylations from ICAM-3. Both ICAM-3 Fc and HIV-1 gp120 were treated with either endo-*N*-acetylgalactosaminidase or peptide-*N*-glycosidase F. The fluorescent bead adhesion assay was performed with the deglycosylated proteins coated on the beads. One representative experiment out of two is shown. *C*, nonglycosylated HIV-1 $gp120_{SF2}$ binds to DC-SIGN, similarly to the glycosylated form of the envelope glycoprotein. Soluble DC-SIGN-Fc binding to both nonglycosylated and glycosylated $gp120_{SF2}$ is determined by an Fc ELISA. Specificity is determined by measuring binding in the presence of blocking antibodies against DC-SIGN or EGTA. One representative experiment out of three is shown.

cells is fully functional (Fig. 1*A*), with a binding activity similar to that observed for DC-SIGN expressed by DC (7, 8). DC-SIGN-mediated adhesion to both ICAM-3 and gp120 is completely inhibited by the blocking antibodies against DC-SIGN, the calcium chelator EGTA, and the polysaccharide mannan (Fig. 1*A*). The Ca^{2+} dependence confirms that ligand binding is mediated by the C-type lectin domain of DC-SIGN, which interacts also with mannose-type carbohydrates, since adhesion to its natural ligands was inhibited by the polycarbohydrate mannan (Fig. 1*A*).

Both ICAM-3 and gp120 are heavily glycosylated (16, 17), and these post-translational modifications could be important for the interaction with DC-SIGN. Enzymatic removal of the *O*-linked glycosylations from both ligands had no effect on their binding to DC-SIGN (Fig. 1*B*). HIV-1 gp120 contains *O*-linked glycosylations (18), as was confirmed by SDS-PAGE analysis after deglycosylation (data not shown), whereas no shift in molecular weight was observed in ICAM-3 after endo-*N*-acetylgalactosaminidase treatment (data not shown), sug-

[2] E. Krieger, unpublished results.

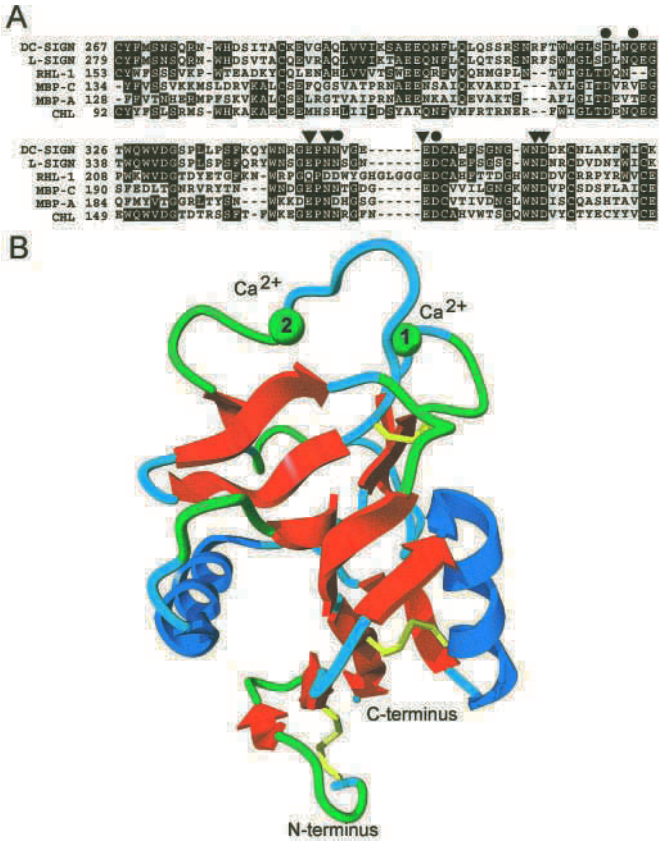

FIG. 2. **The three-dimensional structure of the C-type lectin domain of DC-SIGN.** *a*, amino acid sequence alignment of the CRD of DC-SIGN (AAK20997) with that of other homologous C-type lectins, L-SIGN (AAK20998), RHL-1 (P02706), rat liver MBP-C (P08661), rat serum MBP-A (P1999), and chicken hepatic lectin (*CHL*; P02707). Homologous amino acids involved in Ca^{2+} coordination, both at site 1 (*filled circles*) and 2 (*filled arrows*) and ligand binding (*filled arrows*) are depicted. *B*, a *ribbon diagram* of the CRD of DC-SIGN. The two α-helices are shown in *blue*, the β-strands in *red*, the calcium ions at sites 1 and 2 in *green*, and the three disulfide bridges in *yellow*. Both the N and the C termini are shown at the *bottom*. The structure was determined by molecular modeling. All *images* of the structure were generated using YASARA (available on the World Wide Web at www.yasara.com).

gesting that ICAM-3 does not contain *O*-linked glycosylations. In contrast, removal of the *N*-linked glycosylations from ICAM-3 by peptide-*N*-glycosidase F completely abrogated the adhesion (Fig. 1*B*), demonstrating that *N*-linked carbohydrates are involved in this interaction. In contrast, treatment of HIV-1 gp120 with peptide-*N*-glycosidase F did not result in a decreased interaction. It is possible that the carbohydrates were not completely removed, although it appeared that the *N*-linked glycosylations had been removed from gp120 as determined by SDS-PAGE analysis (data not shown). Therefore, we used nonglycosylated recombinant gp120 and observed that DC-SIGN interacts with this nonglycosylated gp120 in a similar way as with native gp120, derived from mammalian cells (Fig. 1*C*). The interaction with nonglycosylated gp120 was blocked by the anti-DC-SIGN antibodies as well as by EGTA. These results confirm that glycosylations are not necessary for the interaction of HIV-1 gp120 with DC-SIGN, whereas *N*-linked but not *O*-linked oligosaccharides are essential for the binding of ICAM-3.

Structure of DC-SIGN—DC-SIGN belongs to the C-type lectin family (Fig. 2*A*), and we decided to obtain structural information about the C-type lectin domain of DC-SIGN, through comparison with other C-type lectins, to identify crucial amino acid residues involved in ligand binding by site-directed mutagenesis. The CRD of DC-SIGN has 50% identity with that of the H1 subunit of the asialoglycoprotein receptor (rat hepatic lectin-1 (RHL-1)) and 39% with that of the rat serum mannose-binding protein (MBP-A). We performed molecular modeling with the crystal structure of the RHL-1 (12) as a template because of the high identity with RHL-1, and we generated a three-dimensional structure of the C-type lectin domain of DC-SIGN (amino acid residues 267–381; Fig. 2*B*). The CRD of DC-SIGN is a globular protein consisting of 12 β-strands, two α-helices, and three disulfide bridges. The β-strands are arranged in three β-sheets and form the core of the protein with an α-helix on each side of this core. One prominent loop sticks out from the protein surface and forms part of the two Ca^{2+}-binding sites, designated site 1 and site 2. Ca^{2+} is essential to the activity of DC-SIGN (Fig. 1*A*); thus, these sites are probably part of the ligand binding site.

The Primary Ligand-binding Site of DC-SIGN—Amino acid sequence alignment of DC-SIGN with other C-type lectins indicates that four amino acids, Glu^{347}, Asn^{349}, Glu^{354}, and Asn^{365}, interact with the Ca^{2+} at site 2 through their carbonyl groups (Figs. 2*A* and 3*A*). We hypothesized that these four amino acid residues might also participate in the interaction of DC-SIGN with its ligands via hydrogen bonds as was shown for other C-type lectins such as MBP-A (19). Site-directed mutagenesis was used to assess the role of these residues in ligand binding by DC-SIGN. Changing either Glu^{347} into Gln or Asn^{349} and Asn^{365} into Asp resulted in complete loss of both ICAM-3 and gp120 binding (Table I). Structural integrity was retained within these mutants, since the interaction with antibodies against DC-SIGN was unaffected (Table I). Loss of ligand binding due to these mutations could be ascribed to elimination of a single hydrogen bond between DC-SIGN and its ligand through removal of either a hydrogen donor (amide group in Asn) or acceptor (acidic group in Glu) (Fig. 3*A*). Ca^{2+} binding in these mutants will not be affected, since the altered amino acid residues (Glu into Gln and Asn into Asp) are still able to donate a carbonyl group to the interaction with Ca^{2+}, as was also shown for other C-type lectins (19). The amino acid residue Asp^{366} coordinates the Ca^{2+} ion without contributing to the ligand binding (Fig. 3*A*). Mutation of Asp^{366} into Ala resulted in complete loss of ligand binding, which is contributed to loss of Ca^{2+} binding (Table I).

Interaction with Ca^{2+} at the Auxiliary Site—The CRD of DC-SIGN binds two calcium ions (7), and, as we demonstrated, the ligand binding site of DC-SIGN is situated at Ca^{2+} site 2. The Ca^{2+} at site 1, the so-called auxiliary site, is located opposite the protein loop (Fig. 2*B*). Sequence alignment suggests that the amino acid residues Asp^{320}, Glu^{324}, Asn^{350}, and Asp^{355} are involved in the binding of this Ca^{2+} (Fig. 2*A*). This Ca^{2+} is important in the interaction of DC-SIGN with its ligands, since mutating the respective amino acid residues into Ala residues results in complete loss of ligand binding (Table I). These mutants are not able to bind the Ca^{2+} at site 1 because the change of either Glu, Asp, or Asn into Ala removes the carbonyl group at their respective positions necessary for the interaction with Ca^{2+}, as has previously been shown for MBP-A (19). The structure of these mutants is not changed dramatically, since they are still recognized by antibodies against DC-SIGN (Table I). Presumably, the Ca^{2+} at site 1 is essential to the correct positioning of the loops forming the primary ligand binding site as was shown for MBP-A (19).

Antibody Epitopes—We have previously reported two antibodies, AZN-D1 and -D2 (7), that interact specifically with DC-SIGN and completely inhibit the interaction of DC-SIGN with both HIV-1 gp120 and ICAM-3 (Fig. 1*A*). We determined the epitopes of these antibodies by site-directed mutagenesis and subsequent screening of the different DC-SIGN mutants

TABLE I
Functional characterization of the different DC-SIGN mutants

DC-SIGN mutant[a]	Adhesion		Expression		Function
	ICAM-3	gp120	AZN-D1	AZN-D2	
	%	%	%	%	
Wild type	49	39	95	92	
E347Q	0	4	93	92	
N349D	1	0	94	96	Ligand and Ca^{2+} site 2 binding
N365D	2	0	96	97	
D366A	1	3	94	91	Ca^{2+} site 2 binding
D320A	2	7	98	98	
E324A	0	0	95	95	
N350A	1	1	94	92	Ca^{2+} site 1 binding
D355A	0	2	98	97	

[a] Stable K562 transfectants.

for antibody recognition. Mutagenesis of Lys^{368} into Ala abrogated binding by the antibody AZN-D1, whereas AZN-D2 did not bind to the Gln^{323} mutant (Fig. 3*B*). The structure of DC-SIGN shows that the AZN-D1 epitope is located in the active ligand binding site close to Ca^{2+} site 2, whereas AZN-D2 interacts with amino acid residues on the other site of the protein loop close to Ca^{2+} site 1 (Fig. 3*C*). The Ca^{2+} at site 2 constitutes part of the ligand binding, whereas Ca^{2+} site 1 is involved in stabilizing the binding site. Thus, interference with these sites blocks the interaction of DC-SIGN with its ligands, confirming the importance of both Ca^{2+} sites for DC-SIGN function.

Different Binding Sites for HIV-1 gp120 and ICAM-3—The ligand binding site of the CRD of DC-SIGN forms a flat hydrophobic surface resembling a shallow trough, with the amino acid residue Val^{351} forming the edge of the pocket (Fig. 3, *A* and *C*). We investigated the role of this Val^{351} residue in the binding activity of DC-SIGN by changing it into Gly, thereby removing the β-carbon of Val, which has been shown in similar C-type lectins to be important (20). Indeed, this amino acid residue is essential for binding of DC-SIGN to ICAM-3, since the adhesion is completely abolished (Fig. 4*A*). Interestingly, the mutant is still able to bind the HIV-1 envelope glycoprotein gp120 to a similar extent as wild-type DC-SIGN (Fig. 4*A*). The Ca^{2+} dependence of the interaction of the V351G mutant with gp120 is lower than that of wild-type DC-SIGN, but even at high Ca^{2+} concentrations the V351G mutant is unable to interact with ICAM-3 (Fig. 4*B*), confirming a complete loss of ICAM-3 binding activity. These data demonstrate that the interaction of DC-SIGN with ICAM-3 is distinct from that with the HIV-1 envelope glycoprotein gp120 and that the V351G mutant plays an essential role in ICAM-3 but not in gp120 binding.

Previously, we demonstrated that DC capture HIV-1 through DC-SIGN and that DC-SIGN-bound HIV-1 is efficiently transmitted to T cells, producing a vigorous infection of T cells (8). The V351G mutant is able to bind HIV-1 (Fig. 4*A*), and we investigated whether this mutant is able to transmit HIV-1 to T cells. K562 transfectants expressing the V351G mutant were incubated with M-tropic HIV-1 for 2 h, and unbound virus was washed away. Activated T cells were co-cultured with the HIV-1-pulsed K562 transfectants expressing the V351G mutant, and this resulted in a productive infection of T cells similar to that observed with wild-type DC-SIGN (Fig. 4*C*). Similar results were obtained with T-tropic HIV-1 strains (results not shown). Thus, the V351G mutant is able to both capture HIV-1 and transmit the virus to activated T cells. These results also demonstrate that the ICAM-3-binding activity is not essential for transmission of HIV-1 by DC-SIGN.

DISCUSSION

Sexual transmission of HIV-1 involves dissemination of the virus from the mucosal tissues to the lymphoid organs where the target T cells reside. DC are thought to mediate this process (3, 6), and the DC-specific HIV-1 receptor DC-SIGN facilitates capture of HIV-1, its subsequent transport, and the efficient transmission of HIV-1 to T cells (8). DC-SIGN is able to capture both M- and T-tropic HIV-1, HIV-2, and simian immunodeficiency virus and to transmit the viruses to recipient T cells (8, 21). The expression of DC-SIGN on DC in mucosal tissues is consistent with its key function in the early stages of viral infection after sexual transmission (8). Moreover, DC-SIGN might also play a role in chronic HIV-1 infections, since DC-$SIGN^+$ DC are abundantly present in the lymph nodes, where DC-SIGN can contribute to the persistence of T cell infections by HIV-1. Therefore, DC-SIGN is a potential target to inhibit initial mucosal as well as chronic HIV-1 infections. Information about the mechanism of ligand binding is crucial for the development of specific inhibitors, and we investigated in detail the nature of the interaction of DC-SIGN with HIV-1 gp120 as well as with its natural immunological ligand ICAM-3.

To determine the essential amino acid residues involved in the ligand binding, we generated the three-dimensional structure of DC-SIGN CRD by molecular modeling using the crystal structure of the H1 subunit of an asialoglycoprotein receptor (RHL-1) as a template (Fig. 2). The top of the structure consists of several loops that form the functional ligand-binding region of the protein, containing the two Ca^{2+} binding sites. Ca^{2+} binding is one of the hallmarks of C-type lectins, and highly conserved amino acid residues are involved, yet this region does not contain a recognizable secondary structure. This presumably confers ligand specificity, explaining the large differences in this region between different C-type lectins. The pairing of the second N-terminal β-strand (β_2) with the final C-terminal β-strand (β_{12}) in the first β-sheet places the beginning and end of the CRD next to one another (Fig. 2*B*). This "loopout" topology enables CRDs in all membrane-bound members of the C-type lectin family to adopt the same orientation with respect to the cell surface whether they are located at the N terminus of the polypeptide, as in selectins, or at the C terminus of the type II receptors, as in DC-SIGN.

Previously, we demonstrated that DC-SIGN binds two Ca^{2+}, since the calcium dependence of ligand binding is second order (7). Here we confirm and extent this finding and show that the CRD structure of DC-SIGN contains two binding sites for Ca^{2+}, which are separated by a protein loop (Fig. 2*B*). The amino acid residues, which are in close contact with Ca^{2+} at site 2 (Glu^{347}, Asn^{349}, Glu^{354}, and Asn^{365}) form the core of the ligand binding site, and this Ca^{2+} participates in binding by directly ligating

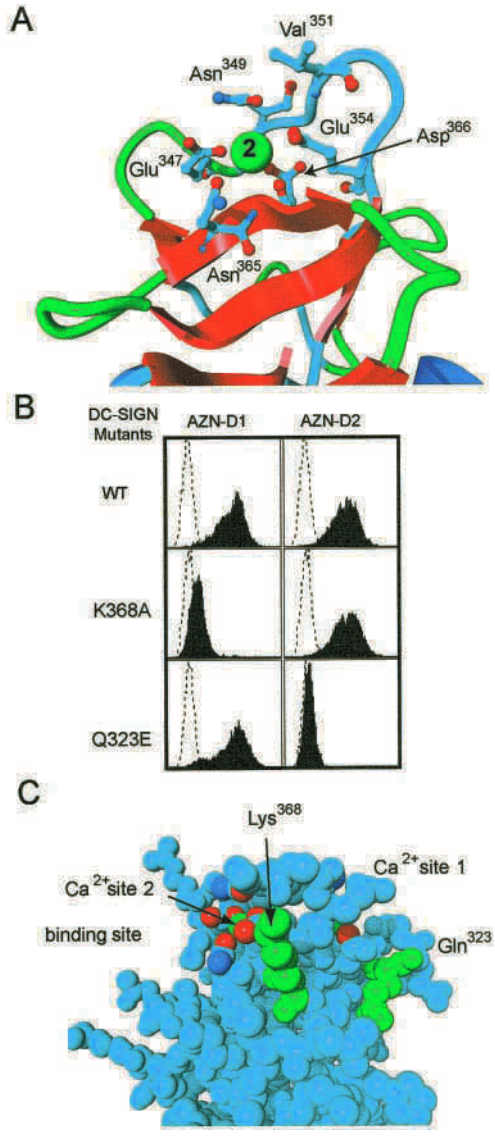

FIG. 3. **The ligand binding site in DC-SIGN is located at the top of the structure and includes the Ca^{2+} at site 2.** *A*, structure of the ligand binding site and Ca^{2+} site 2 in DC-SIGN. Amino acid residues involved in Ca^{2+} binding at site 2 (*green sphere*) are shown. Nitrogen and oxygen atoms are shown in *blue* and *red spheres*, respectively. The five amino acid residues Glu^{347}, Asn^{349}, Glu^{354}, Asn^{365}, and Asp^{366} coordinate binding of the Ca^{2+} ion, and, except for Asp^{366}, they all contribute to the binding of the ligand through hydrogen bonding via acidic or amide groups. *B*, staining of DC-SIGN mutants with the antibodies against DC-SIGN. The Lys^{368} mutant does not interact with AZN-D1, whereas the AZN-D2 binding to the Gln^{323} mutant is abrogated. The *dotted lines* represent the isotype controls, and the *filled black histograms* represent the antibody staining. *C*, diagram of the amino acid residues involved in the epitopes of the blocking antibodies against DC-SIGN. Amino acid residues involved in the epitopes are *blue*. The AZN-D1 epitope contains Lys^{368}, whereas Gln^{323} is essential for the interaction with AZN-D2. The ligand binding site at Ca^{2+} site 2 is also shown. Carbon, nitrogen, and oxygen atoms are shown in *magenta*, *blue*, and *red spheres*, respectively.

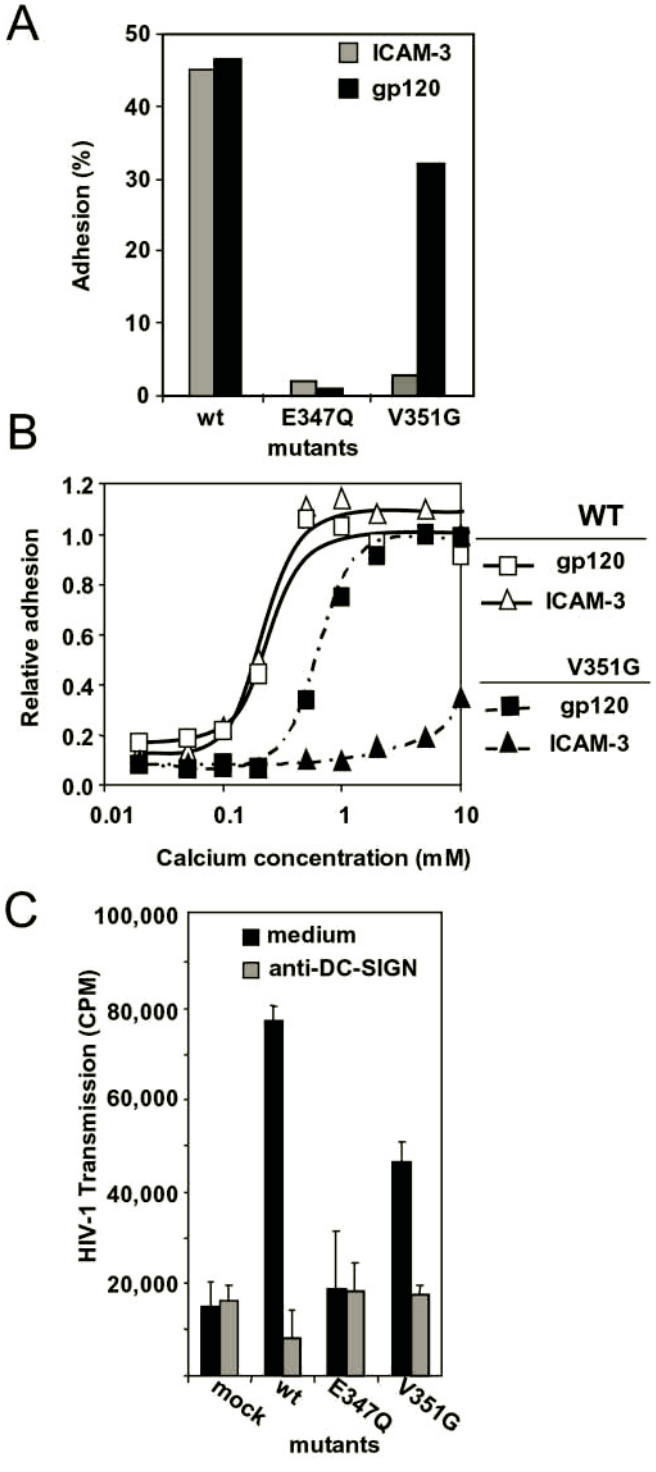

FIG. 4. **DC-SIGN contains different binding sites for HIV-1 gp120 and ICAM-3.** *A*, the amino acid residue Val^{351} is essential for ICAM-3 but not for HIV-1 gp120 binding. Binding of wild-type DC-SIGN and the E347Q and V351G mutants was measured using the fluorescent bead adhesion assay. One representative experiment out of three is shown. *B*, binding of the V351G mutant to ICAM-3 cannot be reconstituted by higher calcium concentrations. Adhesion was determined by the fluorescent bead adhesion assay at different Ca^{2+} concentrations. One representative experiment out of three is shown. *C*, the V351G mutant captures HIV-1 and facilitates infection of HIV-1 permissive cells in *trans*. DC-SIGN transfectants were incubated for 2 h with identical concentrations of pseudotyped M-tropic $HIV\text{-}1_{ADA}$, which contains a luciferase reporter gene. Unbound virus was washed away, and phytohemagglutinin-activated T cells were added. Cell lysates were obtained after 2 days and analyzed for luciferase activity, which is a measure for infection. One experiment out of three is shown.

to the ligand, as has also been demonstrated for other C-type lectins (12, 19, 22). These amino acid residues not only coordinate the Ca^{2+} at site 2 but also form hydrogen bonds with the ligand, thereby creating an intimately linked ternary complex of DC-SIGN, Ca^{2+}, and its ligand (Table I) (see Addendum). The auxiliary Ca^{2+} bound at site 1 correctly orients the protein loop to form the primary ligand binding site, and removal of this Ca^{2+} by mutagenesis completely abrogates ligand binding (Table I, Fig. 2). The importance of the calcium ions is further

underscored by the finding that the epitopes of the blocking antibodies against DC-SIGN, AZN-D2, and AZN-D1 are located in Ca^{2+} sites 1 and 2, respectively (Fig. 3, *B* and *C*).

The inhibition of DC-SIGN binding by the polysaccharide mannan suggests that the interaction with its ligands is mediated by mannose-like carbohydrates (7). Its natural ligand ICAM-3 contains *N*-linked glycosylations consisting of high mannose-type oligosaccharides, which could be involved in DC-SIGN binding (16). Indeed, enzymatic removal of the *N*-linked carbohydrates from ICAM-3 completely abrogates binding to DC-SIGN. The HIV-1 envelope glycoprotein gp120 is also heavily glycosylated and contains high mannose-type oligosaccharides (17, 23). Strikingly, neither *O*- nor *N*-linked glycosylations are vital for the interaction of the HIV-1 gp120 with DC-SIGN, since DC-SIGN interacts with both enzymatically deglycosylated and nonglycosylated gp120. Therefore, the interaction of DC-SIGN with gp120 differs from that with ICAM-3, indicating different binding sites for ICAM-3 and HIV-1 gp120 in DC-SIGN; the C-type lectin DC-SIGN interacts with its ligands either through carbohydrate interactions, such as mannan and ICAM-3, or through protein interactions, such as nonglycosylated gp120. However, the polycarbohydrate mannan is able to inhibit both ICAM-3 and gp120 binding to DC-SIGN by occupying the binding site of DC-SIGN, suggesting that both carbohydrate and protein interactions to DC-SIGN are mediated by overlapping but distinct binding sites in DC-SIGN.

The three-dimensional structure around the ligand-binding site consists of a flat surface representing a shallow trough where the edge of the depression is formed by the Val^{351} residue (Fig. 3, *A* and *C*). The β-carbon of Val^{351} can interact with the ligands located at the binding site, as was shown for the positional equivalent His^{189} in MBP-A and Val^{194} in MBP-C (20). We here demonstrate that this Val^{351} in DC-SIGN denotes the difference between the interaction of DC-SIGN with HIV-1 gp120 and ICAM-3. Replacement of the Val with Gly completely abrogates the binding to ICAM-3, whereas gp120 is still bound (Fig. 4*A*). Changing the MBP-A equivalent His^{189} into Gly resulted in a strong decrease in its affinity of binding to its carbohydrate ligands (20), implying that a similar change in DC-SIGN would influence binding to carbohydrates, as confirmed by the glycosylation-dependent binding to ICAM-3. However, this DC-SIGN V351G mutant is still able to bind HIV-1 gp120, confirming that this interaction is not mediated by carbohydrates, in contrast to that of ICAM-3, as was also observed with deglycosylated ligands (Fig. 1). These findings implicate that, in addition to the ligands interacting with the primary binding site centered around the Ca^{2+} at site 2, the ligands form additional contacts with the surface of DC-SIGN, thereby creating different ligand binding sites, which increases the specificity of ligand binding.

The primary binding site of DC-SIGN is highly homologous to that of other mannose-specific lectins (Fig. 2*A*). Several of these lectins, including mannose-binding proteins, also bind HIV-1 gp120 (24, 25). High mannose type glycans are thought to be involved in the interaction of the mannose binding protein with gp120, as has been shown for certain plant mannose-specific lectins (24). These proteins are potential inhibitors of HIV-1 infection, since their binding to gp120 blocks the CD4-gp120 interaction and neutralizes the HIV-1 infection of T cells. In contrast, DC-SIGN not only binds HIV-1 but also enhances the HIV-1 infection of T cells, confirming the different mechanisms of ligand binding and the unique function of DC-SIGN in HIV-1 pathology. Thus, although C-type lectins have homologous primary binding sites, their ligand specificity and binding mechanisms are clearly distinct due to different secondary surface binding sites. Recently, it was shown that DC-SIGN also differs from other C-type lectins in pronounced clustering of its monomeric form, resulting in a form with a novel tetrameric coiled-coil motif, which provides further ligand binding selectivity (26).

Recently, a human homologue of DC-SIGN was identified called L-SIGN (liver/lymph node-specific ICAM-3-grabbing nonintegrin) (27) or DC-SIGNR (DC-SIGN-related) (28). L-SIGN is about 90% identical to DC-SIGN and is specifically expressed in liver on sinusoidal endothelial cells and in lymph nodes (27, 29). L-SIGN is functionally identical to DC-SIGN in that it has high affinity for ICAM-3 and HIV-1 gp120 (27), and it also enhances HIV-1 infection of T cells in *trans* (27, 29). The high homology with DC-SIGN indicates that the specificity and mechanism of ligand binding of L-SIGN are very similar to that of DC-SIGN. One antibody against DC-SIGN, AZN-D2, cross-reacts with L-SIGN, inhibiting its activity (27), and here we show that this is due to binding of the antibody to the conserved region around the Glu^{335} residue, which is equivalent to Glu^{323} in DC-SIGN (Figs. 2*A* and 3*E*). The antibody AZN-D1 does not cross-react, since its epitope with Lys^{368} is not conserved in L-SIGN (Fig. 2*A*).

Moreover, recently five murine genes were identified that contained CRDs with about 70% homology to that of both DC-SIGN and L-SIGN/DC-SIGNR (30–32). Alignment of their deduced amino acid sequences indicates that these homologues contain two Ca^{2+} binding sites and have a specificity for mannose-like carbohydrates, since the amino acids involved in Ca^{2+} binding at both site 1 and 2 in DC-SIGN are conserved in all of these homologues. Interestingly, the Val^{351} amino acid residue in DC-SIGN, which is involved in ICAM-3 binding, is only conserved in two homologues, indicating a possible diversity in ligand recognition. Thus, structural information about DC-SIGN will also help us to understand the mechanism of ligand binding of the human homologue L-SIGN/DC-SIGNR and the murine homologues.

The different binding sites in human DC-SIGN for ICAM-3 and gp120 are attractive targets for therapeutic intervention of DC-induced immunity and HIV-1 dissemination by DC-SIGN, respectively. The gp120-specific V351G mutant will be used to generate antibodies specific for the gp120 binding site, which should inhibit HIV-1 transmission but not the immune function of DC-SIGN. The development of such inhibitors should greatly facilitate the prevention and dissemination of HIV-1 infections and the treatment of chronic HIV-1 infection, where an efficient immune system is vital. Moreover, the DC-SIGN V351G mutant could be conjugated to toxins to specifically target HIV-1-infected T cell reservoirs. Chimeric toxins such as CD4-PE40 and 3B3(Fv)-PE38 represent a complementary class of agents that already selectively kill productively infected cells, reducing HIV reservoirs in patients with AIDS (33), but the higher affinity of DC-SIGN for gp120 when compared with CD4-gp120 interactions suggests that DC-SIGN-toxin conjugates might be more effective. We anticipate that chimeric toxins based on the V351G mutant will target and kill only HIV-1-infected cells, in contrast to DC-SIGN wild type-based chimeras, which might also target ICAM-3-expressing T cells.

In summary, sequence alignment and site-directed mutagenesis of DC-SIGN highlights important aspects of the HIV-1 gp120 binding site that should facilitate the development of antibodies specifically blocking the interaction of DC-SIGN with gp120 but not with ICAM-3. Blocking the interaction of gp120 with DC-SIGN may be effective in prophylaxis or therapeutic intervention. Identification of the epitopes of the blocking antibodies against DC-SIGN provides potential targets for vaccine strategies aimed at eliciting mucosal antibodies that

inhibit gp120-DC-SIGN interactions. Moreover, the presence of two ligand binding sites facilitates the development of vaccines that specifically inhibit the gp120 interaction without compromising the immune function of DC-SIGN.

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Addendum—The crystal structure of the C-type lectin domain of DC-SIGN bound to an oligosaccharide was solved during the preparation of this manuscript (34). The amino acid residues in the primary binding site that interact with both Ca^{2+} ions, gp120 and ICAM-3, as determined by mutagenesis (Table I) are in accordance with the published crystal structure of DC-SIGN.

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