**N-Glycosylation Is Required for Human CD2 Immunoadhesion Functions**

Michael A. Recny‡, Michael A. Luther§, Maria H. Knoppers, Edith A. Neidhardt, Sanjay S. Khandekar, Michael F. Concino†, Patricia A. Schimke, Marilyn A. Francis, Ulrich Moebius**, Bruce B. Reinhold‡‡, Vernon N. Reinhold‡‡‡, and Ellis L. Reinherz****

From PROCEPT, Inc., Cambridge, Massachusetts 02139, the **Department of Nutrition, Harvard School of Public Health, the Departments of §§Biological Chemistry and Molecular Pharmacology, ¶Pathology and **Medical School, and the **Laboratory of Immunobiology, Dana Farber Cancer Institute, Boston, Massachusetts 02115

The T-lymphocyte glycoprotein receptor, CD2, mediates cell-cell adhesion by binding to the surface molecule CD58 (LFA-3) on many cell types including antigen-presenting cells. Two domains comprise the CD2 extracellular segment, with all adhesion functions localized to the amino-terminal domain that contains a single N-glycosylation site at Asn⁶⁵. We have defined an important role for the N-linked glycans attached to Asn⁶⁵ of this domain in mediating CD2-CD58 interactions and also characterize its N-glycotype structure. Analysis of deglycosylated soluble recombinant CD2 as well as a mutant transmembrane CD2 molecule containing a single Asn⁶⁵-Gln⁶⁵ substitution demonstrates that neither deglycosylated CD2 nor the mutant CD2 transmembrane receptor binds CD58 or monoclonal antibodies directed at native CD2 adhesion domain epitopes. Electrospray ionization-mass spectrometry demonstrates that high mannose oligosaccharides (([Man]ₐ,GlcNAcₐ, n = 5–9) are the only N-glycotypes occupying Asn⁶⁵ when soluble CD2 is expressed in Chinese hamster ovary cells. Based on a model of human CD2 secondary structure, we propose that N-glycosylation is required for stabilizing domain 1 in the human receptor. Thus, N-glycosylation is essential for human CD2 adhesion functions.

CD2 (T11) is a transmembrane glycoprotein receptor that mediates adhesion between T-lymphocytes and antigen-presenting cells (reviewed in Refs. 1 and 2). The T-cell-restricted CD2 molecule binds to the widely distributed CD58 (LFA-3) structure found on many nucleated and non-nucleated cells (3–5). By promoting cell-cell contacts and hence, receptor interactions between apposing membranes of T-lymphocytes and their cognate partners, the CD2-CD58 adhesion pair plays an important role in facilitating CD3-Ti antigen receptor (TCR) recognition of antigens presented via the major histocompatibility complex (6–8). Recent evidence suggests that CD2-CD58 interaction initiates primary cell-cell adhesion prior to TCR engagement (9). Conjugate formation stabilized primarily by CD2-CD58 interactions permits efficient antigen/major histocompatibility complex recognition by the TCR and subsequent up-regulation of the binding affinity between the LFA1-ICAM1 adhesion pair (9–11). Thus, by boosting the efficiency of cell-cell interactions, T-cell activation can effectively be accomplished via specific TCR-major histocompatibility complex binding with much lower antigen concentrations (8). The importance of the CD2-CD58 adhesion pair in immune responses is underscored by studies demonstrating that anti-CD2 monoclonal antibodies (mAbs) which block CD58 binding are directed against a specific CD2 epitope (T11), and these mAbs also inhibit antigen-dependent T-cell proliferation and cytolysic effector functions (12–15).

CD2 also plays a role in T-cell lymphocyte activation. Perturbation of the CD2 extracellular domain with a combination of antibodies against two other CD2 extracellular epitopes (T11₂, T11₃), which are distinct from the T11 site, or interaction of CD2 with CD58 bearing cells together with mAbs specific for the T11₃ epitope can stimulate T-cell proliferation, IL-2 secretion, and clonal expansion (12, 16–19). Stimulation of T-cells through CD2 also transduces activation signals that synergize with those mediated by the TCR (20–21).

We previously established that the structural basis for CD58 recognition lies within the first extracellular domain of the CD2 polypeptide using recombinant soluble CD2 proteins (22). Mutagenesis studies also defined two distinct regions within the CD2 adhesion domain, centered about Lys⁴⁶ and Gly⁶⁰ of the mature polypeptide that are implicated in CD2-CD58 binding (23, 24). However, synthetic peptides spanning 21–30-residue sequences within the adhesion domain between Ile⁴⁶ and Leu⁶⁰ failed to bind either CD2 or anti-CD2 mAbs (22), suggesting that the CD58 binding site on CD2 may be comprised of a combination of nonlinear epitopes on the receptor surface or perhaps requires a conformationally constrained linear polypeptide. The amino acid sequence of human...
N-Glycosylation Regulates Human CD2 Function

RESULTS

Purification and Characterization of sCD2\textsubscript{A5} and sCD2\textsubscript{A8}—
A cDNA construct previously designed to produce a soluble two-domain human CD2 receptor (sCD2\textsubscript{A5}) encoding the first 182 amino acid residues of the molecule (31) was re-engineered in this study to be a new vector for high level expression of sCD2\textsubscript{A5} in Chinese hamster ovary (CHO) cells. Two-domain sCD2\textsubscript{A5} secreted from CHO cells is a mixture of monomeric glycoprotein species with apparent molecular mass between 32 and 36 kDa (Fig. 1, lane 2). The distribution of minor sCD2\textsubscript{A5} protein bands between 31 and 36 kDa varies depending on the preparation and represent multiple isoelectric glycoforms, since

![FIG. 1. SDS-PAGE analysis of recombinant sCD2 proteins.](http://www.jbc.org/)

10 μg of each sample were electrophoresed on 12% polyacrylamide gels under reducing conditions. Lanes 1 and 5, molecular weight standards (Bio-Rad); lane 2, sCD2\textsubscript{A5}; lane 3, sCD2\textsubscript{A5}; lane 4, dN\textsuperscript{36} sCD2\textsubscript{A5}.
treatment of sCD2αβ with neuraminidase collapses these bands to a single species on isoelectric focusing gels (data not shown). Therefore, one or more of the three predicted N-linked glycosylation sites at Asn65, Asn117, and Asn136 contain either hybrid or complex-type glycans capped with variable amounts of sialic acid.

Digestion of sCD2αβ with clostripain produces a homogeneous 105-amino acid residue CD2 adhesion domain (sCD2αβ) resulting from specific cleavage of sCD2αβ at the carboxyl terminus of Arg135 and Arg146 (two of the three predicted sites for clostripain specificity) (Fig. 1, lane 3). An additional clostripain cleavage site within the adhesion domain at Arg48 does not appear readily accessible, as evidenced by the stability of sCD2αβ toward further degradation. This domain fragment is nearly identical in size to the adhesion domain fragment (T11 pep) previously generated by papain digestion of sCD2αβ (22), but does not have a heterogeneous carboxyl terminus found previously with papain-digested T11 pep. Incubation of purified sCD2αβ with peptide N-glycosidase F reduces its molecular mass from ∼16 to ∼12 kDa (Fig. 1, lane 4), consistent with the removal of N-linked glycoconjugates from this site (predicted sCD2αβ polypeptide Mr = 12,430). The sCD2αβ polypeptide sequence contains a single consensus N-linked glycosylation site at Asn65, and based on SDS-PAGE, neither the mobility of multiple preparations of sCD2αβ derived from the two-domain sCD2αβ expressed in CHO cells, this site always appears occupied with N-linked glycans. However, deglycosylated sCD2αβ (dN65αβ-CD2αβ) is completely unreactive in a sandwich ELISA which employs two anti-CD2 mAbs (anti-T11, anti-T112) that recognize distinct functional CD2 adhesion domain epitopes (12–19) (Fig. 2A). Furthermore, when dN65αβ-sCD2αβ is bound directly to ELISA plates and probed separately with either anti-T11, or anti-T112 mAbs, no reactivity is observed with either mAb compared to native sCD2αβ, indicating that both T11, and T112 epitopes are equally disrupted (Fig. 2 B and C).

Deglycosylated sCD2αβ Does Not Bind CD58—The spontaneous aggregation or “rosette” formation between T-lymphocytes and sheep red blood cells (SRBC) originally identified T-lineage cells in the human before the advent of mAbs (37–40). This cell-cell interaction is now known to be the consequence of binding of human CD2 to sheep CD58 (41). In contrast to results obtained with native sCD2αβ which, as expected, inhibit SRBC rosetting with human T-cells in a dose-dependent manner, purified dN65αβ-sCD2αβ fails to bind to sheep CD58 (Fig. 3). Even with doses of dN65αβ-sCD2αβ as high as 100 µM, no inhibition of SRBC rosetting is observed. Therefore, enzymatic removal of the N-linked carbohydrates attached to the single glycosylation site within the human CD2 adhesion domain disrupts binding of dN65αβ-sCD2αβ to CD58 and to two independent anti-CD2 mAbs that recognize native surface structures implicated in CD2 activation and adhesion functions (12–19).

Functional Analysis of a Transmembrane CD2 Glycosylation Mutant—To test whether a transmembrane CD2 receptor lacking N-linked carbohydrate within the adhesion domain could display functional cell-surface epitopes recognized by anti-CD2 mAbs and bind to CD58, a cDNA coding for a full-length mutant CD2 having a single Asn65Gln65 substitution (N65αβGln65αβ-CD2) was constructed. This mutant N65αβGln65αβ-CD2 cDNA was transfected into COS-1 cells and plates were screened for surface expression of N65αβGln65αβ-CD2 by flow cytometry analysis using various anti-CD2 antibodies (Fig. 4A). As shown by indirect immunofluorescence analysis, approximately 50% of COS-1 cells transiently expressing either wild-type transmembrane CD2 (WT-CD2) or mutant N65αβGln65αβ-CD2 specifically react with a rabbit polyclonal anti-CD2 antisera (M32B) raised against two-domain sCD2αβ which recognizes both native and denatured epitopes in SDS-PAGE Western blots. A similar percentage of WT-CD2 COS-1 cells also stain with anti-T11, and anti-T112 mAbs, with a small population staining brightly with both mAbs. In contrast, no staining of COS-1 cells expressing the mutant N65αβGln65αβ-CD2 was observed with either anti-T11, or anti-T112 mAbs. The minor reactivity of the anti-T11 mAb with the mutant N65αβGln65αβ-CD2 population represents nonspecific binding as evidenced by a similar shift in the anti-T11 staining of COS-1 cells transfected with CDM8 vector alone (Fig. 4A). Therefore, mutation of the consensus N-glycosylation sequence at Asn65 which then precludes attachment of N-linked carbohydrate at this site does
not prevent translocation of N\textsuperscript{65},Q\textsuperscript{65}-CD2 to the cell surface, but the mutant N\textsuperscript{65},Q\textsuperscript{65}-CD2 transmembrane molecules lack reactivity with anti-T111 and anti-T112 mAbs. Moreover, when COS-1 cells transfected with N\textsuperscript{65},Q\textsuperscript{65}-CD2 are incubated with SRBC, no rosettes are observed (Fig. 4B). The rosette pattern is indistinguishable from mock transfections with CDM8 vector alone, while parallel analysis of WT-CD2 COS-1 transfectants showed multiple rosettes (Fig. 4B). The latter could be inhibited with micromolar concentrations of soluble sCD2\textsubscript{105} or sCD2\textsubscript{105}, indicating the adhesion dependence of SRBC rosetting on the CD2-CD58 co-receptor pair (data not shown).

**DISCUSSION**

We report here the purification of a recombinant sCD2\textsubscript{105} adhesion domain protein expressed in CHO cells, characterize the glycoform profile of the glycan attached to the single N-linked carbohydrate binding site in sCD2\textsubscript{105}, and examine the functional properties of both transmembrane and soluble CD2 proteins lacking carbohydrate at this site. Analysis of deglycosylated sCD2\textsubscript{105} as well as a mutant transmembrane CD2 molecule containing a single Asn\textsuperscript{65},Glu\textsuperscript{65} substitution demonstrates that neither binds CD58 or monoclonal antibodies directed at native CD2 adhesion domain epitopes. Electrospray ionization-mass spectrometry demonstrates that high mannose oligosaccharides ((Man)_n GlcNAc\textsubscript{2} where n = 5–9) (Fig. 5). The most abundant glycoform species in this sample correspond to intermediate size high mannose glycotypes, i.e. ((Man)_n GlcNAc\textsubscript{2} where n = 5–9 (Fig. 5). The most abundant glycoform species in this sample correspond to intermediate size high mannose glycotypes, Man\textsubscript{3},GlcNAc\textsubscript{2} (18%), Man\textsubscript{1},GlcNAc\textsubscript{2} (31%), and Man\textsubscript{1},GlcNAc\textsubscript{2} (41%). Based on ESI-MS analysis of multiple sCD2\textsubscript{105} samples the high mannose glycoform profile varied in their relative percentages; however, these three glycotypes are consistently the most abundant (>85%). Processed spectra indicate only high mannose glycoform, apparent from peak intervals of m/z 162 Da, with a notable absence of ions indicating hybrid or complex glycotypes (e.g. m/z 256, 291 Da; lactosylamine, hexosylamine, and neuraminyl, respectively). These data suggest that high mannose glycans are the only glycotypes present within the CD2 adhesion domain when two-domain sCD2\textsubscript{105} is expressed in CHO cells. Preliminary ESI-MS evidence has indicated that the second domain of sCD2\textsubscript{105}, which possesses two consensus N-glycosylation sites, contains only complex-type, polyolactosamine neuraminyl capped glycans.

**Fig. 3.** Inhibition of SRBC rosette formation by sCD2\textsubscript{105} and dN\textsuperscript{65},sCD2\textsubscript{105}. Sheep red blood cells (SRBC) were incubated together with varying concentrations of sCD2\textsubscript{105} (closed circles) and dN\textsuperscript{65},sCD2\textsubscript{105} (open circles) for 30 min at 4 °C prior to rosetting with Jurkat T-cells. The percentage inhibition of rosetting was determined by comparing the number of rosettes obtained in the presence or absence of sCD2 proteins.

**Fig. 4.** FACS analysis and SRBC rosetting of WT-CD2 and N\textsuperscript{65},Q\textsuperscript{65}-CD2 mutant expressed in COS-1 cells. Panel A, COS-1 cells were transfected with wild type CD2, mutant N\textsuperscript{65},Q\textsuperscript{65}-CD2, or CDM8 control plasmids and assayed for surface expression of CD2 by staining with Protein-A purified anti-CD2 polyclonal rabbit sera (M32B), anti-T11, mAb (3T48B5), or anti-T112 mAb (10LD2-4C1) followed by fluorescein-conjugated goat anti-mouse or goat anti-rabbit IgG as appropriate. Each panel illustrates staining with the respective antibody overlaid with COS-1 controls. For each experiment, 10,000 cells were analyzed by indirect immunofluorescence flow cytometry on FACSscan (Becton Dickinson). Panel B, COS-1 cells transfected with CDM8 control, human WT-CD2, or N\textsuperscript{65},Q\textsuperscript{65}-CD2 plasmids were incubated with SRBC and rosetting was performed as described (27). Magnification equals 160-fold.
and the protein subsequently purified to homogeneity from urea-solubilized pellets via sequential chromatography.\textsuperscript{3} Nevertheless, the \textit{E. coli} produced CD2 domain 1 protein remained completely unreactive toward anti-CD2 mAbs in both sandwich and antibody capture ELISAs and did not bind CD58 in SRBC rosetting assays. Both sCD2\textsubscript{2a} and sCD2\textsubscript{2b} purified from CHO cells can be reversibly renatured after exposure to strong chaotrophic agents.\textsuperscript{4} However, attempts to renature functional binding activity of the \textit{E. coli} produced human CD2 domain 1 using identical renaturation protocols were unsuccessful. We take these data as additional confirming evidence that N-glycosylation in the human CD2 adhesion domain plays a critical role both in forming and maintaining a functional CD58 binding site.

There are numerous examples whereby oligosaccharides are required for proper folding, transport, and biological function of either secreted glycoproteins or transmembrane glycoprotein receptors (reviewed in Refs. 46 and 47). Early studies with the transmembrane G protein of vesicular stomatitis virus demonstrated that either blockage of N-glycosylation with tunicamycin, elimination of N-glycosylation sites by site-directed mutagenesis, or generation of variants containing novel glycosylation sites resulted in aggregation of nascent vesicular stomatitis virus-G chains in the endoplasmic reticulum and severe impairment of their intracellular transport (48–51). It has also been recently shown that expression of functional human CD4 on the cell surface requires glycosylation at either one of the two N-linked sites within the third Ig-like domain (52, 53). Mutations which eliminate both consensus N-glycosylation sites renders CD4 transport incompetent and improperly folded CD4 (as judged by mAb reactivity) is retained in the endoplasmic reticulum. However, our data indicates that elimination of N-glycosylation within the human CD2 adhesion domain alone does not inhibit surface expression of mutant N\textsuperscript{148:Q\textsuperscript{158}}-CD2, but the CD2 adhesion domain lacking N-carbohydrate is clearly non-native in conformation in either its transmembrane or soluble form.

Carbohydrates also serve directly as molecular determinants responsible for mediating cell-cell adhesion and lymphocyte trafficking via binding to the selectin family of adhesion receptors (54). ELAM-1 regulates adhesion of leukocytes to vascular endothelium by recognition of Sialyl-Le\textsubscript{X} (sialyl-Lewis X) (55, 56), a carbohydrate ligand found on cell-surface glycoprotein and glycolipid groups of neutrophils. In contrast, N-linked glycosylation within domain 3 of ICAM-1 appears to specifically shield the ligand binding site for the leukocyte integrin MAC-1 (CD11a/CD11b) in that reagents which interfere with N-carbohydrate biosynthesis (or mutations that eliminate N-glycosylation sites in ICAM-1) enhance the binding of ICAM-1 to MAC-1 (57).

Based on the assumption (given \textasciitilde 50% amino acid identity between rat and human CD2) that the three-dimensional structure of human CD2 domain 1 resembles its rat CD2 counterpart, Ig \(\beta\)-strand folding patterns for human CD2 were proposed (Fig. 6) (43). Preliminary data obtained on human CD2 domain 1 (sCD2\textsubscript{2a}) by multidimensional NMR spectroscopy indeed suggests that the CD2 adhesion domain adopts an overall conformational structure characteristic of an Ig-fold.\textsuperscript{6} Taken together, these observations suggest that the N-glycans attached to Asn\textsuperscript{148} project outward from a tight loop connecting \(\beta\)-strands D and E of a classical Ig \(\beta\)-sandwich structure.

\textsuperscript{3}M. Recny, M. Luther, E. Neidhardt, M. Knoppers, and S. Khandekar, unpublished observations.

\textsuperscript{4}M. Luther, M. Knoppers, and M. Recny, manuscript in preparation.

\textsuperscript{5}D. Wyss, J. Withka, and G. Wagner, personal communication.

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**N-Glycosylation Regulates Human CD2 Function**

**Fig. 5.** ESI-MS analysis of sCD2\textsubscript{2a}.\textsuperscript{5} Panel A, electrospray ionization-mass spectra of sCD2\textsubscript{2a} showing an envelope of multiply charged ion clusters generated from the single glycopeptide. Molecular weight and satellite peak intervals within each ion cluster are a function of charge state and characteristic of the glycans' glycosyl and glycoform. Since these interval measurements are identical between all ion clusters, pattern recognition algorithms may be applied to assign glycopeptide mass and glycoform (panel B). Panel B, cluster deconvolution of glycopeptide electrospray raw data (panel A) accomplished by pattern recognition using a relative or level-2 entropy algorithm (37). Processing of the raw data in this manner collapses the spectra shown in panel A to a "root" glycopeptide glycoform and improves signal to noise. The prominent base ion, 1,530 Da larger than the known polypeptide mass of Tlllos (43). This receptor fragment was prepared by fusing amino acid residues 1–99 of the rat CD2 sequence to glutathione-S-transferase and expressing the fusion protein in \textit{E. coli}, where it fractionated in the soluble lysate of \textit{E. coli} cells. Rat CD2 domain 1 was judged to be folded correctly after cleavage from its fusion partner by its reactivity with distinct mAbs raised to specifically shield the ligand binding site for the leukocyte integrin MAC-1 (CD11a/CD11b) in that reagents which interfere with N-carbohydrate biosynthesis (or mutations that eliminate N-glycosylation sites in ICAM-1) enhance the binding of ICAM-1 to MAC-1 (57).
domain. Depending on the length and flexibility of this D/E loop, and the orientation of the asparaginyl side chain within this loop, high mannose N-glycans attached to Asn6 might be positioned alongside β-strands B, D, and E in the face of the β-sheet opposite to the proposed binding site for CD58. Thus, in this regard the high mannose N-glycans would not serve directly as a ligand in mediating CD58 binding but could influence the integrity of the CD58 binding site by some, as yet undefined, long-range stabilization effects. Alternatively, these high mannose N-glycans might project upward from the top of the D/E loop and stabilize flexible turn regions between either β-strands B and C or possibly C′ and C′′ via hydrogen-bonding with specific amino acid side chains residues within these loops. Based on our data, the presence of this N-glycan is clearly required for both the formation and maintenance of native structure. The precise role that N-linked carbohydrate plays in mediating the conformational stability of the human CD2 adhesion domain, and thereby influencing molecular recognition of CD58, may be clarified once the three-dimensional structure of human CD2 is solved.

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