An N-Linked Glycan Modulates the Interaction between the CD1d Heavy Chain and β2-Microglobulin∗

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Human CD1d molecules consist of a transmembrane CD1 (cluster of differentiation 1) heavy chain in association with β2-microglobulin (β2m). Assembly occurs in the endoplasmic reticulum (ER) and involves the initial glycan-dependent association of the free heavy chain with calreticulin and calnexin and the thiol oxidoreductase ERp57. Folding and disulfide bond formation within the heavy chain occurs prior to β2m binding. There are four N-linked glycans on the CD1d heavy chain, and we mutated them individually to ascertain their importance for the assembly and function of CD1d-β2m heterodimers. None of the four were indispensable for assembly or the ability to bind α-galactosyl ceramide and to present it to human NKT cells. Nor were any required for the CD1d molecule to bind and present α-galactosyl ceramide after lysosomal processing of a precursor lipid, galactosyl-(α1-2)-galactosyl ceramide. However, one glycan, glycan 2 at Asn-42, proved to be of particular importance for the stability of the CD1d-β2m heterodimer. A mutant CD1d heavy chain lacking glycan 2 assembled with β2m and transported from the ER more rapidly than wild-type CD1d and dissociated more readily from β2m upon exposure to detergents. A mutant expressing only glycan 1 dissociated completely from β2m upon exposure to the detergent Triton X-100, whereas a mutant expressing only glycan 2 at Asn-42 was more stable. In addition, glycan 2 was not processed efficiently to the complex form in mature wild-type CD1d molecules. Modeling the glycans on the published structure indicated that glycan 2 interacts significantly with both the CD1d heavy chain and β2m, which may explain these unusual properties.

The human CD15 (cluster of differentiation 1) family consists of five transmembrane glycoproteins encoded by linked genes (1). They are divided into two groups based on amino acid sequence homology; group 1 includes CD1a, -b, and -c, and group 2 consists of CD1d, the only isoform present in mice and rats. The fifth member of the family, CD1e, has an amino acid sequence intermediate between the two groups. CD1 heavy chains are structurally similar to MHC class I molecules and possess a short C-terminal cytosolic tail, a hydrophobic transmembrane region, and an extracellular region that interacts non-covalently with β2-microglobulin (β2m). The role of CD1 molecules is to bind lipid antigens and present them to T cells, and the α1 and α2 domains of the extracellular region fold in a similar manner to the analogous domains in MHC class I molecules to generate the lipid binding site.

CD1 heavy chain folding and association with β2m occurs in the endoplasmic reticulum (ER). After exiting the ER, the assembled CD1 molecules pass through the secretory pathway and reach the plasma membrane. From there, with the exception of CD1a, they enter the endocytic system by adaptor protein (AP)-dependent internalization using tyrosine-based endocytic motifs (YxxΦ, Φ = any amino acid and Φ = bulky hydrophobic amino acid). Similar to MHC class II molecules, antigen loading occurs mainly in the endocytic system (1). The affinity of CD1 molecules for lipid antigens is explained by the architecture of their antigen binding sites, which are more hydrophobic and deeper than those of MHC class I molecules. They accommodate the alkyl chains of the lipids, leaving the more polar regions exposed for recognition by T cell receptors (2–4).

CD1d molecules are expressed by many cell types, in particular by dendritic cells, macrophages, B cells, and thymocytes (1). They present lipid antigens to a special subset of T cells that express the NK1.1 marker. The majority of these NKT cells are called invariant NKT cells because they express fixed rear-

The abbreviations used are: CD1, cluster of differentiation 1; β2m, β2-microglobulin; ER, endoplasmic reticulum; CNX, calnexin; CRT, calreticulin; α-GaLCer, α-galactosyl ceramide; GaLGaLCer, galactosyl-(α1-2)-galactosyl ceramide; TBS, Tris-buffered saline; PE, phycoerythrin; NKT, natural killer T cells; MHC, major histocompatibility complex; Endo H, endoglycosidase H; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; AP, adaptor protein; HPLC, high pressure liquid chromatography; PNGase F, peptide N-glycosidase F.
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ranged T cell receptor α chains (Vα14-Jα18 in mice and Vα24-Jα18 in humans) that are associated with different Vβ chains. After activation, they rapidly secrete Th1 and Th2 cytokines, suggestive of an immunoregulatory function (5). An exception-

After activation, they rapidly secrete Th1 and Th2 cytokines, exogenous lipid antigens, e.g. from *Sphingomonas* and *Ehrlichia* species, can be presented to NKT cells by CD1d molecules (8–15). Loading of exogenous lipids occurs mainly in the endocytic system and is catalyzed by saposins or the GM2 activator protein, which are small lipid transfer proteins (16, 17). Initial endogenous lipid loading occurs in the ER (8) and is facilitated by the action of microsomal lipid transfer protein (18–20).

CD1d molecules have four occupied N-glycosylation sites (five in the case of mouse CD1d) and six Cys residues, four of which are involved in the formation of intrachain disulfide bridges. Correct folding of the heavy chain depends on glucose trimming (21) and involves the chaperones calnexin (CNX) and calreticulin (CRT) and the oxido-reductase ERP57. After release from the CNX/CRT cycle, the heavy chain binds non-covalently to β₂m before trafficking to the plasma membrane (21). In addition to gaining access to the endocytic pathway by AP2- and AP3-dependent endocytosis, CD1d molecules can also be directed there by association with the invariant chain, normally responsible for the endocytic localization of MHC class II mole-

ules, or by interaction with MHC class II invariant chain complexes (22, 23). CD1d undergoes multiple rounds of recycling between the endocytic system and the plasma membrane.

The four N-linked glycans of human CD1d are localized in the α1 and α2 domains, effectively surrounding the antigen binding site, and in this study, we have focused on the role of these glycans in the assembly and stability of CD1d molecules. We show that in mature human CD1d molecules, one of the glycans (at position Asn-42) exists mainly in a form that is sensitive to the enzyme endoglycosidase H (Endo H), likely the unprocessed high mannose form, and that its deletion affects the stability of the heavy chain-β₂m heterodimer. We propose a role for this glycan in mediating interactions between the heavy chain and β₂m.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The monoclonal mouse antibodies (mAbs) to human CD1d, CD1d51, and D5 have been previously described (24, 25). The polyclonal rabbit anti-human CD1d, CD1d51, and D5 have been previ-

ously described (24, 25). The polyclonal rabbit anti-human CD1d antibody was from Dako Cytomation. The rat anti-human Grp94 mAb was from Stressgen. The reagents used for sandwich ELISA (anti-human interferon-γ [IFN-γ] mAb, biotinylated anti-human IFN-γ mAb (clone 45B3), and avidin horseradish peroxidase conjugate) were from Pharmingen. The glycosidases Endo H and PNGase F were from New England Biolabs. α-GalCer and galactosyl-(α1-2)-galactosyl ceramide (GalGalCer) were synthesized as described (26).

**NK T Cell Clone**—The human Vα24/Vβ11 CD4+ NKT clone 6F5 was isolated by staining peripheral blood mononuclear cells from a normal human subject with mAbs specific for CD4 (OKT4; hybridoma obtained from ATCC, Manassas, VA) and the invariant T cell receptor α-chain expressed by CD1d-restricted NKT cells (6B11, Pharmingen). Cells staining positive for both mAbs were sorted as individual cells directly into wells of sterile 96-well plates using a MoFlo high speed fluorescence activated cell sorter with autoclone module (Dako-Cytomation). CD4+ NKT cell clones, including clone 6F5, were expanded using irradiated (50 grays) allogeneic peripheral blood mononuclear cell as feeders with PHA and recombinant interleukin-2 as described previously (27).

**Generation of N-Glycosylation Mutants of Human CD1d**—Mutant constructs were generated using a human CD1d cDNA expression plasmid, pSRα-neo.CD1d. CD1d cDNAs containing single N-glycosylation site mutations were generated by the PCR method using primers that mutated each of the codons encoding Asn to generate Ala substitutions. CD1d mutants containing two glycosylation site mutations were generated using single mutants as templates for second round PCR-based mutagenesis and so forth for mutants at three and eventually all four glycosylation sites.

**Generation of Stable Transfectant C1R Cells**—C1R cells were transfected with the vector pSRα-neo containing the gene of interest by electroporation at 230 mV/960 microfarads and selected for neomycin resistance at 1.8 mg/ml G418 (Invitro-

gen). The cells were assayed for CD1d surface expression by flow cytometry. Cells were maintained in Iscove’s modified Dulbecco’s medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), penicillin/streptomycin (Invitrogen), and G41B at 37 °C in 5% CO₂ atmosphere.

**Flow Cytometry**—C1R cells stably expressing wild-type or mutant CD1d molecules were washed in cold phosphate-buff-
ered saline followed by incubation with phycoerythrin (PE)-

 conjugated mouse anti-human CD1d mAb (clone CD1d42) recognizing heavy chain associated with β₂m or PE-rat anti-

mouse IgG₁ (A85-1) as an isotype control (Pharmingen).

**NK T Cell Stimulation Assay**—C1R cells transfected with wild-type and mutant CD1d constructs were pulsed for 6 h with α-GalCer or GalGalCer (100 ng/ml in Me₂SO) or Me₂SO as control. After three washes with phosphate-buffered saline, the cells were fixed in 0.05% glutaraldehyde. Cells were washed twice in Iscove’s modified Dulbecco’s medium and once in NKT cell medium before use. Triplicate samples of 75,000 cells were co-cultured with 25,000 6F5 NKT cells in 96 U-bottom well plates for 22 h. The medium was supplemented with phorbol 12-myristate 13-acetate (1 ng/ml) (Sigma) and interleukin-2 (1 μg/ml) (Preprotech). Secreted human IFN-γ was detected by sandwich ELISA.

**Metabolic Labeling and Immunoprecipitation**—Labeling with [³⁵S]methionine/cysteine (ICN, PerkinElmer Life Sci-

ences) and immunoprecipitations were performed as described (21). Reimmunoprecipitation was performed by heating the washed immunoprecipitates in 1% SDS, 5 mM dithiothreitol for 3 min at 100 °C before diluting 10-fold in 1% Triton X-100 in 0.15 M NaCl, 0.01 M Tris, pH 7.4 (TBS), 1.5 mM iodoacetamide. The supernatants were then used for reimmunoprecipitation with D5 antibody. The samples were boiled in SDS sample buffer and separated by SDS-PAGE (12% acrylamide) before autoradiography. When Endo H digestion was performed, the samples were incubated overnight with 50 units of Endo H according to the manufacturer’s protocol.
Purification of CD1d from C1R.CD1d Cells—5 × 10^9 C1R.CD1d cells were lysed in 2% CH_3COOH in TBS. The lysate was loaded onto a CD1d51 immunoaffinity column in tandem with a precolumn coupled with mouse IgG to remove nonspecific contaminating proteins (21). After washing with 0.1% C_12E_9/TBS, the proteins were eluted in acidic conditions (0.1% C_12E_9, 0.1M NaCl, 0.05M sodium acetate, pH 3.5). The fractions were neutralized with 1M Tris, and the proteins precipitated with ethanol. The proteins were then solubilized in 0.2% SDS.

Western Blotting—The proteins were resolved by SDS-PAGE and electrophoretically transferred to Immobilon-P membranes (Millipore). Blots were incubated with D5 mAb (1:5000) or rat anti-human Grp94 mAb (1:10,000) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000) antibodies or alkaline phosphatase-conjugated goat anti-mouse or goat anti-rat IgG (1:5000) for quantitative analysis. The proteins were detected by enhanced chemiluminescent Western blotting (SuperSignal West Pico, Pierce) in the first case or goat anti-rat IgG (1:5000) for quantitative analysis. The proteins were resolved by SDS-PAGE.

Peptide Analysis—After overnight digestion of the purified CD1d-β_m complexes with 100 units of Endo H, heavy chains were separated by SDS-PAGE. The proteins were stained with Coomassie Blue, and the bands were excised from the gel. The gel samples were washed in 50% acetonitrile, 10–50 mM NH_4HCO_3 dried, and incubated for 24 h at 37°C with 0.1 μg of trypsin/15 mm^2 gel in 10 mM NH_4HCO_3. The peptides were extracted in 0.1% trifluoroacetic acid, 60% acetonitrile, dried, and resuspended in 0.05% trifluoroacetic acid, 5% acetonitrile. Fractionation of the samples was performed on a Hewlett-Packard 1090 HPLC system equipped with an Isco Model 2150 peak separator and a 1 mm × 25 cm Vydac C-18 (5-μm particle size, 300-μm pore size) reverse phase column equilibrated with 98% buffer A (0.06% trifluoroacetic acid) and 2% buffer B (0.052% trifluoroacetic acid, 80% acetonitrile). Peptides were eluted at 50 μl/min (gradient program: 0–60 min (2–37% buffer B), 60–90 min (37–75% buffer B), and 90–105 min (75–98% buffer B) and detected by their absorbance at 210 nm. Fractions were collected, and the peaks of interest were subjected to Edman sequencing using an ABI Precise 494 cLC instrument with an on-line HPLC for detection of the phenylthiohydantoin amino acids.

Molecular Modeling of Glycosylated CD1d—The model of glycosylated CD1d was generated starting from the crystal structure of human CD1d complexed with the α-GalCer, Protein Data Bank code 1zt4 (3), in which the four glycan structures are not resolved in the electron density map. The glycans attached by in silico modeling to the heavy chain core of CD1d were of the GlcNAc_2Man_α type in a configuration consistent with the most populated conformer derived from a data base of experimental glycan structures (28). Glycan attachment, conformational search, and clash analysis were performed with Glyco-Pack, an in-house software package designed for glyco-protein structural analysis. Refined modeling was performed using the Insight II software from Accelrys using the Discover module with the cvff force field for the simulated annealing and the energy minimization. The solvent accessibility was calculated with Naccess 2.1.1 with a 1.5 Å radius probe.

RESULTS

None of the Four N-Linked Glycans Are Essential for Recognition by NKT Cells—We then wished to determine whether the absence of any of the N-linked glycans interferes with lipid loading and presentation to CD1d-restricted NKT cells. α-GalCer can bind to cellular CD1d by direct exchange at the plasma membrane or in the endocytic system (6, 7). A derivative, Gal-G5GalCer, can bind to CD1d but cannot stimulate NKT cells unless the terminal galactose residue is removed by lysosomal α-galactosidase to generate α-GalCer (30). C1R cells expressing wild-type or mutant CD1d molecules were incubated for 6 h at 37°C with vehicle control (Me2SO) or lipid (100 ng/ml α-GalCer or GalGalCer), fixed, and then co-cultured with the 6F5 NKT cell line for 22 h. The culture supernatants were assayed for secreted IFN-γ by a sandwich ELISA. All the CD1d mutants were able to present both α-GalCer and GalGalCer to the NKT cell line to about the same extent as wild-type CD1d (Fig. 2). Moreover, even in the absence of exogenously added antigen, IFN-γ release was readily detected, indicating that endogenous lipid loading by the mutants was normal. It was also evident from these experiments that the reduced surface expression of the mutant forms, seen in Fig. 2C, did not result in proportionate reductions in NKT cell recognition. Thus the absence of any single glycan had no discernable effect on the lipid binding and T cell recognition characteristics of CD1d.

Intracellular Transport of the CD1d Single N-Glycosylation Mutants—N-glycans can facilitate glycoprotein folding and trafficking through the secretory pathway (31). The expression of the CD1d mutants on the cell surface showed that they were transported but did not address whether the absence of any of the individual glycans affects the efficiency of folding or the rate of transport. To examine this question, the C1R transfectants were labeled with [35S]methionine/cysteine for 15 min, chased up to 8 h, and lysed in 1% Triton X-100 in TBS. The proteins were immunoprecipitated with the monoclonal antibodies

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S. J. Hubbard and J. M. Thornton, personal communication.

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CD1d51 or D5, which recognize CD1d-β₂m heterodimers or free heavy chains, respectively (25). Transport was monitored by acquisition of resistance to Endo H treatment. The transport properties of the mutants lacking glycan 1, 3, or 4 were similar to that of wild-type CD1d. The glycans of the CD1d-β₂m dimers began to acquire Endo H resistance at 2 h of chase, and by 8 h, the majority was in the mature, complex form (Fig. 3). The mutant lacking glycan 2 was transported more rapidly, with Endo H-resistant mature glycans detectable at 1 h of chase. Control immunoprecipitation using the anti-HLA-A2 monoclonal antibody BB7.2 gave no bands upon SDS-PAGE (data not shown).

Wild-type CD1d molecules with mature glycans were predominantly associated with β₂m, measured by efficient immunoprecipitation with the CD1d51 mAb as compared with the D5 mAb. This is best seen at the 4- and 8-h time points, looking at the Endo H-treated lanes of Fig. 3. The mutants lacking glycan 3 or 4 resembled wild-type CD1d. In contrast, more of the mature forms of the mutants lacking glycan 1 or 2 reacted with the D5 mAb, consistent with a lack of associated β₂m. The effect was most pronounced for the mutant lacking glycan 2. CD1d heavy chains associate with β₂m in the ER, as suggested by Fig. 3 and as described previously (21), and surface CD1d molecules are predominantly β₂m-associated. Therefore these observations suggested either that CD1d heavy chains lacking glycan 1 or 2 can be transported from the ER efficiently without β₂m association or that the heterodimers they form dissociate more readily upon detergent solubilization.

**CD1-β₂m Dimers Lacking Glycan 2 Dissociate Readily in Detergents**—Different detergents have variable effects on protein-protein interactions. For example, the interaction of transporter associated with antigen processing (TAP) with the transmembrane glycoprotein tapasin is preserved in digitonin but disrupted in Triton X-100 (32). We therefore
compared the stability of wild-type and mutant CD1d-β₂m dimers in digitonin and Triton X-100. Transfected C1R cells were labeled for 15 min with [35S]methionine/cysteine and chased for 4 h to ensure that the majority of the molecules had exited the ER. Aliquots of the cells were extracted in either 1% Triton X-100 or 1% digitonin in TBS, and immunoprecipitations were performed with the D5 mAb or a rabbit anti-human β₂m antibody to compare the amounts of free heavy chain and CD1d-β₂m dimers. Control experiments using untransfected C1R cells showed little background in immunoprecipitations using these antibodies (Fig. 3). For the mutant lacking glycan 2, there was considerable free mature heavy chain even in digitonin, suggesting that this glycan has a critical role in maintaining the stability of the CD1d-β₂m heterodimer upon detergent extraction.

To confirm the results in Fig. 4A and to rule out a role for differences in the extraction capacity of the two detergents, cells were similarly labeled and extracted in 1% digitonin, and the CD1d-β₂m heterodimers were immunopurified with anti-β₂m antibody and protein A-Sepharose beads. The beads were then incubated for 4 h either in TBS or in TBS containing 1% digitonin or 1% Triton X-100 followed by centrifugation. The extent to which free heavy chains dissociated from β₂m upon incubation in the various conditions was assessed by immunoprecipitation of the supernatants with the D5 antibody. As shown in Fig. 4B, for all the species, Triton X-100 induced substantial heavy chain dissociation from β₂m as compared with incubation in the absence of detergent or in the continued presence of digitonin. As expected from the data in Fig. 4A, the most pronounced effect was observed for CD1d-β₂m dimers lacking glycan 2.

To investigate the effect of the presence of glycan 1 or 2 on CD1d interactions with β₂m, we generated triple N-glycosylation mutants of human CD1d and expressed them in C1R cells. Mutants expressing only glycan 1 or only glycan 2 were readily detected at the plasma membrane in association with β₂m, although at lower levels than wild-type CD1d (Fig. 4C). Mutants expressing only glycan 3 or 4 were expressed at even lower levels, and it was very difficult to isolate significant quantities of radiolabeled CD1d-β₂m heterodimers from these cells. Similarly, a mutant CD1d lacking all four glycans was not detectably surface-expressed at all. Therefore these mutants were not further analyzed (data not shown). For the mutants expressing glycan 1 or 2, we used the same experimental approach as in Fig. 4B to examine the stability of the singly glycosylated CD1d-β₂m dimers. The dimers were immunopurified using the anti-β₂m antibody from digitonin extracts. After incubation without detergent or in digitonin or

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![Image](49x429 to 408x363)

**FIGURE 2.** Mutant CD1d molecules lacking any individual glycan can load lipid antigen and present it to NKT cells. C1R cells expressing CD1d or the mutants were incubated for 6 h with α-GalCer (100 ng/ml), GaGalCer (100 ng/ml), or vehicle control (Me2SO). After washing and fixing, the cells (75,000) were co-cultured with human 6F5 NKT cells (25,000) in triplicate. Secretion of IFN-γ into the medium was analyzed by sandwich ELISA. Results are expressed as mean and standard deviation of triplicate values. WT, wild type.

**FIGURE 3.** Intracellular transport and N-glycan processing of CD1d and the N-glycosylation mutants. A, cells stably expressing wild-type (WT) CD1d or the mutants lacking the individual N-linked glycans were pulse-labeled with [35S]Met/Cys for 15 min, chased up to 8 h, and extracted in 1% Triton X-100. CD1d-β₂m heterodimers or free CD1d heavy chains were immunopurified with the CD1d51 or D5 mAbs, respectively. After heating in SDS, the free heavy chains from both samples were reimmunoprecipitated with the D5 mAb, and half of each sample was digested overnight with Endo H. The samples were separated by SDS-PAGE and analyzed by autoradiography. B, quantitation of the data in panel A for the mutant CD1d, presented as the percentage of Endo H resistance at each time point (▲). Each panel includes the curve for wild-type CD1d (●) for ease of comparison, and they represent the Δ1, Δ2, Δ3, and Δ4 mutants from the top to the bottom, respectively.
Triton X-100, the free heavy chains released were quantitated. The results, shown as the percentage of the total amount of heavy chain that remains \(\beta_2\text{m}-\text{associated}\), are presented in Fig. 4D. In Triton X-100, \(\sim 55\%\) of the wild-type CD1d heavy chains remained \(\beta_2\text{m}-\text{associated}\). If only glycan 2 was expressed, the amount was \(\sim 20\%\). However, if glycan 2 was absent and only glycan 1 was expressed, virtually none of the CD1d heavy chain remained \(\beta_2\text{m}-\text{associated}\). The data suggest that glycan 2 is essential for the stability of the CD1d-\(\beta_2\text{m}\) complex in the presence of Triton X-100.

**Glycan 2 Is Not Processed to Complex Forms in Wild-type CD1d**—Endo H digestion of mature wild-type CD1d heavy chains reproducibly results in a slight increase in electro-
To identify the Endo H-sensitive glycan, CD1d heavy chains, untreated or digested with Endo-H or PNGase F, detected by the D5 mAb. The peaks were sequenced by Edman degradation. In the upper panel, the inset shows a Western blot of the purified CD1d heavy chains, untreated or digested with Endo-H or PNGase F, detected by the D5 mAb. The upper panel shows a Western blot of the purified CD1d heavy chains, untreated or digested with Endo-H or PNGase F, detected by the D5 mAb.

The mobility shift correspondingly observed upon Endo H digestion was apparent (Fig. 5, inset). Deglycosylation was complete when glycopeptides containing glycan 2 were analyzed by reverse phase HPLC using a C18 column. As shown in Fig. 5, comparison of the two samples revealed only a single peak with an altered retention time. The peak from the Endo H-treated sample had a longer retention time (84 min) than the one from the control sample (82 min). The peptides eluting in these peaks were N-terminally sequenced by Edman degradation. The peak from the control sample contained a mixture of two peptides with the N-terminal sequences TDGLAXLQELQTH and LYPXELQVSAGXEVK, where X is an undetermined amino acid. The first sequence was dominant and corresponded to the peptide containing glycan 2 (residues 28–48, with the anticipated glycosylated Asn residue at position 42). The second corresponded to the peptide containing glycan 3 (residues 90–121, with the anticipated glycosylated Asn residue at position 108). The specific peak from the Endo H-treated sample contained a single peptide with the N-terminal sequence TDGLAXLQELQTH, clearly corresponding to the peptide derived from the glycopeptide containing glycan 2. The residual small peaks from the Endo H-treated sample eluting at 82–83 min were also subjected to Edman sequencing. This revealed a low level mixture of the two peptides found in the untreated sample (data not shown). The data clearly show that glycan 2 in the CD1d heavy chain remains predominantly Endo H-sensitive, suggesting that it may be relatively inaccessible to the Golgi glycosyl transferases responsible for the formation of complex glycans. A likely explanation is that the glycan is obscured in the transport-competent folded form of the glycoprotein.

**Glycan 2 Interacts with the Surface of β2m**—To gain insight into the possible orientation of glycan 2 in the assembled CD1d-β2m dimer, we performed molecular modeling using the published structure of the CD1d-β2m-α-GalCer complex (3). For simplicity, the glycans were all modeled in the GlcNAc2Man3 form. The analysis revealed that glycan 2, positioned at Asn-42, exhibited potentially significant glycan-protein clashes, whereas the three remaining sites could accommodate glycans without any stereochemical conflicts. Further extensive conformational searches showed that to avoid clashes with any heavy chain or β2m, the allowed configuration of the Asn-42-glycan linkage was severely restricted (Fig. 6A). This is due to the very low accessibility of this site, which restricts the glycan in relation to heavy chain, and also its loca-
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FIGURE 6. Glycan 2 resides at the interface between the CD1d heavy chain and β2m. A, Ramachandran type plot of Asn side chain angles (χ1,χ2). The circled X shows the experimental side chain angles of Asn-42 derived from crystallographic coordinates in Protein Data Bank 1zt4; the circled period shows the optimal configuration used for modeling glycosylated Asn-42. Blue areas cover Asn side chain configurations giving no “hard” clashes (i.e. backbone atoms and Cβ) with CD1d heavy chain. Magenta areas cover configurations giving clashes with β2m. The black dots are experimentally documented glycosylated Asn side chain configurations; one of these is very close to the optimal values (86,294) used to generate the model. B, two orientations (frontal and lateral, rotated 90 degrees counterclockwise) of the CD1d-β2m dimer, showing the modeled glycan 2 in the space-filling form (orange). The CD1d heavy chain is shown in blue, and β2m is shown in magenta. α-GalCer (green and red) is just visible in the orientation on the right. Glycans 1, 3, and 4 (Asn-20, Asn-108, Asn-163) are shown in a stick representation in orange.

tion with respect to the associated β2m. Finally, as a starting point for modeling, an Asn-42-glycan linkage configuration was chosen that was the best compromise between a minimal deviation from the experimental Asn-42 side chain conformation, a minimal deviation from the closest experimentally documented Asn-glycan linkage conformation, and constraints imposed by the nature of clashes and the reduction of their number: for example, the need to avoid superposition of backbone atoms and side chain β-carbons.

The starting model with glycans attached in the experimental configuration for Asn-20 (glycan 1), Asn-108 (glycan 3), and Asn-163 (glycan 4) and the optimal configuration at Asn-42 (glycan 2) was subjected to repeated rounds of local simulated annealing followed by energy minimization, with constraints imposed on α-carbons and further constraint-free energy minimization. Modeling the structure around site Asn-42 needed careful attention because of the very limited allowed conformational freedom. To avoid steric clashes with the CD1d heavy chain, the glycan had to be persistently oriented toward β2m, and only a narrow space available at the surface of β2m avoided clashes with both heavy chain and β2m. In contrast, glycan 1 at Asn-20 is relatively distant from β2m and thus forms fewer contacts with it. The location of the remaining two glycans in the structure makes contacts with β2m impossible. A structural model with glycan 2 added is presented in Fig. 6B.

Differential solvent accessibility analysis of the model also reveals extensive contacts between glycan 2 and the protein core of CD1d, including both heavy chain and β2m. As described above, this glycan practically sits on the surface of β2m (Fig. 6B), but more importantly, it shelters the interface between heavy chain and β2m. For example, four residues located at this interface, heavy chain residues Arg-25 and Asp-27 and β2m residues Asp-35 and Leu-55, show a direct and consistent reduction of their solvent accessibility when the glycan is attached. These observations suggest a possible explanation for the experimental data showing that this glycan retains sensitivity to Endo H and for the complex behavior in detergents of CD1d-β2m dimers in the mutants lacking glycan 2.

DISCUSSION

The addition of N-linked glycans to newly synthesized glycoproteins is thought to occur during translocation into the ER, shortly after the three-residue glycosylation site (NXT or NXS, where X is any amino acid except proline) enters the ER lumen (33). At this stage, conformational restraints are limited, and consequently, almost all available glycosylation sites are occupied. In their monoglucosylated form, the glycans can serve as substrates for CNX or CRT binding, facilitating glycoprotein folding by the CNX/CRT/ERp57-dependent folding cycle in which the enzymes glucosidases I and II and UDP-glucose glycoprotein transferase work together to ensure the correct conformation and disulfide bonding pattern (31). Once folding is complete, the glycoproteins typically leave the ER and transit the Golgi apparatus on their way to the plasma membrane. During transport through the Golgi, multiple additional modifications and sugar additions take place, resulting in the generation of complex-type glycans terminating in sialic acid residues. Characteristically, N-linked glycans are surface-exposed, and because of this, the majority of them mature to the complex type. Occasionally, a particular N-linked glycan in a glycoprotein remains in the immature form. Steric constraints that are imposed after folding and consequent shielding of the glycan from the trimming enzymes and glycosyl transferases present in the Golgi that are responsible for the later stages of glycan maturation are a plausible explanation for this course of events (34).

The initial stages of human CD1d folding and disulfide bond formation are glucose trimming-dependent and involve CNX, CRT, and ERp57 (21). Notably, disulfide bond formation is complete before the association of the heavy chain with β2m, and the chaperones do not interact with CD1d-β2m dimers. Therefore a likely scenario based on what we have observed is
that glycan 2, which remains almost exclusively Endo H-sensitive after CD1d transport to the cell surface, becomes inaccessible after β2m association. The structural model presented in Fig. 6B is entirely consistent with this hypothesis; glycan 2 substantially interacts with both the heavy chain and β2m in the folded structure. Glycans 3 and 4 are not predicted to interact with the folded protein, and only minor interactions are possible for glycan 1. It is also interesting to note that the CD1d mutant lacking glycan 2 is transported more rapidly from the ER, based on the rate at which it acquires Endo H resistance (Fig. 3). Based on the model structure in Fig. 6B, it is likely that the significant structural constraints imposed on the CD1d heavy chain-β2m interaction by glycan 2 slow down the rate of dimer formation and subsequent exit from the ER. The prolonged residence in the ER provoked by glycan 2 may be important in facilitating lipid association with CD1d molecules in the ER, which is mediated by microsomal lipid transfer protein (18).

The proximity of glycan 2 to the region of the CD1d-β2m interaction presumably in some way also explains the ready detergent-induced dissociation observed in the absence of this glycan (Fig. 4). Mutants lacking glycan 2 that express only glycan 1 or glycans 1, 3, and 4 dissociate more readily in Triton X-100 than wild-type CD1d-β2m dimers or dimers incorporating mutants that express glycan 2. The interactions of glycan 2 with both the heavy chain and β2m, as predicted by the model, could play a role in stabilizing the dimer. There are two possible mechanisms that could explain this. First, detergent monomers could interact with the lipid binding site. The first structure obtained for a CD1 molecule with a bound ligand, that of CD1b, had a detergent molecule and a lipid molecule present simultaneously in the binding groove (35), and we have observed detergent-dependent dissociation of CD1d-bound lipids in the laboratory (data not shown). Conceivably, eliminating associated lipid could reduce the stability of the CD1d-β2m dimer, similar to the way that lack of peptide association reduces the stability of MHC class I-β2m dimers (36), and eliminating the interactions of glycan 2 with the protein components could exacerbate this. Alternatively, monomeric detergent molecules could insert more readily into the site of interaction of the CD1d heavy chain and β2m when glycan 2 is absent. The solvent accessibility constraints imposed by the presence of glycan 2 that we observed during the modeling procedure would be consistent with this idea. The hydrophobic domain of digitonin is a rigid, steroid-based structure with a covalently bound pentameric sugar forming the hydrophilic portion of the detergent. On the other hand, Triton X-100 is relatively flexible with an octylphenol hydrophobic component and a polyethoxylate hydrophilic component. The flexibility of Triton X-100 may allow it to insert more readily than digitonin into the CD1d-β2m interaction site, accounting for the differential abilities of the two detergents to induce dissociation (Fig. 4). Alternatively, and for similar reasons, Triton X-100 may be better able than digitonin to induce lipid dissociation from the binding site. Currently, no data are available that would allow us to distinguish between these two hypotheses.

The proposed roles of N-linked glycans are varied, ranging from protein folding (31), intracellular transport (37), and protection from degradation (31, 38). However, there are few examples in the literature where defined interactions between glycans and the proteins that bear them have demonstrated biological significance. One interesting example is in the proform of the lysosomal enzyme cathepsin Z, where an interaction between a high mannose oligosaccharide and a β-hairpin loop in the protein appears to maintain the glycan in a conformation better able to interact with the cargo-receptor ERGIC-53 (39). Elimination of the glycan or the loop by mutation reduces the rate of export of the proenzyme from the ER. Interestingly, maintenance of the loop is required to prevent the maturation of the glycan into the complex form. This is similar to the suggestion from our own work that its interaction with the CD1d-β2m interface prevents the maturation of glycan 2.

CD1d molecules use the glycan-dependent CNX/CRT cycle during folding, although assembly can still proceed in the absence of any single glycan, and indeed, it can occur to some extent if only glycan 1 or 2 is present (Fig. 4C). Assembly is seriously impeded, however, if only glycan 3 or 4 is present, and in the complete absence of glycans, no assembly or surface expression of heavy chain-β2m dimers occurs (data not shown). This is likely to reflect a requirement for the glycan-dependent CNX/CRT folding cycle. Glycan 1 is the only glycan absolutely conserved among all the CD1d family members and therefore may be of particular importance. However, CD1d molecules clearly fold to some extent and they maintain function in its absence. Glycan 2 is not present in CD1b or CD1c, but a similarly placed glycan is present in CD1a. Whether this glycan interacts with β2m in CD1a remains unknown.

CD1d molecules recycle continuously through late endocytic compartments and are therefore exposed to proteases for prolonged periods. Presumably because of this, they have evolved to be highly protease-resistant (29, 40), allowing their survival in this environment. The presence of four N-linked glycans flanking the lipid binding site may play a role in this stability. However, the pulse-chase data presented in Fig. 3 do not indicate that any of the mutants lacking a single glycan are more rapidly turned over than the wild-type molecule. Thus the reason why CD1d molecules have evolved to express four glycans in the particular positions they occupy is not clear. Currently, no data are available that would allow us to distinguish between these two hypotheses.

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