Identification of an N-Linked Glycosylation in the C4 Region of HIV-1 Envelope gp120 That Is Critical for Recognition of Neighboring CD4 T Cell Epitopes[1]

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The heavy glycosylation of HIV-1 envelope gp120 shields this important Ag from recognition by neutralizing Abs and cytolytic CD8 T cells. However, very little work has been done to understand the influence of glycosylation on the generation of gp120 epitopes and their recognition by MHC class II-restricted CD4 T cells. In this study, three conserved glycans (linked to N406, N448, and N463) flanking the C4 region of gp120 that contains many known CD4 T cell epitopes were disrupted individually or in combination by asparagine-to-glutamine substitutions. The mutant proteins lacking the N448 glycan did not effectively stimulate CD4 T cells specific for the nearby C4 epitopes, although the same mutants were recognized well by CD4 T cells specific for epitopes located in the distant C1 and C2 regions. The loss of recognition was not due to amino acid substitutions introduced to the mutant proteins. Data from trypsin digestion and mass spectrometry analyses demonstrated that the N448 glycan removal impeded the proteolytic cleavage of the nearby C4 region, without affecting more distant sites. Importantly, this inhibitory effect was observed only in the digestion of the native nondenatured protein and not in that of the denatured protein. These data indicate that the loss of the N448 glycan induces structural changes in the C4 region of gp120 that make this specific region more resistant to proteolytic processing, thereby restricting the generation of CD4 T cell epitopes from this region. Hence, N-linked glycans are critical determinants that can profoundly influence CD4 T cell recognition of HIV-1 gp120. ***The Journal of Immunology,*** **2008, 180: 4011–4021.**

The importance of CD4 helper T lymphocytes in controlling various virus infections is well documented in the literature (1–7). A vigorous CD4 T cell response is also considered to be essential for the control of HIV infection (reviewed in Ref. 8). These cells provide help to both B cells and CD8 T cells for the development and the maintenance of their effector functions (9–12). CD4 T cells can also mediate direct antiviral activities by killing infected cells and/or by secreting cytokines and chemokines that suppress infection (13–15).

CD4 T cells typically recognize short peptide fragments derived from exogenous Ags that are internalized and processed by professional APCs and presented as MHC class II (MHC-II)[3]-peptide complexes on the cell surface (reviewed in Ref. 16). Thus, Ag processing, conducted by an array of endolysosomal proteases, is a critical step frequently required for the generation of most CD4 T cell epitopes (17). Specific aspartic and cysteine proteases have been shown to be necessary for the processing of certain Ags, such as the asparagine-specific endopeptidase required in the initial cleavage of tetanus toxoid C fragment (18), but little is currently known about the complete range of enzymes and enzymatic processes involved in processing individual Ags. The processing of antigenic proteins with posttranslational modifications is even less well understood, even though many of the peptide epitopes recognized by T cells are derived from proteins that are posttranslationally modified by acetylation (19), isoaspartylation (20), phosphorylation (21), or glycosylation (22, 23).

Many viral Ags that are important targets for the immune system are glycoproteins. The HIV envelope gp120, for example, is so highly glycosylated that the glycans (20–30 per molecule) account for half of the m.w. of this molecule. Although CD4 T cells have been found occasionally to recognize epitopes that contain few sugar residues or other modifications (22–24), most CD4 T cell epitopes are typically devoid of large carbohydrates. Indeed, heavy glycosylation on Ags, such as tumor Ags MUC1 and HER-2/neu, has been shown to hinder intracellular transport and processing in dendritic cells, rendering these Ags to be poorly immunogenic (25, 26). Interestingly, the presence of an N-linked glycosylation located outside the T cell epitope has also been shown to interfere with CD4 T cell recognition of influenza hemagglutinin Ag (27, 28), although the mechanisms by which such a glycan exerts its effect are not completely clear. In addition to steric hindrance that prevents peptide binding to MHC-II or restricts the proteolytic enzymes from having access to the proteolytic cleavage sites, the presence of large carbohydrate moieties on the protein surface also affects the folding and secondary or tertiary conformation of the

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[3] Abbreviations used in this paper: MHC-II, MHC class II; CHO, Chinese hamster ovary; MS, mass spectrometry; tPA, tissue plasminogen activator; WT, wild type.

protein Ag (29), thereby influencing the way the Ag is processed and presented on MHC-II (30).

Although many studies have shown the profound effects of N-linked glycans on the envelope gp120 of HIV or SIV on Ab and CD8 T cell responses to these Ags (31–38), very little is known about how these glycans affect gp120 Ag processing and its MHC-II presentation to the CD4 T cells. Previous studies have shown that CD4 T cell epitopes tend to cluster at specific nonglycosylated gp120 regions that are flanked with multiple N-linked glycosylation sites (39, 40), but it is not known how these N-glycans actually influence CD4 T cell recognition of gp120 epitopes. Botarelli et al. demonstrated that 20% of the CD4 T cell clones obtained from humans immunized with a recombinant, nonglycosylated form of gp120 failed to respond to glycosylated gp120 of the same HIV-1 isolate (41). They further identified that one of the epitopes recognized by such clones contained two asparagines that were glycosylated in the native glycosylated gp120 and therefore were not efficiently recognized by the CD4 T cells (41), indicating that the presence of N-linked glycans within or near to an epitope can have a serious impact on its recognition by CD4 T cells. However, the obstructive effects of N-linked glycosylation on CD4 T cell recognition are not a universal phenomenon (22–24).

In this study, we investigated the contribution of three conserved glycosylation sites flanking the CD4 T cell epitope cluster in the C4 region of gp120 (designated G1, G2, and G3 at positions 406, 448, and 463, respectively). The glycosylation sites at N406 and N463 have been shown to bear complex oligosaccharides, while high mannose or hybrid glycans have been found at N448 (42). Previously, elimination of these three N-linked glycans was shown not to increase exposure of known neutralizing epitopes or uncover new B cell epitopes (43, 44). However, immunization with gp120 lacking these three glycans resulted in the loss of T cell responses to a specific epitope located in the nearby C4 region (45), although the mechanism(s) by which the N-linked glycans affect the T cell recognition were not at all understood. To investigate this issue, we produced gp120 proteins lacking single or triple glycans at these three positions and examined their recognition by CD4 T cell lines specific for the C4 epitopes or other distant epitopes. We identified that the N-linked glycan at position 448 was absolutely necessary for the CD4 T cell recognition of the C4 epitopes. We further demonstrated by trypsin digestion and MALDI-TOF mass spectrometry (MS) that the loss of this particular N-glycan rendered the C4 region of gp120 more resistant to proteolytic digestion, thereby hindering the release and presentation of the C4 epitopes. Hence, N-glycans located near CD4 T cell epitopes on HIV gp120 are critical determinants for efficient processing, generation, and consequently the recognition of these epitopes by the T cells.

Materials and Methods

Recombinant proteins, synthetic peptides, and mAbs

Recombinant baculovirus-derived $gp120_{IIIB}$ purchased from ImmunoDiagnostics was used for in vitro antigenic stimulation to expand the primary CD4 T cells specific for the different gp120 epitopes. $Gp120_{BH10}$ proteins with the wild-type (WT) sequence and with various N-to-Q mutations tested in this study were generated in our laboratory as described below. Synthetic gp120 peptides were provided by the National Institutes of Health AIDS Reagent Repository or the National Institute for Biological Standards and Control Centre for AIDS Reagents, except for the gp120 peptide (residues 432–452) with an N-to-Q mutation at position 448, which was purchased from Global Peptide. Peptides AQUA-C4 (MYAPPISGQI*R) and AQUA-C2 (VSFEPIPIHYCAPAGFAIL*K) bearing ^{13}C and ^{15}N-labeled K or R residues were purchased from Sigma-Genosys. Human gp120-specific mAbs used in this study were generous gifts from Drs. Susan Zolla-Pazner (New York University School of Medicine) James Robinson (Tulane University School of Medicine), and Abraham Pinter (Public Health Research Institute), as well as from Drs. Hermann Katinger (2G12), Dennis Burton, and Carlos Barbas (b12) through the National Institutes of Health AIDS Reagent Repository Program.

CD4 T cells

Primary HIV-1 gp120-specific CD4 T cell lines PS01, PS02, and PS05 were generated from PBMCs of chronically infected HIV-1 patients and have been maintained as short-term cultures (~8 wk) by in vitro stimulation with irradiated autologous PBMCs treated with $gp120_{IIIB}$ in the presence of IL-2 (Roche) (46). These human CD4 T cells recognize the dominant epitopes in the C1, C2, and C4 regions of gp120 (Fig. 1). All subjects whose cells were used to establish the T cell lines gave informed consent, and the study was reviewed and approved by the Veterans Affairs New York Harbor Healthcare System Institutional Review Board. A gp120-specific mouse CD4 T cell clone T1 was provided by Drs. Jeffrey Ahler and Jay Berzofsky (National Institutes of Health). This line recognizes an epitope in C4 region of gp120 that partially overlaps with that of PS05 (Fig. 1) (47). Gp120 peptide-pulsed irradiated syngeneic mouse splenocytes were used as APCs to stimulate the T cells.

Construction of gp120-expression plasmids and site-directed mutagenesis

The pEE14 vector expressing glutamine synthetase and HIV-1 glycoprotein $gp120_{BH10}$ genes was obtained from Dr. Simon Jeffs (Division of Medicine, Imperial College, London U.K.) via Dr. Luigi Buonaguro (Istituto Nazionale Tumori, Fondazione "G. Pascale", Napoli, Italy) (48). The $gp120_{BH10}$ gene and an upstream tissue plasminogen activator (tPA) signal peptide sequence were amplified using the Expand Long Template PCR System (Roche). The primers introduced unique *Hin*dIII and *Eco*RI restriction sites upstream and downstream of the amplified fragment. The tPA-$gp120_{BH10}$ cassette was then inserted into pUC19 to make a template construct for introducing the N-to-Q mutations that remove N-linked glycosylation sites at positions 406, 448, and 463 (designated G1, G2 and G3) (Fig. 1). The three pairs of primers designed to introduce these mutations are as follows: pair 1: 5′-TGGAGTACTAAAGGGTCACA GAACACTGAAGGAAGTGACA-3′ and 5′-TGTCACTTCCTTCAGT GTTCTGTGACCCTTTAGTACTCCA-3′; pair 2: 5′-TGGACAAATT AGATGTTCATCACAGATTACAGGGCTGCTATTAACAAG-3′ and 5′-CTTGTTAATAGCAGCCCTGTAATCTGTGATGAACATCTAAT TTGTCCA-3′; pair 3: 5′-TGGTGGTAATAGCAACCAGGAGTCCG AGATCTTCAGAC-3′ and 5′-GTCTGAAGATCTCGGACTCCTGGT TGCTATTACCACCA-3′). Site-directed mutagenesis was performed with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The entire gp120 gene of each mutant was sequenced to confirm that the specific mutations were introduced successfully without any other changes. To introduce multiple mutations, each mutagenesis was done sequentially. After the mutations were introduced, the tPA-$gp120_{BH10}$ cassette was then removed from pUC19 and inserted back to the pEE14-expressing vector for transfection and expression of soluble gp120 in Chinese hamster ovary (CHO) cells.

Expression and purification of gp120 proteins

Transfection of CHO-L761h cells with the pEE14 expression vector was performed by calcium phosphate precipitation. The selection of stable clones was conducted by glutamine depletion (48). Briefly, transfected cells were selected by growing in glutamine-free DMEM media (SAFC Biosciences) supplemented with 10% of glutamine-free FCS and nonessential amino acids. Additionally, L-methionine sulfoximine was added to inhibit the endogenous L-glutamine synthesis gene. Detection of gp120 in the culture supernatant was done by an in-house sandwich ELISA with sheep anti-C5 Ab (Cliniqa) and a mAb specific to the V3 region of gp120 (694-98D). Clones with stable gp120 expression were selected for large-scale gp120 production.

Stably transfected clones were expanded in T75 flasks in the presence of 200 μM of L-methionine sulfoximine. Supernatants were collected every 3–4 days and stored at −80°C. Gp120 proteins were purified from the supernatants by affinity chromatography using the anti-V3 mAb 694-98D coupled to an AminoLink plus coupling gel column (Pierce Biotechnology). Fractions containing most of the gp120 proteins were collected and dialyzed against PBS. Purified proteins were aliquoted and stored at −80°C until use. Purity of each gp120 preparation was assessed by SDS-PAGE and Coomassie blue staining, and their concentrations were determined by the Bio-Rad AC DC protein assay.

mAb reactivities and function of gp120 proteins

ELISA to detect mAb binding to gp120 proteins was done as previously described (49). Gp120 proteins were captured onto the wells by sheep

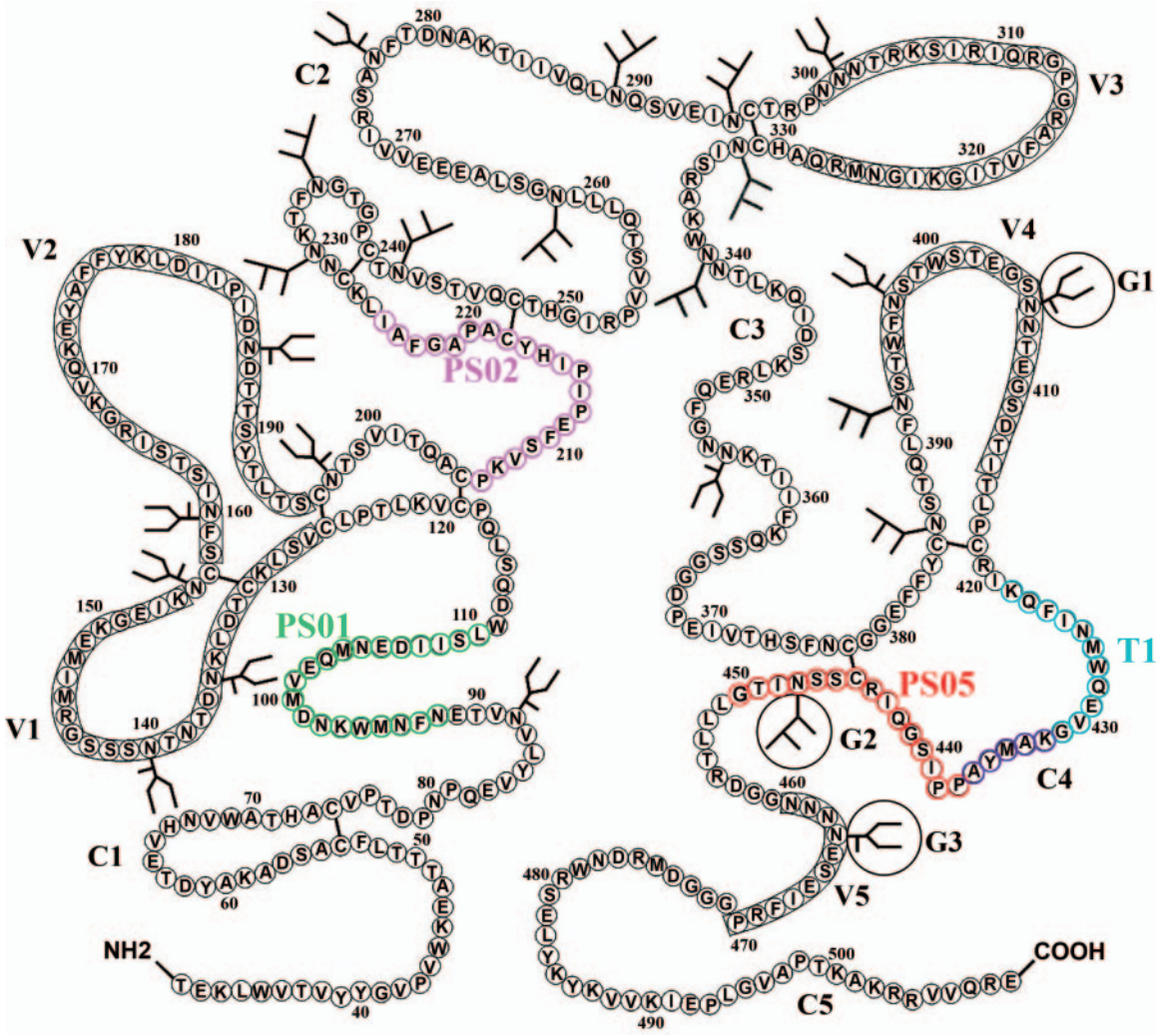


FIGURE 1. Site-specific N-linked glycan deletions introduced to gp120 and their positions relative to CD4 T cell epitopes examined in the study. N-to-Q substitutions were introduced to $gp120_{BH10}$ to remove N-linked glycans at positions 406, 448, and 463 (marked by circles and designated G1, G2, and G3, respectively). Locations of the epitopes recognized by gp120 CD4 T cells tested in this study were highlighted by different colors: green for the PS01 epitope in the C1 region, pink for the PS02 epitope in the C2 region, blue for the T1 epitope in the C4 region, red for the PS05 epitope in the C4 region, and purple for the overlap between the T1 and PS05 epitopes. This diagram (modified from Leonard et al. (42)) also shows the relatively conserved (C1–C5) and variable (V1–V5) regions of the gp120 protein, while the 30-amino acid signal peptide at the N terminus was omitted.

anti-C5 Abs (Cliniqa) and reacted with different human anti-gp120 mAbs. Detection of the mAb binding was done using alkaline phosphatase-conjugated anti-human IgG (Sigma-Aldrich). CD4 binding was detected similarly using the mouse anti-CD4 mAb OKT4 and alkaline phosphatase-conjugated anti-mouse IgG.

The capacity of the WT or mutant proteins to mediate virus entry was determined using single-cycle pseudovirions in U87-$CD4^+CXCR4^+$ and TZM-bl cells. A 580-bp *Bgl*II fragment of $gp120_{BH10}$ was cloned into the HXB2-env backbone (50). The pseudovirions were then generated by transfecting 293T cells with the envelope constructs along with pSV-rev (51, 52) and Env-defective pNL4-3.LucR-E- (53, 54). Virus infectivity was assessed by infecting the target U87 or TZM-bl cells with each of the pseudovirions at the same amounts of p24 and/or RT. The luciferase activity was measured 48 h after infection. The following reagents were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health): pNL4-3.Luc.R-E- from Dr. Nathaniel Landau, HXB2-env from Dr. Kathleen Page and Dr. Dan Littman, and pSV-rev from Dr. Marie-Louis Hammarskjold and Dr. David Rekosh.

T cell proliferation assay

T cell proliferation was assessed by the standard [^{3}H]thymidine incorporation assay as described previously (49). Autologous PBMCs or mouse splenocytes were irradiated (12,000 rad) and treated with Ags for 18–22 h before use as APCs in the assay. In some experiments, EBV-transformed autologous B lymphoblastoid cells treated with 0.5% of formaldehyde were used as APCs. T cell responses to APCs alone in the absence of any Ag were determined in each assay as background proliferation. Each experimental condition was tested in triplicate, and all experiments were performed at least twice.

In vitro proteolysis of gp120

Digestions of gp120 by endolysosomal fractions isolated from B lymphoblastoid cell lines were done according to a previously published protocol (55). Gp120 proteins were incubated with lysosomal fraction (0.75 million cell equivalents per μg of gp120) overnight at 37°C in 0.08 M of sodium acetate buffer at pH 5. The kinetics of gp120 digestion were determined by measuring intact gp120 remaining over time in the reaction using a sandwich ELISA with Abs specific for the C and N termini of gp120 (49).

Trypsin digestion and MALDI-TOF MS analysis

Denatured and native nondenatured gp120 proteins (2.5 μg) were digested by trypsin (0.5 μg) overnight at 37°C in 10 μl of 25 mM NH_4HCO_3 buffer (pH 8). Before digestion, all samples were filtered using C18 ZipTips (Millipore). To denature the proteins, samples were dried, reconstituted with urea, and incubated with DTT for 1 h at 56°C. The samples were then treated with iodoacetamide in the absence of light for 30 min.

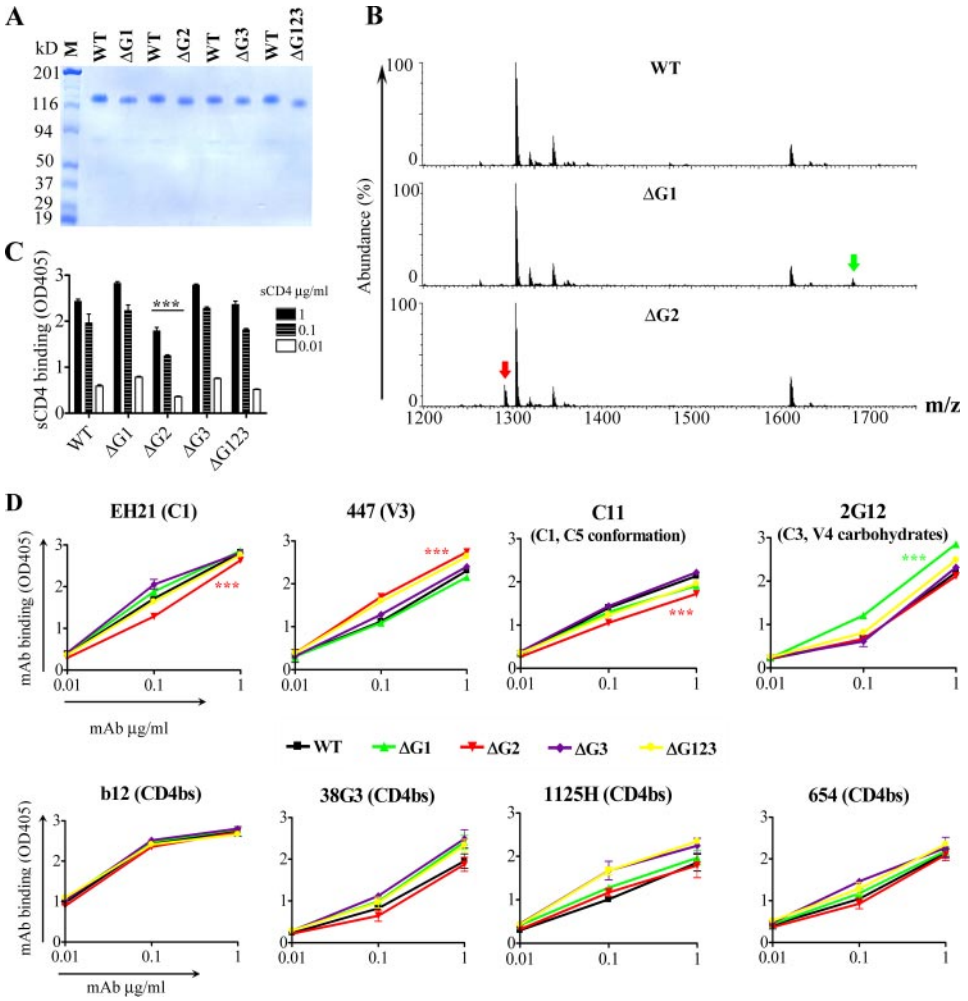


FIGURE 2. Characterization of gp120 proteins with N-glycan deletions. *A*, Recombinant gp120 proteins bearing either single N-to-Q mutations at positions 406 (ΔG1), 448 (ΔG2) or 463 (ΔG3) or triple mutations at all three positions (ΔG123) were purified by affinity column and run under a reduced condition on 10% SDS-PAGE alongside of the WT protein. The gel was stained with Coomassie brilliant blue to assess the apparent molecular masses and the purity of each protein preparation. Lane M shows the migration of standard molecular mass markers. The amount of protein loaded in each lane of this gel was not normalized, but the protein concentration of each sample was determined using the Bio-Rad AC DC protein assay before use in all subsequent experiments performed for this study. *B*, MS spectra of tryptic peptides derived from the WT and mutant gp120 proteins. gp120 proteins (WT, ΔG1, and ΔG2) were denatured and treated with trypsin as described in *Materials and Methods*. Unique peptides generated from ΔG1 and ΔG2 are indicated by a green arrow and a red arrow, respectively. These peptides correspond to GSQNTEGSDTITLPCR (m/z of 1679.8) and CSSQITGLLTR (m/z of 1291.7) in the ΔG1 and ΔG2 spectra, respectively. *C*, The CD4 binding capacity of the mutated gp120 proteins was determined by ELISA. Mutant and WT gp120 proteins were captured onto ELISA plates and incubated with varying concentrations of soluble CD4 (sCD4). The CD4 binding was detected by mouse anti-CD4 mAb OKT4 and alkaline phosphatase-conjugated anti-mouse IgG. Means and SDs of OD_{405} values from triplicate wells are shown. The data are representative results from one of two independent experiments. ***, $p < 0.001$ for ΔG2 when compared with WT and the other mutants. *D*, Ab reactivities of the mutant and WT gp120 were analyzed by ELISA. Gp120 proteins (1 μg/ml) were captured onto ELISA plates and reacted with human anti-gp120 mAbs at the designated concentrations. Alkaline phosphatase-labeled anti-human IgG was used to detect mAb binding. Means and SDs from triplicate wells are presented in the graphs. Representative results from one of two or more experiments are shown. ***, $p < 0.001$ as compared with WT.

Digestion reaction was terminated by adding formic acid. The digested products were then analyzed by MALDI-TOF MS (TofSpec 2E, Waters Micromass).

To quantify the amounts of specific gp120 peptides generated by trypsin digestion, AQUA peptides were synthesized with identical amino acid sequences but bearing ^{13}C- and ^{15}N-labeled K or R terminal residues. A known concentration of the AQUA peptide was mixed with the gp120 digestion products, filtered using C18 ZipTips, and analyzed by MS as described above. The amounts of gp120 peptides in the digestion reactions were calculated according to the following equation: $Area_{AQUA}/Concentration_{AQUA} = Area_{sample}/Concentration_{sample}$, with $Area_{AQUA}$ and $Area_{sample}$ representing the areas under the monoisotopic peaks for the AQUA or sample peptides in the MALDI-TOF spectra.

Statistical analysis

Statistical analyses for comparing mutant proteins with the WT gp120 were performed using one-way ANOVA with Dunnett's posttests or two-way ANOVA with Bonferroni's posttests (GraphPad Prism 4).

Results

Production of recombinant gp120 proteins lacking single or triple N-linked glycans

Because most CD4 T cell epitopes cluster in gp120 regions that are flanked by N-linked glycosylation sites, we sought to determine the role of these N-linked glycans in the generation and recognition of the

CD4 T cell epitopes. In this study, we focused on three highly conserved N-linked glycosylation sites at positions 406, 448, and 463 that flank the C4 epitope cluster, and we examined the effects of removing each of these glycans on CD4 T cell recognition of the C4 epitopes vs the more distant C1 or C2 epitopes (Fig. 1). Soluble recombinant gp120 proteins were produced with single N-to-Q mutations at each of the respective glycosylation sites (ΔG1, ΔG2, and ΔG3) or with triple mutations (ΔG123). The proteins were secreted from stably transfected CHO cell lines and affinity purified from the culture supernatant to attain >90% purity as confirmed by SDS-PAGE (Fig. 2*A*). Fig. 2*A* also shows the electrophoretic mobilities of the mutant proteins as compared with the WT protein. The ΔG1, ΔG2, and ΔG3 proteins migrated slightly faster than did the WT protein, consistent with a reduction of ~2–3 kDa in their apparent molecular mass due to the loss of one glycan, while the ΔG123 protein had the lowest apparent molecular mass, confirming the lack of three N-linked glycans in this protein. MS analysis of the tryptic fragments from ΔG1 and ΔG2 also confirmed the loss of N-linked glycans at positions 406 and 448, respectively (Fig. 2*B*). The WT and mutant gp120 proteins all displayed similar MS spectra, except for the appearance of peptides GSQ_{406}NTEGSDTITLPCR (m/z of 1679.8) and CSSQ_{448}ITGLLTR (m/z of 1291.7) in the ΔG1 and ΔG2 spectra, respectively. The corresponding WT peptides with N406 and N448 were not detected in the MS spectrum, due to the additional mass of the attached oligosaccharides at G1 or G2. These data confirm both the amino acid changes and the specific glycan removals in the ΔG1 and ΔG2 proteins.

Gp120 proteins lacking single or triple N-glycans bind to CD4 and anti-gp120 mAbs

To determine whether the removal of single or triple N-glycans caused significant alterations in gp120 conformation and function, the ability of mutated proteins to bind to soluble CD4 was tested in ELISA. All of the mutants retained their capacity to bind CD4, although ΔG2 consistently displayed a small decrease in CD4 binding as compared with WT and the other mutants ($p < 0.001$) (Fig. 2*C*). Interestingly, the ΔG123 mutant had CD4 binding comparable to the WT protein, possibly reflecting the compensatory effects of the ΔG1 and/or ΔG3 on the CD4-binding activity of the ΔG2 mutant. Nevertheless, these changes are relatively minor; the loss of N-glycans at positions 406, 448, and 463, either singly or jointly, did not have detrimental effects on the capacity of gp120 to bind CD4. We also observed that when these mutations were introduced to the envelope of pseudovirions, the viruses remained infectious. In fact, when tested in U87-CD4$^+$CXCR4$^+$ and TZM-bl cell lines, the infectivity levels of pseudovirions bearing the mutated envelopes (ΔG1, ΔG2, ΔG3, and ΔG123) were enhanced, ranging from 134 to 216% as compared with those of the WT virus. These data are consistent with previous findings from other laboratories (56, 57); however, whether these mutant viruses also showed comparable or slightly enhanced infectivity in primary target cells such as PBMCs remains to be determined. Hence, the minor changes in CD4 binding capacity of these mutants do not appear to seriously alter the envelope functions and virus infectivity.

To further probe for structural changes in the mutated gp120 proteins, the mutant proteins were examined for their reactivity with mAbs directed to different regions of gp120, including the conformation-dependent C1/C5 epitope (C11), the CD4 binding site (b12, 38G3, 1125H, and 654), the N-terminal C1 region (EH21), the V3 loop (447), and an epitope involving mannose residues in the C3 and V4 regions (2G12). The mutant and WT gp120 proteins displayed similar reactivities with most of the mAbs tested (Fig. 2*D*). Only small, albeit statistically significant, alterations were observed in the reactivities of ΔG2 with mAbs EH21, 447, and C11, as well as in the reactivity of ΔG1 with mAb

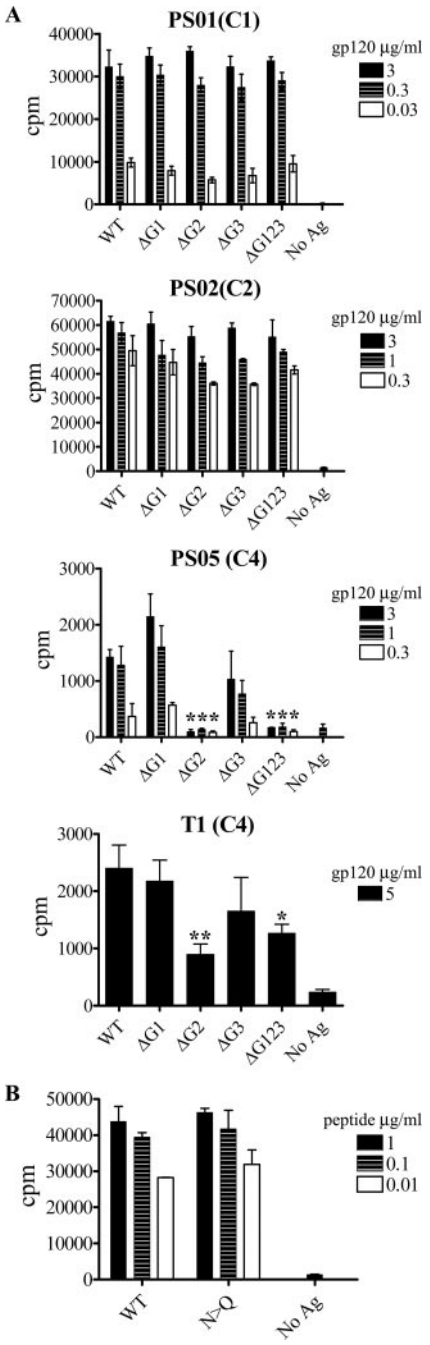


FIGURE 3. CD4 T cell responses to mutated gp120 proteins and peptide. *A*, Recognition of gp120 lacking site-specific N-glycans was assessed using CD4 T cell lines specific for a C1 epitope (PS01), a C2 epitope (PS02), or two overlapping C4 epitopes (PS05 and T1). T cell proliferation was measured in the standard [^{3}H]thymidine incorporation assay. Autologous human PBMCs or mouse I-E^{k+} splenocytes were used as APCs after irradiation and treatment with WT and mutant gp120 proteins at the indicated concentrations. *B*, An N-to-Q substitution at position 448 does not affect recognition of the C4-specific CD4 T cell line PS05. Peptide 432–452 with either the WT sequence or with an N-to-Q substitution at position 448 was tested for recognition by the CD4 T cell line PS05 in the [^{3}H]thymidine incorporation assay. Means and SDs of cpm from triplicate wells are presented in all of the graphs. The data are from one of two or more repeated experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (when compared with WT).

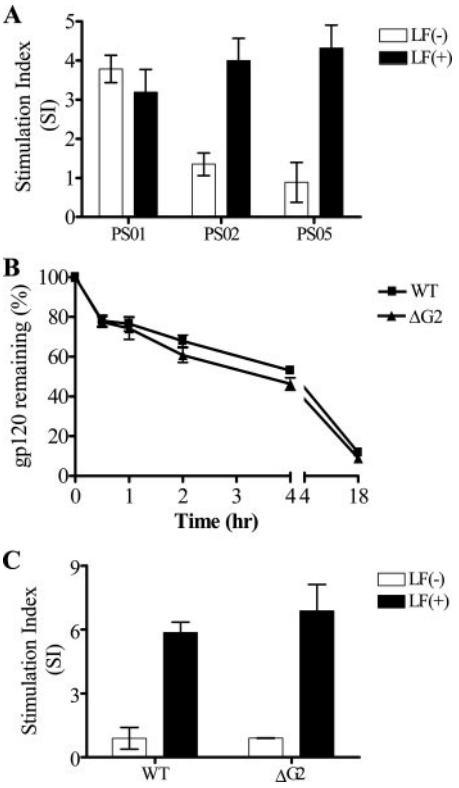


FIGURE 4. In vitro digestion of gp120 by lysosomal enzymes and the presentation of the digested gp120 to gp120-specific CD4 T cells by fixed APCs. *A*, Ag processing requirement for MHC-II presentation of the different gp120 epitopes was assessed. WT gp120 were digested in vitro with B lymphoblastoid lysosomal fraction (LF^+) for 18 h or left untreated (LF^-), and then incubated with fixed APCs. The proliferation of the CD4 T cell lines PS01, PS02, and PS05 in response to the fixed APCs treated with undigested or digested gp120 (1 μg/ml) was assessed in the standard [^{3}H]thymidine incorporation assay. Autologous formaldehyde-treated B lymphoblastoid cells were used as APCs. Background proliferation of the T cells in response to APCs treated with no gp120 was measured in each experiment. Stimulation indices (SI) were calculated as the ratios of the [^{3}H]thymidine incorporation (cpm) in experimental wells over the background cpm. Representative results of two independent experiments are shown. *B*, The kinetics of digestion of WT gp120 and the ΔG2 mutant by lysosomal enzymes were compared. gp120 WT and ΔG2 were digested by lysosomal enzymes, and the percentages of intact gp120 remaining at different time points were measured by ELISA as described in *Materials and Methods*. *C*, The presentation of the C4 epitope generated after in vitro lysosomal digestion of WT or ΔG2 proteins was evaluated. gp120 WT or ΔG2 were digested for 18 h with lysosomal enzymes or left untreated, and then incubated with formaldehyde-fixed B cell lines used here as APCs. The responses of the C4-specific T cell line PS05 to these APCs were assessed by [^{3}H]thymidine uptake as described above. Means and SDs from triplicate wells are shown.

2G12. Interestingly, unlike the single ΔG1 and ΔG2 mutants, the ΔG123 mutant did not show any significant difference in its reactivities with the mAbs tested, suggesting the potential compensatory effects of these three mutations. Overall, these results indicate that removal of the single or triple N-glycans and the N-to-Q substitutions introduced at the three conserved glycosylation sites did not greatly alter the overall gp120 conformation or function.

CD4 T cell recognition of gp120 proteins lacking single or triple N-linked glycans

To examine whether removal of N-glycans flanking the CD4 T cell epitope-rich area in the C4 region affected CD4 T cell recognition of gp120, we measured CD4 T cell proliferation in response to the mutant vs WT gp120 proteins. Three human CD4 T cell lines (PS01, PS02, and PS05) and a mouse CD4 T cell clone (T1) specific for different gp120 epitopes were tested (Fig. 3). The C1-specific PS01 and C2-specific PS02 lines recognized each of the four mutants as well as the WT ($p > 0.05$). However, the C4-specific PS05 line failed to respond to the ΔG2 and ΔG123 mutants at all concentrations tested ($p < 0.001$ when compared with WT). This particular line also responded poorly to ΔG2 when tested at 5 μg/ml (with stimulation index of 2, as compared with stimulation index of 11.5 for WT). Reduced recognition of ΔG2 and ΔG123 was also observed with the C4-specific clone T1 ($p < 0.01$ and $p < 0.05$, respectively). In contrast, PS05 responded well to both ΔG1 and ΔG3 at each of the concentrations tested ($p > 0.05$ as compared with WT). The T1 responses to ΔG1 and ΔG3 were also not significantly different from those against the WT protein ($p > 0.05$). These results demonstrate that the ΔG2 mutation at position 448 specifically affected CD4 T cell epitopes located in the nearby C4 region, and not those located in the more distant C1 or C2 regions.

To examine whether the N-to-Q mutation introduced at position 448 directly affected PS05 recognition, we introduced the same N-to-Q mutation in a 20-mer peptide covering the epitope recognized by T cell line PS05 (residues 432–452) (Fig. 1). Fig. 3*B* shows that the PS05 line responded to a peptide containing a glutamine (Q) at position 448 as well as the WT peptide ($p > 0.05$), demonstrating that the failure of PS05 to recognize ΔG2 is not likely due to the N-to-Q substitution introduced to position 448 of the mutant gp120. Rather, the data support the idea that the loss of an N-linked glycan at this C4 site may affect the way CD4 T cell epitopes derived from this particular region of gp120 are processed and presented.

Ag processing requirement for the different gp120 epitopes recognized by CD4 T cells

To determine whether gp120 Ag processing is necessary for the recognition of this Ag by CD4 T cells, we evaluated the CD4 T cell responses to whole undigested gp120 vs enzymatically digested gp120 that were presented by fixed APCs. WT gp120 proteins

Table I. *Tryptic peptide fragments identified by MS overlap with synthetic CD4 T cell epitope peptides*

gp120 Regions	Tryptic Fragments Detected by MS	Synthetic Peptides Recognized by T Cell Lines
C1	NDMVEQMHEDIISLWDQSLKPCVK[a]	NFNMWKNDMVEQMHEDIISL (PS01)
C2	VSFEPIPIHYCAPAGFAILK[b]	PKVSFEPIPIHYCAPAGFAI (PS02)
C4	AMYAPPISGQIR[c]	KAMYAPPISGQIRCSSNITG (PS05)
C4	QIINMWQEVGK	KQIINMWQEVGKAMYA (T1)

[a] Underlined letters indicate common amino acid sequences found in the tryptic fragments detected by MALDI-TOF MS and the synthetic peptides used to map the T cell epitopes.
[b] This fragment is designated peptide C2.
[c] This fragment is designated peptide C4.

were left untreated or were pretreated in vitro overnight with lysosomal enzymes isolated from the B lymphoblastoid cell line that have been routinely used as APCs, and then they were incubated with formaldehyde-treated B cells. The fixed B cells do not ingest and process exogenous Ag but retain the capability to present preprocessed peptides in the context of the proper MHC-II. The requirement of gp120 processing for CD4 T cell lines PS01, PS02, and PS05 was evaluated. The results show that PS05 did not recognize undigested gp120 presented by the fixed APCs, but it responded to gp120 that was predigested. Similar data were obtained with the PS02 line that recognized the C2 epitope. Surprisingly, the C1-specific PS01 cell line recognized both the digested and undigested gp120 equally well (Fig. 4*A*). These results demonstrate that while the C1 epitope recognition can occur without any processing, the proteolytic digestion of gp120 is absolutely required for the generation and presentation of CD4 T cell epitopes derived from the C2 and C4 regions of gp120. The reason for the unusual mode of CD4 T cell recognition of the C1 epitope is not known at this point; however, the crystallographic analyses of different HIV and SIV gp120 structures demonstrate that the C1 region is a part of the inner domain of gp120 that is exposed on the protein surface and is capable of undergoing large conformational shifts and rotations (32, 58). This fragment may be sufficiently surface-exposed and mobile to interact with MHC-II without any processing, although further investigations are necessary to examine this issue.

Generation of the C4 epitope by in vitro proteolysis of gp120 WT proteins and ΔG2

Because MHC-II presentation of the C4 epitope to the CD4 T cell line PS05 requires proteolytic processing, we investigated the effects of the ΔG2 mutation on the processing and generation of this particular epitope from in vitro digestion of the gp120 Ags by lysosomal enzymes. First, the kinetics of proteolytic digestion of WT and ΔG2 gp120 proteins by lysosomal enzymes were determined by measuring the amounts of intact undigested gp120 remaining in the reaction over time. Fig. 4*B* shows that both proteins were digested at comparable rates, and after overnight digestion, very little intact gp120 proteins, either WT or ΔG2, were detected. These data indicate that overall these two proteins were similarly sensitive to proteolysis by lysosomal enzymes. Nevertheless, there might be differences in proteolytic cleavage at specific gp120 regions that could not be detected in this assay. We subsequently evaluated if the C4 epitope recognized by PS05 could be generated and presented properly following in vitro digestion of ΔG2 as compared with that of the WT protein. After the gp120 proteins were digested overnight by lysosomal enzymes, the digestion products were incubated with fixed B cells used as APCs, and the PS05 responses to these fixed APCs were measured. Fig. 4*C* shows that the C4 epitope was efficiently presented to the PS05 cells after in vitro lysosomal digestion of either ΔG2 or WT proteins. These results rule out the possibility that the ΔG2 mutation might have destroyed the C4 epitope by either exposing or introducing new proteolytic sites in this region of gp120. The data also suggest that when the ΔG2 protein is processed by multiple lysosomal enzymes simultaneously, as occurs in the in vitro digestion condition, a sufficient level of the C4 epitope can be generated and presented on MHC-II to stimulate the PS05 cells. In contrast, the C4 epitopes may not be efficiently released following the stepwise Ag processing that is expected to take place inside the APCs.

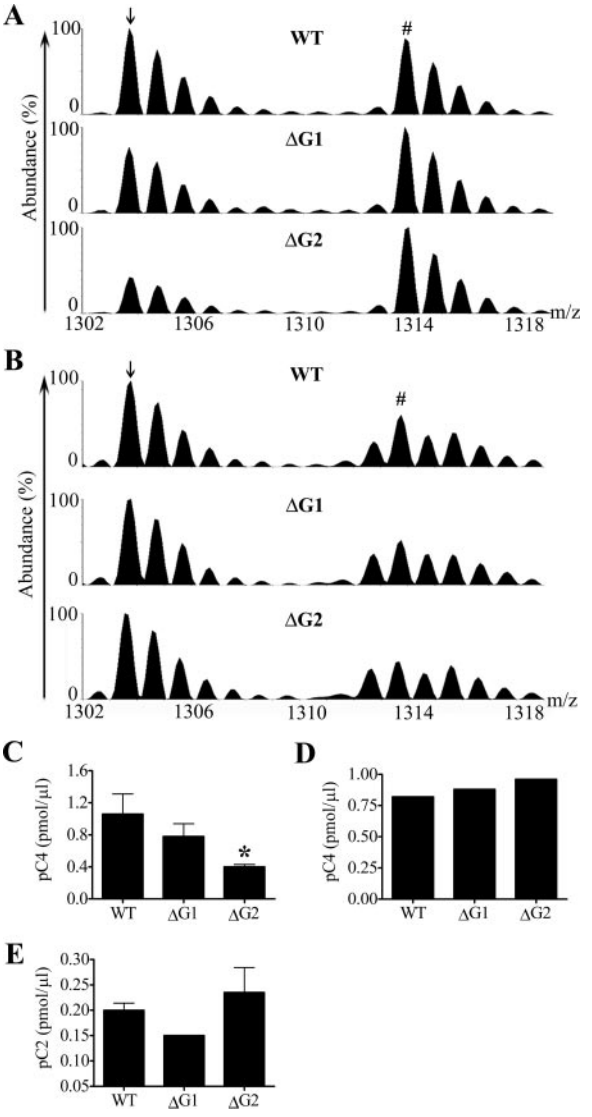


FIGURE 5. The ΔG2 mutation retards the trypsin digestion of the C4 region, but not the C2 region, of gp120. *A*, The amounts of peptide C4 produced following trypsin digestion of gp120 WT, ΔG1, and ΔG2 were quantified using the internal standard AQUA-C4 peptide. Each gp120 protein (2.5 μg) was digested with trypsin (0.5 μg) for 18 h. A known concentration of the AQUA-C4 peptide (0.5 or 1 pmol/μl) was added to the digestion products and analyzed by MALDI-TOF MS. The peaks of peptide C4 (m/z of 1303.8, marked with ↓) generated from the trypsin digestion of gp120 WT, ΔG1, and ΔG2 are shown together with the peaks of the AQUA peptide (m/z of 1313.8; marked with #). Representative spectra from one of two or more experiments are shown. *B*, The amounts of peptide C4 generated from the different gp120 proteins that were denatured before trypsin digestion were similarly measured. Each of the gp120 proteins was irreversibly denatured by treatment with urea, DTT, and iodoacetamide sequentially, then digested by trypsin and analyzed by mass spectrometry as described above. *C* and *D*, The calculated yields of peptide C4 (pC4) produced from trypsin digestion of the native nondenatured gp120 proteins (*C*) and denatured gp120 proteins (*D*) are presented. *, $p < 0.05$ as compared with WT. *E*, The yields of peptide C2 (pC2) generated from digestion of native nondenatured WT, ΔG1, and ΔG2 gp120 proteins were measured using the AQUA-C2 peptide standard. The average concentrations and SDs from two to three experiments are shown.

Effect of the N-linked glycan deletion ΔG2 on gp120 proteolytic cleavage

The Ag processing mechanisms necessary for generating CD4 T cell epitopes from the C2 and C4 regions of gp120 are not yet

known. In fact, none of the proteases involved in gp120 proteolytic processing within APCs has been defined. Hence, to test the idea that the loss of N448-linked glycan due to the ΔG2 mutation exerts a specific impact on the proteolysis of the C4 regions of gp120, we examined the relative sensitivity of this region as compared with the more distant C2 region to proteolytic cleavage by trypsin. MALDI-TOF MS was used to detect and quantify the different gp120 peptide fragments derived from the trypsin digestion. Gp120 tryptic fragments detectable by MS included peptides from the C1, C2, and C4 regions of gp120. Interestingly, these peptides partially overlap with each of the four CD4 T cell epitopes studied (Table I). These results confirmed previous findings by Brown et al. (59) showing that tryptic fragments of gp120 indeed overlapped with many of the immunodominant CD4 T cell epitopes already identified.

To assess if the ΔG2 mutation had significant effects on proteolytic cleavage of the C4 region of gp120, a C4-derived tryptic peptide fragment (designated peptide C4) that partially overlaps with the PS05 epitope was selected for the initial analyses. We compared the amounts of peptide C4 produced following trypsin digestion of the ΔG2, ΔG1, or WT gp120 proteins as a measure of the relative efficiencies by which the C4 region of the different gp120 proteins was proteolytically processed into smaller peptide fragments. Quantitative MS analyses were performed using a known quantity of an AQUA internal standard that had an identical sequence to peptide C4 but contained a ^{13}C- and ^{15}N-labeled arginine. Digestion of the ΔG2 protein produced a lower amount of peptide C4 ($p < 0.05$ as compared with WT) (Fig. 5, *A* and *C*). In contrast, the amount of peptide C4 released from ΔG1 digestion was not significantly different ($p > 0.05$). For comparison, the amounts of the C2 peptide generated from the trypsin digestion of the WT or mutant gp120 proteins were also analyzed and found to be comparable ($p > 0.05$) (Fig. 5*E*). However, when the gp120 proteins were irreversibly denatured and then digested, similar amounts of peptide C4 were produced from each of the three proteins (Fig. 5, *B* and *D*). These results suggest that the ΔG2 mutation at position 448 induces conformation changes in the C4 region of gp120 that render this particular region more resistant to proteolytic cleavage, impeding the generation of the peptide fragments from this region. The same mutation did not affect proteolytic cleavage of the more distant C2 region, and thus had no effect on the presentation of the C2 epitope. Notably, the reduction of the C4 peptide yield from the nondenatured ΔG2 is only ~67%, but this experiment was performed with trypsin and under an in vitro condition that does not replicate the processing environment inside APCs. The processing of the C4 region from ΔG2 inside APCs may be much more reduced, since the C4-specific CD4 T cells PS05 did not respond well to ΔG2, even when the amounts were increased from 0.3 to 5 μg/ml.

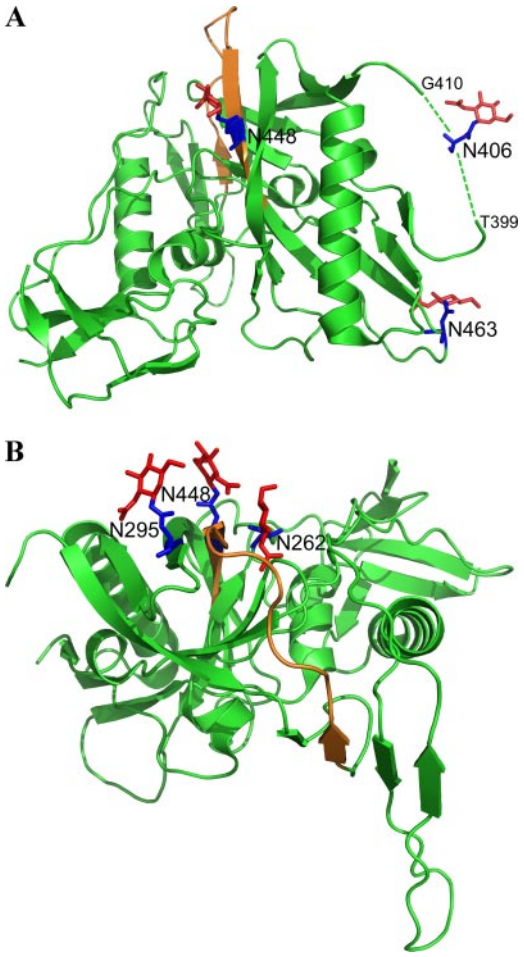


FIGURE 6. The location of N448-linked glycan (G2) on the gp120 three-dimensional structure in relation to the C4 epitope recognized by the CD4 T cell line PS05 and to other N-linked glycans described in this study. *A*, The positions of G1, G2, and G3 (linked with N406, N448, and N463, respectively) are shown in the context of the crystal structure of HIV-1 gp120 resolved by Zhou et al. (65). Because the V4 region containing residue N406 was not resolved, the approximate position of N406 is shown on a dashed line linking T399 and G410. *B*, The N448-linked glycan (G2) is flanked by two glycans linked to N262 and N295. These three N-glycans cluster together on the gp120 surface, forming "sugar towers" that rise over the C terminal portion of the PS05 epitope. The PS05 epitope is highlighted in orange, while asparagine and sugar residues are shown in blue and red, respectively. Only the proximal sugar units present in the crystal structure are shown.

Discussion

This study demonstrates that the presence of a conserved glycan (linked to N448) in the C4 region of HIV-1 gp120 is critical for efficient processing and generation of the neighboring C4 epitopes, thereby determining the recognition of these epitopes by MHC-II-restricted CD4 T cells. Hence, unlike the obstructing effects of N-linked glycans that have been previously reported on CD4 T cell recognition of various glycoprotein Ags (25–28), our data provide evidence that the large carbohydrate moieties on glycoproteins can also serve as a key structural determinant that dictates whether a particular CD4 T cell epitope on a glycoprotein Ag is processed, presented, and therefore recognized by the T cells. Specifically, our data clearly show that the removal of the essential N448-linked glycan from the C4 region of gp120 did not enhance the presentation of the nearby CD4 T cell epitopes; instead, the loss of this glycan abrogated or diminished CD4 T cell recognition of these epitopes. The absence of this particular glycan was also associated with increased resistance of the C4 region to proteolysis. These data provide an explanation for a previous study by Sjölander et al. (45) that showed the loss of T cell responses to the C4 region of gp120 near N448 in mice immunized with gp120 lacking the same three glycans studied here. We consider the possibility that these observed changes are simply due to the N-to-Q mutation introduced to remove the glycan, but this seems unlikely since this amino acid substitution has no direct effect on T cell recognition of the C4 epitope (Fig. 3*B*). There is also no evidence that the substitution creates an unfavorable motif that compromise the proteolytic activity of trypsin or endolysosomal enzymes examined in this study (60–64).

Of note, the N448 glycan removal affected specifically the nearby C4 epitopes, and its effect was most dramatic on the C4 epitope closest to N448 (i.e., the PS05 epitope) (Fig. 1). The effect

on a C4 epitope located slightly upstream (T1 epitope) was less profound, while the distant C1 or C2 sites were not at all affected, indicating the highly localized impact of this particular glycan. In contrast, the removal of two other glycans (linked to N406 and N463) flanking the C4 epitopes did not cause any significant alterations in CD4 T cell recognition of these epitopes. The reason for this difference is that even though the N406- and N463-linked glycans appear in the linear gp120 sequence to flank the C4 epitopes, they are not in the immediate vicinity of the C4 region in the three-dimensional structure of gp120 (Fig. 6*A*). Of the three glycans studied here, only the N448-linked glycan is located directly over the C4 region, while N406- and N463-linked glycans protrude from different faces of the gp120 surface. These glycans are shown in Fig. 6*A* in the context of the CD4-bound gp120 structure that has been determined at a relatively high resolution (65), and they are similarly positioned on the unliganded gp120 protein (58).

Although the N448 glycan is highly conserved among HIV-1 of subtype B, ~6% of these viruses lack this particular glycosylation site. One intriguing question that remains to be addressed is whether the induction of the CD4 T cell response targeting the C4 epitopes leads to selection of viruses that lack the N448-linked glycan. In most HIV-1-seropositive subjects, either chronically or acutely infected, CD4 T cell responses to C4, and other gp120 epitopes are usually weak or undetectable (46, 66–69) and thus are not likely to exert sufficient selective pressure for the emergence of escape mutants. Nevertheless, robust CD4 T cells specific for the C4 epitopes were stimulated in an HIV-seronegative recipient of the recombinant gp120 vaccine (70), and upon exposure with the virus challenge, these cells may contribute to the generation of escape mutants. The data presented here show that one effective strategy that the virus can exploit to escape immune surveillance by CD4 T cells is by disposing of a glycan, such as the N448-linked glycan, which does not substantially affect virus–receptor interactions and may even enhance virus infectivity. Nevertheless, the viruses lacking this glycan may also become more susceptible to Ab-mediated neutralization and/or CD8 T cell recognition (35, 57).

Why does the removal of N448-linked glycan lead to the loss of CD4 T cell recognition of the nearby C4 region? Previous studies have shown that some T cells are specific for glycosylated peptides and fail to recognize the deglycosylated version of the same peptides. The capacity of each of the gp120-specific CD4 T cell lines studied here, including the C4-specific PS05 and T1 lines, to respond to nonglycosylated synthetic peptides, which were utilized in the first place to map the epitopes, argues against this possibility. We also consider the possibility that the removal of N448-linked glycan may introduce or expose proteolytic sites that destroy the C4 epitopes during proteolysis. However, in vitro digestion of the ΔG2 protein lacking the N448-glycan by APC-derived endolysosomal enzymes efficiently generated the C4 epitope for MHC-II presentation to the CD4 T cell line PS05 (Fig. 4*C*). These results indicate that the PS05 epitope is not destroyed following digestion of the ΔG2 mutant by endolysosomal proteases, although the enzymatic processes that take place during gp120 Ag processing inside the APCs are not completely understood and may be distinct from the endolysosomal enzyme digestion performed in vitro. Considering that N-linked glycosylation is known to play an important role in determining protein folding and conformation, we propose an alternative hypothesis that the loss of the N448-linked glycan G2 induces a structural change in the proximal C4 region, which hinders the proteolytic cleavage of this particular region. This idea is supported by the data from trypsin digestion and MALDI-TOF MS analysis showing that trypsin digestion of the ΔG2 mutant generated a lower amount of peptide from the C4 region as compared with digestion of the WT or ΔG1 mutant, although this C4 region was equally sensitive to trypsin cleavage when the ΔG2 mutant was denatured before digestion (Fig. 5). A larger peptide fragment (m/z of 2580) containing peptide C4 that was not cleaved at its C terminus was also detected in the ΔG2 digestion, but not in the digestion of the WT or ΔG1 mutant (data not shown). In contrast, the ΔG2 mutation has no effect on proteolytic cleavage of the unrelated C2 region. These results are consistent with the differential effects of the ΔG2 mutation on CD4 T cell recognition of the C4 epitopes vs the other gp120 epitopes.

The nature of structural changes induced by the loss of N448-linked glycan G2 has yet to be defined. The ELISA data demonstrate that the ΔG2 mutant had a small, but consistent, decreased CD4-binding activity as compared with the WT or other mutants (Fig. 2*C*). However, the envelope bearing this mutation remained functional and could efficiently mediate virus entry. This mutant also showed slight alterations in reactivity with mAbs specific for the C1 or C1/C5 regions (EH21 and C11) and the V3 region of gp120 (447), although its reactivities with mAbs to other gp120 regions were not altered. Hence, the loss of the G2 glycan does not cause global conformational changes on the gp120 protein, but it may simply induce localized structural changes in the C4 region. The loss of N-linked glycans has typically been associated with reduced rigidity of the nearby amino acids, thereby lowering entropy for protein–protein interactions and increasing sensitivity to enzyme activities (29). However, in the case of the ΔG2 mutant, the removal of a glycan actually brings about the opposite results. Glycosylation is also known to influence protein folding, and the loss of the G2 glycan may induce alterations in the β-strand-turn-β-strand structure found in the C4 region. Currently, there is no evidence for such alterations, and further studies are needed to explore this possibility. Another possibility is that the removal of the G2 glycan alters the structural arrangement of the neighboring glycans. Previous studies demonstrate that the reactivity of anti-gp120 mAb 2G12, which recognizes a cluster of oligomannose sugars on the gp120 surface, was affected by the removal of glycans that are not directly bound by mAb but are in close proximity to the epitope (71–75), indicating the contribution of glycan–glycan interactions in maintaining the conformation of glycans on the gp120 surface. On the three-dimensional structure of gp120, the N448-linked G2 glycan is flanked on each side by N295-linked and N262-linked glycans. These three glycans protrude to form a cluster of sugar towers that rise over the C4 region recognized by the CD4 T cells (Fig. 6*B*). The loss of the G2 glycan in the middle of this cluster may cause the two flanking glycans to collapse or bend over the C4 region, blocking access of proteolytic enzymes to this particular region. Studies are in progress to test this hypothesis by preparing additional gp120 mutants lacking the N262- and N295-linked glycans, as well as the N448-glycan.

In summary, this study demonstrates that N-linked glycosylation is an important determinant for modulating the processing and MHC-II presentation of HIV envelope Ag gp120, and consequently CD4 T cell responses to this Ag. While the presence of glycans on gp120 may shield some epitopes, this also allows the presentation and recognition of other epitopes, thus shaping the repertoire of the immune responses against this viral Ag. A better understanding about the roles of glycans positioned in the different surfaces of gp120 will help us design immunogens to target the immune responses toward the crucial epitopes on this viral envelope.

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Disclosures

The authors have no financial conflicts of interest.

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