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Contribution of *N*-Linked Glycans to the Conformation and Function of Intercellular Adhesion Molecules (ICAMs)*

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The crystal structures of the glycosylated N-terminal two domains of ICAM-1 and ICAM-2 provided a framework for understanding the role of glycosylation in the structure and function of intercellular adhesion molecules (ICAMs). The most conserved glycans were less flexible in the structures, interacting with protein residues and contributing to receptor folding and expression. The first *N*-linked glycan in ICAM-2 contacts an exposed tryptophan residue, defining a conserved glycan-W motif critical for the conformation of the integrin binding domain. The absence of this motif in human ICAM-1 exposes regions used in receptor dimerization and rhinovirus recognition. Experiments with soluble molecules having the N-terminal two domains of human ICAMs identified glycans of the high mannose type *N*-linked to the second domain of the dendritic cell-specific ICAM-grabbing nonintegrin lectin-ligands ICAM-2 and ICAM-3. About 40% of those receptor molecules bear endoglycosidase H sensitive glycans responsible of the lectin binding activity. High mannose glycans were absent in ICAM-1, which did not bind to the lectin, but they appeared in ICAM-1 mutants with additional *N*-linked glycosylation and lectin binding activity. *N*-Linked glycosylation regulate both conformation and immune related functions of ICAM receptors.

Most of the cellular receptors that populate the cell surface are glycosylated so that heterogeneous glycan moieties decorate the extracellular regions of the membrane bound proteins, building the so-called glycocalyx. The glycoproteins can contain both *N*-linked (Asn-linked) and *O*-linked (Ser/Thr-linked) glycans of variable length and composition. Complex, hybrid, and high mannose glycans are usually present in glycoproteins. The glycosylation pattern appears protein, *N*-linked site, and cell specific so that a diverse glycosylation repertoire is present on cell and viral surfaces (1–3). The local conformation of the proteins and the overall multidomain arrangement can influence the processing of a glycan at an individual *N*-linked site (1). Variations in the carbohydrate structure linked to individual sites lead to heterogeneity in glycoprotein samples.

It is generally accepted that glycans shield protein surfaces and prevent nonspecific protein-protein interactions, providing protease protection and increasing glycoprotein stability (3). Viruses such as human immunodeficiency virus exploit glycosylation by using it to mask protein epitopes from antibody recognition (4). Some glycans have a great influence on the conformation of the receptor proteins to which they are linked, modulating their ligand binding activity (5, 6). Those glycans have a particular relation with protein residues such as that described for the inmunoadhesin CD2, where a glycan located on the opposite side of the ligand binding surface counterbalances a cluster of positive-charged residues and stabilizes the immunoglobulin domain for cell adhesive interactions (5). Glycans in some viral receptors are known to stabilize the virus binding surface (7, 8).

The members of the ICAM[1] subfamily are heavily glycosylated cell surface receptors of the immunoglobulin superfamily (IgSF). They share functional and structural homologies and mediate cell-cell adhesion interactions relevant for the function of the immune system (9). Adhesive interactions between members of the ICAM subfamily and leukocyte integrins LFA-1 and Mac-1 mediate leukocyte trafficking through inflamed and uninflamed tissues and contribute to antigen-specific T-cell responses. The recently reported binding of ICAM-2 and ICAM-3 to the C-type lectin dendritic cell-specific ICAM-grabbing nonintegrin (DC-SIGN) is relevant for the function of dendritic cells (10). Binding of DC-SIGN to ICAM-2 regulates emigration of dendritic cells, rolling in shear flow, and transmigration through endothelium (11). Alternatively, the interaction of DC-SIGN with ICAM-3 establishes initial contact between dendritic cells and resting T-cells during antigen presentation (11). Whereas the integrin binding surface in ICAMs is glycan-free (12, 13), the recognition of DC-SIGN is dependent on carbohydrates (10). The lectin binds with high affinity to high mannose and fucose-containing carbohydrates (14).

Six members of the ICAM subfamily have been described so far, all binding to the integrin LFA-1 ($\alpha L\beta 2$) but showing large variation in tissue distribution (15). The ICAMs are type I membrane proteins with a single transmembrane segment and concatenated IgSF domains in the extracellular region. The LFA-1 integrin binds to the first, N-terminal domain (D1) of the receptors (13, 16). There are large differences in the number of extracellular domains among the members of the subfamily, ranging from two in ICAM-2 and ICAM-4 to nine in ICAM-5. Most of the IgSF domains have *N*-linked glycans, and some are heavily glycosylated. The role of those glycans in receptor folding and ligand recognition is poorly understood. Ablation of the

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[1] The abbreviations used are: ICAM, intercellular adhesion molecule; IgSF, immunoglobulin superfamily; DC-SIGN, dendritic cell-specific ICAM-grabbing nonintegrin; HRV, human rhinovirus; MES, 4-morpholineethanesulfonic acid; endo-H, endoglycosidase H.

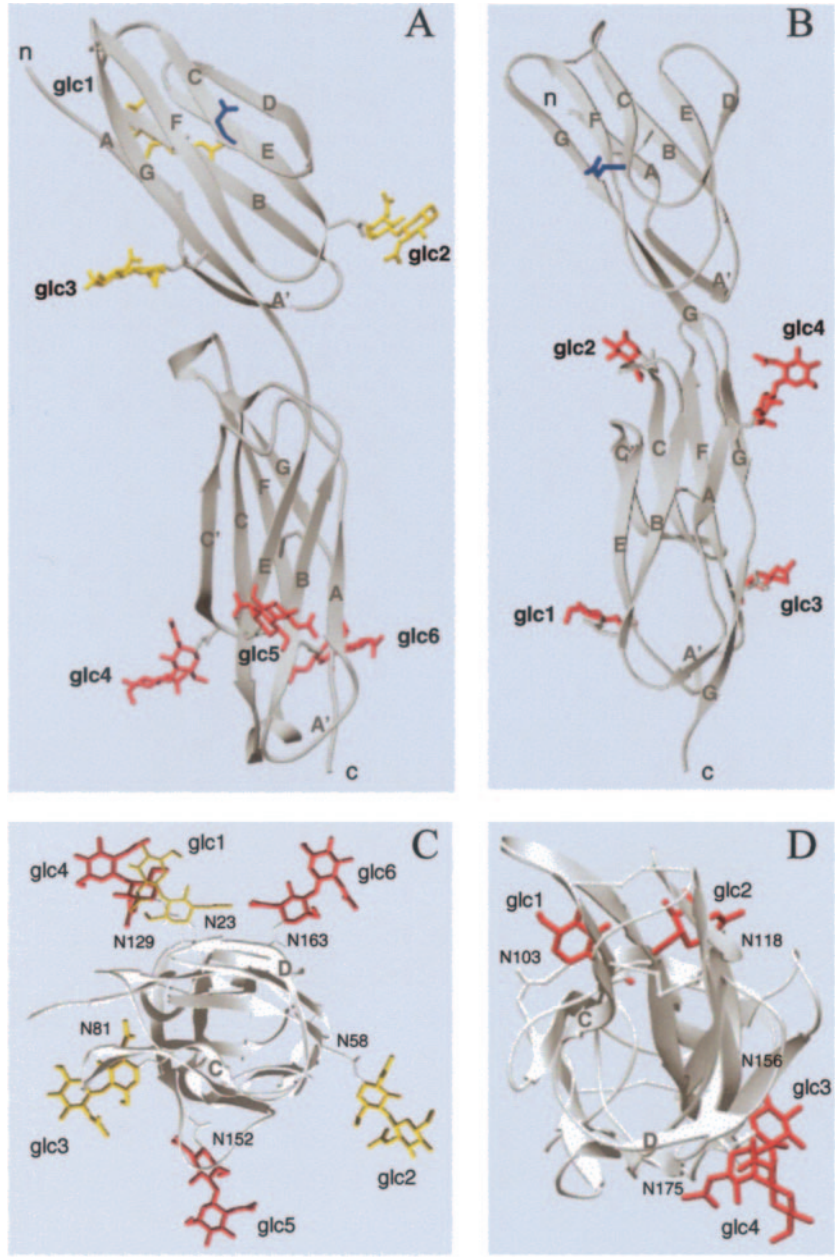

FIG. 1. **Crystal structures of ICAM-1 and ICAM-2.** A *ribbon model* with the N-terminal two IgSF domains of ICAM-2 (*A* and *C*) and ICAM-1 (*B* and *D*) colored *silver* and with *N*-linked glycans attached to first and second domain in *yellow* and *red*, respectively. Side (*A* and *B*) and top views (*C* and *D*) are shown. β-strands are labeled, and critical glutamic acid residue for integrin LFA-1 recognition is *blue*. Glycans have been numbered as they appear in the protein sequence. Asparagine residues to which glycans attach are also numbered.

first *N*-linked glycosylation site in ICAM-3 disrupted several antibody epitopes and LFA-1 recognition, whereas deletion of other glycans in the first domain had no effect on ligand binding (17). Mutation of several *N*-linked glycans in the second domain of ICAM-1 had small effects on LFA-1 binding (16).

The crystal structures of the glycosylated N-terminal two domains of ICAM-1 and ICAM-2 showed the location of the glycans in the IgSF domains (18, 19). The conformation of the first and in some cases the second GlcNAc residue of the chitobiose core was defined by the electron density maps (see Fig. 1). No traceable electron density was observed for the third mannose residues in the *N*-linked glycans, suggesting their high flexibility. The distribution of the *N*-linked glycans differs in ICAM-1 and ICAM-2 (see Fig. 1). In ICAM-2 the glycans linked to the two IgSF domains distribute around the ICAM-2 molecule (see Fig. 1*C*), whereas in ICAM-1 they locate on two opposite sides of the second domain (see Fig. 1*D*). The glycans are linked to the bottom of the domains in ICAM-2 and have a tripod-like distribution in the second domain (see Fig. 1, *A* and *C*), suggesting that they can be used to orient the receptor molecule on the cell surface (18). In ICAM-1 the first domain lacks *N*-linked glycans (Fig. 1*B*), providing additional interacting surfaces for cis-dimerization and nonintegrin ligands, such as human rhinovirus (HRV) and *Plasmodium falciparum*-derived proteins (19, 20). Here we report and discuss the contribution of the *N*-linked glycosylation to protein conformation and ligand recognition in members of the ICAM subfamily of adhesion receptors.

MATERIALS AND METHODS

Plasmids and Mutagenesis—Mutagenesis of ICAM-1 and ICAM-2 was carried by the overlap PCR technique using PfuI polymerase (Stratagene) and the cDNAs cloned in the unique XbaI site of pAprM9 (12, 17). Mutants were subcloned either in the pAprM9 vector for cell surface expression or in the pEF vector (27) for the preparation of soluble proteins.

Cell Surface Expression and Binding Assays with Antibodies, LFA-1, and HRV—Wild type and mutant ICAM-1 and ICAM-2 proteins were expressed on the surface of COS cells. Transfections using the DEAE-dextran method were essentially as described (12). Each experiment included transfection with 0.1–8.0 μg of wild type and 6 μg of mutant DNAs.

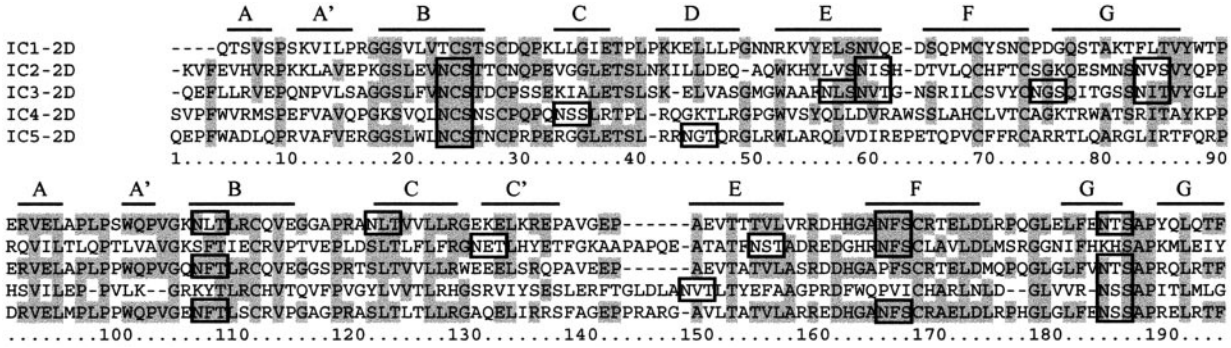

FIG. 2. **Alignment of ICAMs and *N*-linked glycosylation in the subfamily.** Structural and sequence alignment of the N-terminal two domains (2D) of human ICAMs (*IC1* to *IC5*) with the *N*-linked glycosylation sites *boxed*. Alignments were done with ClustalW and Modeler (30). First domain (D1) is at the *top*, and second domain (D2) is at the *bottom*. *Lines* comprise residues included in the indicated β-strands either in the crystal structure of ICAM-1 or ICAM-2.

Antibody binding to ICAM molecules on the cell surface was monitored by indirect immunofluorescence and flow cytometry (fluorescence-activated cell sorter). About 1×10^5 cells were incubated with anti-ICAM antibodies at 10 μg/ml. The percentage of cells expressing antibody epitopes was determined after subtraction of background staining from cells mock transfected with pAprM9 (12).

A cell binding assay with purified and plastic coated LFA-1 was carried out as described in detail elsewhere (12). The percentage of transfected cells bound to LFA-1 was determined for wild type and mutant ICAM molecules and corrected by the subtraction of background binding of mock transfected cells.

Virus binding to transfected cells was done with ^{35}S-labeled HRV3 and 14 (28). Transfected cells (1×10^5 cells/well) with wild type (2–8 μg) and mutant (4 μg) recombinant cDNAs were reseeded 48 h post-transfection into 96-well plates and cultured 24 h at 37 °C. [^{35}S]HRV3 or HRV14 (2×10^4 cpm) in 80 μl of phosphate-buffered saline, 5% fetal calf serum were added in duplicate to wells and incubated for 1 h at 35 °C. In antibody blocking experiments, cells were preincubated 15 min with the anti-ICAM-1 RR1/1 antibody (30 μg/ml) (16). Virus solution was aspirated, and wells were washed two times with 50 μl of phosphate-buffered saline, 5% fetal calf serum. Cells and bound viruses were harvested with 30 μl of 1% Triton X-100 and scintillation counted. Counts from virus bound to cells were corrected by background binding to mock transfected cells. A binding ratio was determined from the counts bound to cells expressing similar amounts of mutant and wild type ICAM-1 surface molecules, determined by fluorescence-activated cell sorter with the anti-D4 monoclonal antibody CL203 (16).

Soluble Fc Fusion Protein Preparation—Fusion proteins were engineered by cloning the ICAM cDNA coding for the indicated domains upstream of the human IgG1-Fc (Fc) genomic DNA in the mammalian expression vector pEF. They comprise mature protein residues 1–187 of ICAM-1 (IC1–2D), residues 1–193 of ICAM-2 (IC2–2D), and residues 1–86 or 189 of ICAM-3 (IC3–1D or IC3–2D). A control IgSF fusion protein (Ig) was prepared with the cDNA coding for the N-terminal two domains of Nectin-1 (23). Proteins were transiently expressed in 293T cells transfected with the calcium phosphate method. The Ig-Lec protein was prepared from Chinese hamster ovary Lec 3.2.8.1 cells using the glutamine synthetase expression system (25). Protein secretion into the cell supernatant was monitored and quantified by a sandwich enzyme-linked immunosorbent assay with antibodies against the Fc region (DAKO). Purified Fc protein of known concentration was used as standard to determine protein concentration in supernatants, which ranged from about 20–100 μg/ml.

DC-SIGN Preparation—The DC-SIGN lectin domain was expressed using a bacterial system. A recombinant cDNA coding for the DC-SIGN lectin domain (residues Glu^{250}-Leu^{392}) was cloned in the NheI and XhoI sites of the vector pET-27b (+) (Novagen). Protein expressed in BL21 *Escherichia coli* cells formed inclusion bodies. Urea solubilized protein under denaturing conditions (25 mM MES, pH 6.5, 8 M urea, 10 mM EDTA, 1 mM dithiothreitol) was subjected to refolding *in vitro* by 1:100 dilution into refolding buffer (0.1 M Tris-HCl, pH 8.5, 400 mM L-arginine, 2 mM EDTA, 6.3 mM cysteamine, 3.7 mM cystamine, 0.1 mM phenylmethylsulfonyl fluoride). The protein solution (1 liter) was concentrated to 3 ml and run through a Superdex-75 (Amersham Biosciences) size exclusion column in Tris-buffer, pH 8.0, with 100 mM NaCl. DC-SIGN protein fractions coming out at the expected retention volume were pooled and run through a Hi-Trap Mono-Q column (Amersham Biosciences). Highly purified protein was eluted from the ion-exchange column with 250 mM NaCl in Tris-buffer, pH 8.0. The purified protein was recognized by the anti-DC-SIGN MR-1 monoclonal antibody (29).

Binding of ICAMs to DC-SIGN—To analyze binding of ICAMs to DC-SIGN we designed an *in vitro* assay with plastic bound DC-SIGN and supernatants containing soluble Fc fusion proteins. 96-Well plates (Maxi Sorp, Nunc) were precoated with 50 μl of DC-SIGN (10 μg/ml) in 20 mM Tris-HCl, 100 mM NaCl, pH 7.5, with 2.5 mM $CaCl_2$ (binding buffer) overnight at 4 °C. The plates were then incubated for 1 h at 37 °C, washed four times with binding buffer, and blocked with 2% bovine serum albumin for 1 h at 37 °C. Cell supernatants with the Fc fusion proteins at about 25 μg/ml were diluted with 1% bovine serum albumin in binding buffer at concentrations from 25–1 μg/ml added to wells with coated DC-SIGN and incubated for 1 h at 37 °C. After washing, horseradish peroxidase-labeled anti-Fc antibody (DAKO) diluted 1:500 in binding buffer with 1% bovine serum albumin was added to the wells. Binding was monitored by OD at 490 nm using a Benchmark reader (Bio-Rad).

Endoglycosidase Treatment of ICAM-Fc Proteins and Analysis—Endoglycosidase H (endo-H, New England Biolabs) or enzyme buffer was added to supernatants (200 μl) with the Fc fusion proteins (20 μg/ml), and the mixture was incubated overnight at 30 °C. DC-SIGN binding to enzyme- and buffer-treated samples was carried as described above. Size change in the protein samples related to glycan hydrolysis was analyzed by electrophoresis and Western blotting. Endo-H-treated Fc proteins were immunoprecipitated at 4 °C with protein-A-Sepharose (Amersham Biosciences). Samples were run in a 10% SDS-PAGE under reducing conditions and transferred to a Hybond-P membrane (Amersham Biosciences). After overnight blocking with 5% nonfat dried milk and 0.1% (v/v) Tween-20 in phosphate-buffered saline, the membrane was incubated with a horseradish peroxidase-labeled anti-Fc antibody (DAKO) and bound antibody detected by chemiluminescence using the ECL detection system (Amersham Biosciences). The percentage of protein sensitive to endo-H was determined with a Fluor-S Multimager device (Bio-Rad). The fluorescence counts in the area corresponding to the endo-H sensitive or resistant protein fractions were determined in the tracks with samples treated with endo-H or buffer. The ratio between counts in the endo-H sensitive area and the total counts in the enzyme treated protein sample were determined and corrected by the background ratio determined from the buffer-treated protein.

RESULTS

N-Linked Glycans in the ICAM Subfamily and Their Contribution to Receptor Expression and LFA-1 Recognition—The N-terminal two domains of all human ICAMs are heavily glycosylated with the number of *N*-linked glycans ranging from four in ICAM-1 and ICAM-4 to seven in ICAM-3 (Fig. 2). The first and last glycans, respectively, in β-strand B of domain 1 and β-strand G of domain 2 are highly conserved. The first glycan in ICAM-2 (glc1, Fig. 1, *A* and *C*) had the lowest temperature factor among all the glycans in the ICAM-2 structure (Table I), related to a particular contact with protein residues discussed below. The chitobiose core of the glycan in the β-strand G of the second domain of ICAM-1 (glc4, Fig. 1*B*) runs parallel to the protruding FG-loop in the ICAM-1 structure and

TABLE I
LFA-1 and antibody binding activity of ICAM-2 glycomutants

LFA-1 and antibody binding to cell surface expressed ICAM-2 mutants. The LFA-1 binding was normalized to the wild type receptor with same amount of expression as described under "Materials and Methods" and elsewhere (12). Cell surface expression was determined with the CBR-IC2/1 antibody. Binding of antibodies to the mutants were normalized to the percentage of cells expressing wild type ICAM-2 receptor, transfected with the same amount (6 μg) of DNA. Cell binding in the presence of anti-ICAM-2 CBR-IC2/2 (+IC2-Ab) and anti-LFA-1 TS1/18 (+LFA-Ab) antibodies are included. The average from three experiments is shown with standard deviation in parentheses. Deleted (Δ) glycans have been numbered as in Fig. 1, and mutations carried are in parentheses. Temperature factors (B) quantify thermal-associated flexibility for the first GlcNAc of the glycans reported in the crystal structure of ICAM-2 (Protein Data Bank accession code 1ZXQ).

Mutant	B	LFA-1	IC2/1	IC2/2	BT-1	6D5
AprM9		0	0	0	0	0
ICAM-2		100	100	100	100	100
+IC2-Ab		5.4 (7)				
+LFA-Ab		2.5 (5)				
Δglc1 (N23A)	54	1.5 (2)	25 (14)	2 (2)		37 (22)
Δglc1 (N23T)	54	3.4 (3)	26 (8)	6 (6)	3 (1)	34 (4)
Δglc1 (S25A)	54	1.1 (1)	23 (16)	3 (0)	5 (4)	44 (2)
W51A		1.5 (2)	31 (2)	4 (4)	2 (0)	3 (6)
Δglc2 (S60A)	69	139.3 (32)	65 (22)	56 (30)	64 (20)	74 (34)
Δglc3 (S83A)	61	74.1 (14)	90 (16)	82 (16)	84 (18)	104 (44)
Δglc4 (T131A)	69	125.6 (2)	84 (24)	84 (14)	67 (11)	47 (0)
Δglc5 (T154A)	76	76.5 (18)	85 (13)	73 (27)	78 (16)	59 (29)
Δglc6 (S165A)	64	66.5 (6)	69 (13)	65 (10)	57 (15)	67 (25)
Δglc4,5		44.8 (24)	51 (10)	54 (12)	46 (10)	52 (2)
Δglc5,6		10.0 (2)	12 (4)	20 (5)	8 (3)	38 (2)

is linked to the interdomain interface. The first GlcNAc stacks onto the side chain of Thr[85] in all reported crystals structures (13, 19, 20), and additional contacts of the second GlcNAc with Tyr[83] and Phe[173] residues are also observed in some structures (13, 19) (data not shown). This glycan had the lowest temperature factor and therefore the lowest flexibility among glycans in the four molecular structures reported for the fully glycosylated ICAM-1 (13, 19), quite likely because of the interaction with protein residues. Deletion of glc4 reduced expression of a soluble ICAM-1 with the two N-terminal domains and had some effect on LFA-1 binding (16, 20).

The contribution of some *N*-linked glycans to receptor expression and LFA-1 binding has been studied by mutagenesis in ICAM-1 and ICAM-3 (16, 17), although no data have been reported for ICAM-2. Glycans linked to the first domain of ICAM-2 are conserved in ICAM-3, but those in the second domain are not (Fig. 2). We deleted *N*-linked glycans in ICAM-2 to determine their contribution to protein processing and expression on the cell surface and to integrin LFA-1 recognition. Cellular expression was monitored with a panel of four monoclonal antibodies (Table I). Deletion of the conserved glc1 linked to N23 in ICAM-2 with anyone of three different mutations largely decreased receptor expression and LFA-1 binding. The effect observed here was even more marked than that reported for deletion of the homologous glycan in ICAM-3 (17). Single deletion of any other glycan had only a marginal effect on ICAM-2 expression on the cell surface. LFA-1 binding to those mutants correlated with the level of expression, although deletion of glc2 and -4 slightly increased cell adhesion (Table I). Double mutations of glycans in the second domain further decreased receptor expression (Table I), whereas very low cell surface expression was monitored for the triple mutant (data not shown).

A Conserved Glycan-Protein Motif in the Integrin Binding Domain of ICAMs—The crystal structure of glycosylated ICAM-2 showed several peculiarities for the conserved glc1 in ICAMs (Figs. 1 and 2). It locates in the middle region of the IgSF domain, differing from the remaining *N*-linked glycans in ICAM-2, which locate in the bottom of D1 and D2 (Figs. 1*A* and 3). It had the lowest temperature factor among all glycans (Table I), indicating restricted flexibility because of the interaction with a conserved protein residue (Fig. 3). The structure showed an evident contact between glc1 and the conserved W51 residue (Fig. 3), with the first GlcNAc stacking on the exposed

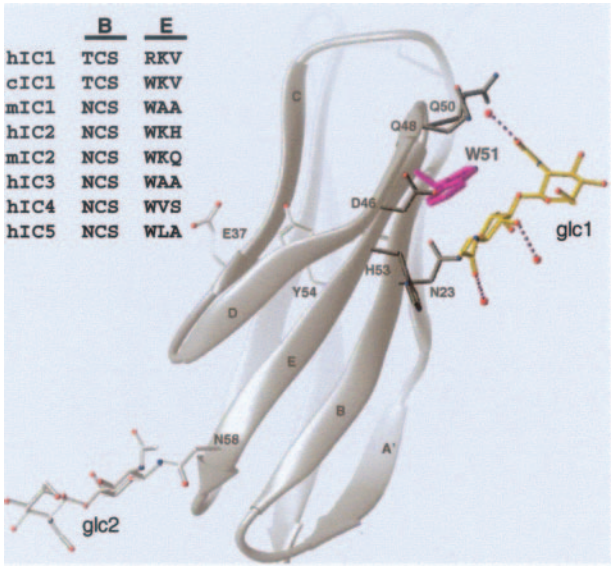

FIG. 3. **The conserved glycan-W motif in the integrin binding domain.** A *ribbon model* of the N-terminal domain of ICAM-2 with the interacting glc1 (*yellow*) and tryptophan (*magenta*) residues. Residues surrounding W51 are colored *dark gray*, and the critical LFA-1 binding residue E37 is *white*. β-strands are labeled. Oxygen and nitrogen atoms are *red* and *blue spheres*, respectively. Water molecules hydrogen bonded to glc1 are *red spheres*. The *inset* presents the glycan-W motif in human ICAMs (hIC1 to hIC5), chimpanzee ICAM-1 (cIC1), and mouse ICAMs (mIC1,2).

aromatic ring of the side chain. Indeed, solvent accessible surface calculations with SURFACE (21) showed that atoms within the first carbohydrate ring of glc1 were not solvent exposed (probe radius of 1.4–1.8 Å), differing from the other GlcNAc residues in the ICAM-2 structure, which were solvent-accessible (data not shown). Additionally, the methyl group of the first GlcNAc lays at ~4 Å of the indole ring of His[53] (Fig. 3). Because of those contacts, glc1, rather than project perpendicular to the main axis of the domain (see glc2 in Fig. 3), runs parallel to it toward the top of the first domain. Trp[51] appears surrounded by the glycan on one side and the aliphatic side chains of Gln[48] and Gln[50] on the other side, preventing solvent exposure of all but 1 atom of the aromatic ring.

Both glc1 and the interacting tryptophan residue (glycan-W motif) are highly conserved in the ICAM subfamily (Fig. 3,

TABLE II
LFA-1, HRV, and antibody binding activity of ICAM-1 glycomutants

LFA-1, HRV, and antibody binding to cell surface expressed ICAM-1 mutants. Binding was normalized to that obtained with transfected cells having the same wild type receptor expression, determined with the anti-D4 CL203 monoclonal antibody as described under "Materials and Methods." Mutant and wild type binding ratios for the CL203 antibody were determined from the percentage of cells expressing the epitope, transfected with the same amount of mutant and wild type DNA. LFA-1 and HRV binding in the presence of the anti-ICAM-1 RR1/1 antibody (ICI-Ab) is included. The mutations carried in the wild type human ICAM-1 identified the mutant receptors. The average from at least three experiments (except for the T20/A mutant) is shown, with standard deviation in parentheses.

MUTANT	LFA-1	HRV	CL203	RR1/1	R6.5	LB2	YH370	7F7
AprM9	0	0	0	0	0	0	0	0
ICAM-1	100	100	100	100	100	100	100	100
+IC1-Ab	22 (15)	3 (4)						
T20N	59 (12)	78 (11)	101 (23)	87 (5)	90 (9)	62 (6)	58 (8)	0 (0)
T20A		123 (5)	82	115	110	100		
R49W	94 (38)	92 (7)	96 (38)	10 (3)	100 (3)	98 (4)	96 (16)	102 (11)
T20N-R49W	110 (9)	46 (11)	102 (34)	103 (6)	104 (7)	24 (3)	39 (8)	5 (7)

inset). All ICAMs except ICAM-1 from human and chimpanzee have the glycan-W motif, so it must have a conserved role in the subfamily. Mutation of either the glycan or the Trp residue in ICAM-2 reduced significantly both surface expression of the receptor and integrin binding (Table I). A similar phenotype was reported with human ICAM-3 (17), showing that the glycan-W motif is critical for the proper conformation of the integrin binding IgSF domain in the ICAM subfamily. Quite interestingly this motif evolved in primates, disappearing in humans. However, a mutant ICAM-1 molecule having the glycan-W motif (T20N/R49W) expressed on the cell surface as well as the wild type human ICAM-1 receptor, as monitored by the anti-D4 monoclonal antibody CL203 (Table II). LFA-1 binding was almost identical in the mutant and wild type ICAM-1 receptor molecules, showing proper folding of the domain for integrin recognition. Introduction of either the glycan or the Trp residue in ICAM-1 did not have a significant effect on expression, although some effect on integrin binding was observed for the T20N mutant (Table II).

As with the CL203 epitope in D4, the R6.5 binding site, which includes epitopes from D1 and D2 of ICAM-1 (16), appeared unaffected by the addition of the glycan-W motif in the N-terminal domain (Table II). Inclusion of the glycan (T20N mutant) blocked 7F7 antibody binding and partially reduced binding of LB2 and YH370 antibodies. However, binding of those two antibodies to the ICAM-1 mutant with the glycan-W motif was further reduced despite the lack of effect for the R49W mutation (Table II). This behavior suggests a change in glycan conformation. In the absence of the Trp residue the glycan must run perpendicular to the first IgSF domain as glc2 in ICAM-2 (Fig. 3). However, the orientation of the glycan in the double mutant must be similar to that described by the crystal structure of ICAM-2 (Fig. 3) covering the LB2 and YH370 epitopes. Additionally, we would then expect that the critical rhinovirus binding region at the top of the first IgSF domain (22) will be decorated by the glycan in the human ICAM-1 molecule carrying the glycan-W motif, implying some effect on virus binding. We observed that inclusion of the glycan in the middle of the first IgSF domain of ICAM-1 (T20N mutant) slightly decreased virus binding to the receptor, whereas addition of the W residue (T20N/R49W mutant) further decreased binding (Table II).

DC-SIGN Recognition by the ICAM Subfamily—It has been reported that the DC-SIGN lectin binds specifically and with high affinity to the N-terminal two domains of ICAM-2 and ICAM-3 in the presence of calcium, whereas binding to ICAM-1 was undetectable (10). To gain further insights on recognition of DC-SIGN by ICAMs, we developed a protein binding assay using purified and plastic coated DC-SIGN protein and soluble ICAM molecules with their N-terminal two domain fused to the Fc used as binding reporter by labeled antibodies (see "Materials and Methods"). Structural integrity of the ICAM region in the fusion proteins was assessed with anti-ICAM monoclonal antibodies (data not shown). A control protein with the two N-terminal IgSF domains of Nectin-1 (23) and high mannose carbohydrates (Ig-Lec) bound to DC-SIGN (Fig. 4*A*), consistent with the described binding specificity of the lectin (24). By contrast, the Ig protein expressed in 293T cells (Ig-293T) and therefore bearing complex carbohydrates did not bind to DC-SIGN, even though this protein has 5 *N*-linked glycans. Soluble proteins expressed in 293T cells and having the N-terminal two domains of ICAM-2 (IC2–2D) and ICAM-3 (IC3–2D) bound to DC-SIGN (Fig. 4*A*), whereas molecules with the homologous domains of ICAM-1 (IC1–2D) or the single N-terminal domain of ICAM-3 (IC3–1D) did not bind to the lectin. DC-SIGN binding activity was also monitored with an homologous ICAM-5 soluble molecule (data not shown). Quite interestingly, ICAM-1 molecules with two domains and with an additional *N*-linked glycan in three different locations of the first domain bound quite efficiently to DC-SIGN (Fig. 4*B*). DC-SIGN binding was abolished by chelating the calcium with EDTA, by treatment with endo-H, or by competition with high concentrations of mannan (Fig. 4*C*), suggesting that the ICAM molecules use high mannose or hybrid type of glycans for adhesion to the DC-SIGN lectin.

Further analysis of the protein samples binding to DC-SIGN included electrophoresis of proteins treated with endo-H (Fig. 5). Complete size reduction of the Ig-Lec protein sample was consistent with the expected high mannose composition of the *N*-linked glycans in glycoproteins expressed in Chinese hamster ovary Lec cells (25). No size reduction upon endo-H treatment was observed for Ig-293T, showing absence of high mannose carbohydrates and therefore lack of DC-SIGN binding activity. Similarly, the endoglycosidase treatment of IC1–2D showed no high mannose *N*-linked glycans (Fig. 5), as for IC3–1D (data not shown). By contrast, about 40% of the other ICAM samples analyzed had reduced size upon endo-H treatment, consistent with the DC-SIGN binding activity related to those proteins samples (Fig. 4, *A* and *B*). The smaller percentage of Fc fusion proteins bearing high mannose glycans in IC2–2D and IC3–2D than in Ig-Lec explains their lower lectin binding activity and the differences in binding competition with mannan (Fig. 4, *A* and *C*).

ICAM-2 N-Linked Glycans in DC-SIGN Recognition—Identification of glycans linked to ICAM-2 critical for binding to DC-SIGN was carried by mutation. Single glycan mutation had no effect on binding of soluble ICAM-2 to the lectin (data not shown), whereas double mutants showed decreased but still significant binding (Fig. 6*A*). Deletion of all first domain glycans gave 50% binding reduction in binding, whereas no lectin binding activity was detected by deletion of all three glycans linked to the second domain. Consistently, high mannose car-

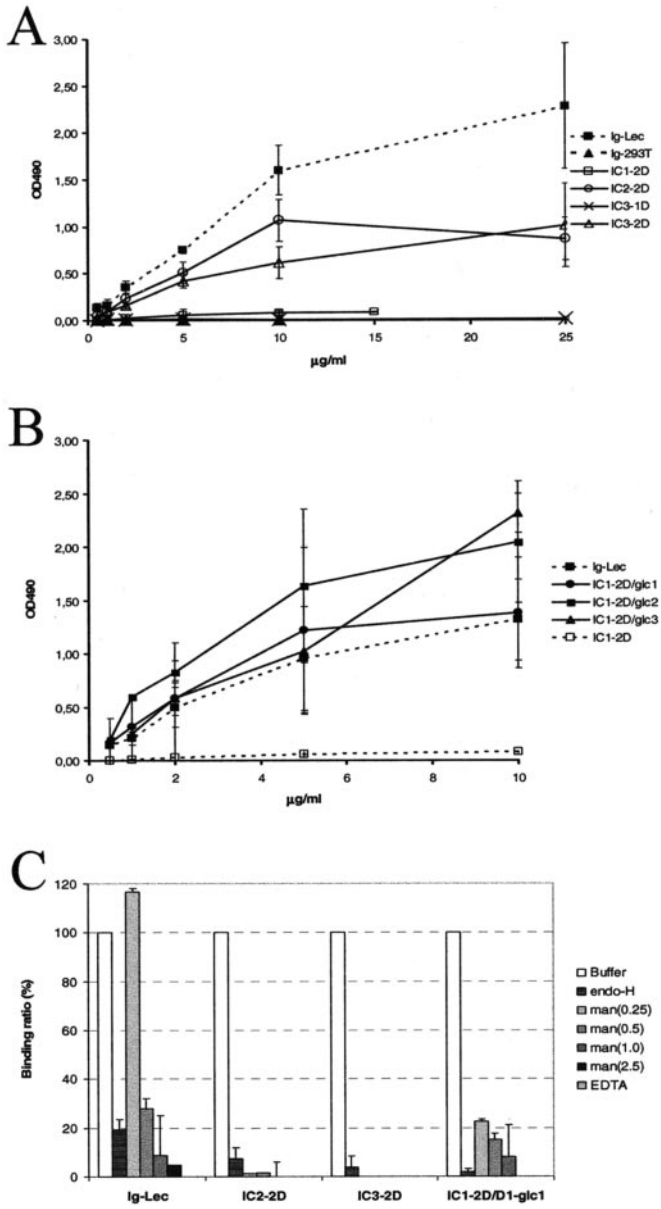

FIG. 4. **ICAM binding to DC-SIGN.** Binding of soluble Fc fusion proteins to plastic coated DC-SIGN was carried and monitored as described under "Materials and Methods." Average OD_{490} and standard deviations for a range of protein concentrations from at least three different experiments are shown in *A* and *B*. *A*, proteins expressed in 293T cells and having domains 1 and 2 of ICAM-1 (IC1–2D), ICAM-2 (IC2–2D), ICAM-3 (IC3–2D), or domain 1 of ICAM-3 (IC3–1D) were analyzed. Binding assays were carried also in parallel with a fusion protein having the N-terminal two IgSF domains of Nectin-1 (23) expressed either in Chinese hamster ovary Lec (Ig-Lec) or in 293T (Ig-293T) cells. *B*, binding assays done in parallel with Ig-Lec, IC1–2D, and ICAM-1 mutants with a single glycan in the N-terminal domain, attached either at position 20 (IC1–2D/glc1), 56 (IC1–2D/glc2), or 79 (IC1–2D/glc3). *C*, specificity on soluble ICAM binding to DC-SIGN. Binding ratios (%) were calculated from the measured OD_{490} for treated and untreated proteins. DC-SIGN binding assays were carried with the indicated protein at 10 μg/ml treated with buffer or endo-H or in the presence of increasing concentration (mg/ml) of mannan or EDTA (25 mM). No detectable binding was monitored for the EDTA-treated samples. Average and standard deviations from three independent experiments are shown.

bohydrates sensitive to endo-H were only absent in the ICAM-2 mutant lacking *N*-linked glycans in the second domain (Fig. 6*B*), indicating that they must be responsible of the lectin binding activity shown by ICAM-2 (Fig. 4*A*). The smaller size of the Δglc1,2,3 compared with the Δglc4,5,6 mutant observed in

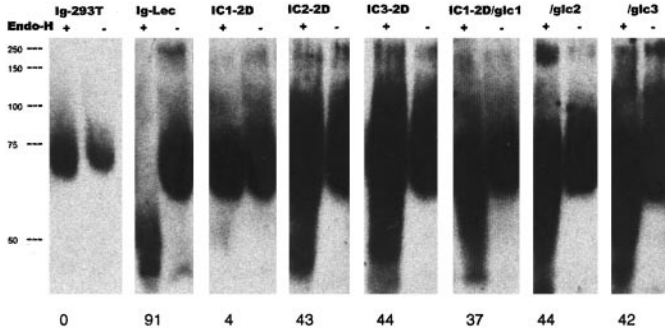

FIG. 5. **Sensitivity of soluble ICAM proteins to endo-H.** Western blotting of Fc fusion proteins presented in Fig. 4 treated in the absence (−) or presence of endo-H (+) as described under "Materials and Methods." The percentage of protein sensitive to endoglycosidase is presented at the bottom of each track.

the electrophoresis (data not shown) suggested a shorter length for glycans linked to the second domain, indicative also of high mannose type glycosylations. The lack of DC-SIGN binding by IC3–1D (Fig. 4*A*) suggests that the high mannose type glycans must be linked to the second domain of ICAM-3 as well.

DISCUSSION

The results presented have shown how *N*-linked glycans regulate the function of the ICAM subfamily of cellular receptors. Analysis of the available crystal structures of the glycosylated ICAM-1 and ICAM-2 showed that the most conserved glycans contact protein residues and had the lowest flexibility among the *N*-linked carbohydrate residues (Table I), being critical for IgSF domain conformation and consequently ligand recognition. Therefore it is likely that glycans having low temperature factors (similar to protein residues) modulate the three-dimensional structures of cell and virus glycoproteins. Non-conserved glycans, such as those linked to the second domain of ICAM-2, did not have a significant contribution to receptor structure, but they were involved in ligand recognition.

The high conservation of the glycan-W motif in the middle of the N-terminal domain of the ICAM subfamily and its absence in human and chimpanzee ICAM-1 (Figs. 2 and 3) is striking. As shown here for ICAM-2 (Table I) and elsewhere for ICAM-3 (17), the deletion of this motif largely affected integrin binding, even though it is on the opposite side of the interacting surface. Similar conformational effect by a glycan in receptors of the IgSF was described in CD2, although in that case the glycan contacts a cluster of positive-charged residues (5). The glycan-W motif appeared critical to ensure correct folding of the IgSF domain and conformation of the integrin binding surface, and it could confer certain domain stability required for cell adhesion interactions mediated by the ICAM subfamily. The absence of the conserved glc1 in the middle of the first domain of human ICAM-1 uncovered the ABED β-sheet of the domain for molecular interactions. This surface is used in receptor dimerization optimal for multivalent interaction with the integrin LFA-1 (13, 19), which increases binding avidity and provides a cell adhesive interaction further specialized in the firm adhesion of leukocytes to endothelium (9).

The lack of glycosylation in the N-terminal domain of human ICAM-1 exposed a larger protein surface for protein-protein interactions than in other ICAM receptors, which could have a positive influence in the selection of ICAM-1 as a receptor by pathogens. Indeed, the presence of the conserved glc1 present in human ICAM-2 could certainly affect recognition by *P. falciparum* derived proteins because they bind to nearby residues

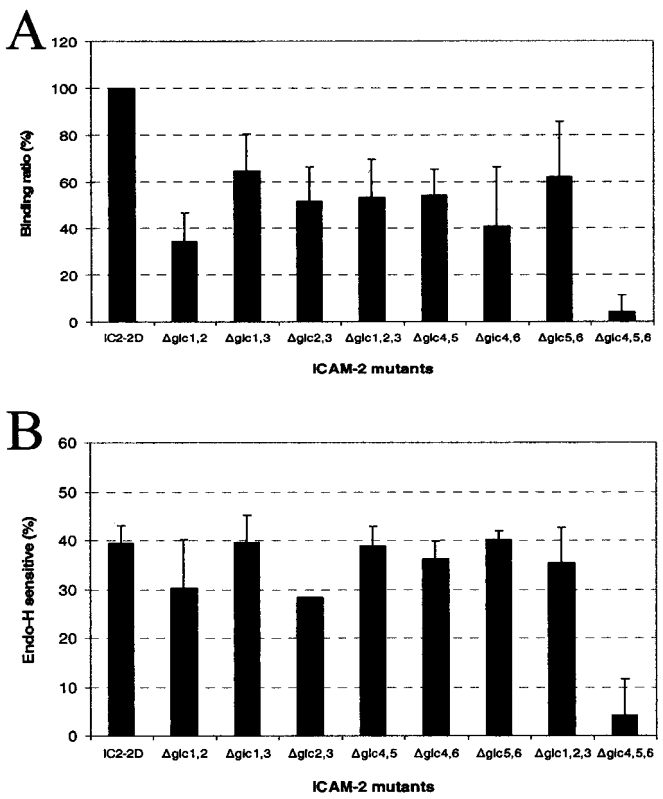

FIG. 6. **Recognition of ICAM-2 glycosylation mutants by DC-SIGN and sensitivity to endo-H.** *A*, normalized lectin binding activity of ICAM-2 mutants lacking the indicated glycans, deleted by mutations presented in Table I. Binding ratios (%) were calculated from the measured OD_{490} in binding assays with plastic coated DC-SIGN and soluble mutant and wild type proteins as described in Fig. 4. The average of five experiments carried out with three protein concentrations (10, 5, and 2 μg/ml) is shown with standard deviations. *B*, percentage of each protein sample sensitive to endo-H treatment determined from Western blotting as described in Fig. 5. The average of two experiments and standard deviations are plotted.

in the β-strand B of ICAM-1 (19). Furthermore, we show here that inclusion of the glycan-W motif in human ICAM-1 sterically hindered binding to major group human rhinoviruses (Table II), so that N-terminal glycosylated ICAM-1 would function poorly as a rhinovirus receptor.

The crystal structures of ICAM-1 and ICAM-2 showed the location of the *N*-linked glycans in the N-terminal two domains for the ICAM subfamily (Fig. 1). Most glycans in all human ICAMs locate near the top or bottom of the IgSF domain (Figs. 1 and 2) close to interdomain interfaces. This preferred location suggests that they can influence interdomain orientation and flexibility as well as orient the receptor molecules on the cell surface. Evidences are provided by the glycan linked to the upper region of the β-strand G in D2 of ICAM-1 (glc4), which participates in the interdomain interface by interacting with several protein residues. The critical contribution of glc4 to the folding and conformation of the N-terminal two domain module of ICAM-1 is supported by mutagenesis data (16, 20). Its conservation in the subfamily (Fig. 2) suggests that it will build up interdomain junctions in related ICAM receptors.

Beside the structural role discussed for some *N*-linked glycans in the ICAM subfamily, we showed that they are used in ligand recognition, providing specificity for binding to the lectin DC-SIGN. Using a protein-protein binding assay we found specific lectin binding to ICAM-2 and ICAM-3 molecules with the N-terminal two domains and lack of lectin binding to ICAM-1 (Fig. 4) as reported elsewhere (10). Experiments with endo-H further extend those findings, showing that the lectin binding activity in ICAMs relates to high mannose carbohydrates specifically linked to the second domains of ICAM-2 and ICAM-3 (Figs. 4–6). However there is heterogeneity in the glycan composition of ICAM-2 and ICAM-3, and only about 40% of the molecules bear an endo-H-sensitive high mannose type of glycans (Fig. 5) responsible for DC-SIGN binding (Fig. 4*C*). The calculated percentage of ICAM molecules having high mannose type oligosaccharides is similar to that reported for ICAM-3 (26). Our results prove also that there are not significant interactions of ICAM protein residues with the DC-SIGN lectin.

Soluble proteins with the N-terminal two domains of ICAM-1 lacked endo-H-sensitive glycans and consequently did not bind to the lectin DC-SIGN. Quite interestingly, inclusion of a single glycan in three different locations of domain 1 gave a population of molecules with high mannose glycans, suggesting a threshold glycosylation number for expression of properly folded receptor molecules bearing simple carbohydrates. Nevertheless, the presence of high mannose carbohydrates was ICAM-specific as they were absent in the two N-terminal domains of the IgSF Nectin-1 receptor, even though it has 4 and 1 *N*-linked glycan, respectively, in domains 1 and 2. Those differences could be related to differences in IgSF fold between Nectin and ICAM domains as well as to differences in the interdomain relations.

Besides conferring protection to proteases and prevent nonspecific interactions, *N*-linked glycans regulate both structure and function of immune receptors, either by interacting with protein residues to which they are linked, modulating the accessibility of protein surfaces, or participating in ligand recognition. As we showed here with ICAMs, the extent and heterogeneity of the *N*-linked glycosylation influence conformation, oligomerization state, and ligand recognition scope of this family of cellular receptors. Glycosylation appears to be intimately linked to the functional properties of cellular and viral glycoproteins.

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