Peptide anchor residue glycosylation: Effect on class I major histocompatibility complex binding and cytotoxic T lymphocyte recognition

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[1] Molecular Immunology Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, GB
[2] Glycobiology Institute, Department of Biochemistry, University of Oxford, Oxford, GB

Peptide anchor residue glycosylation: effect on class I major histocompatibility complex binding and cytotoxic T lymphocyte recognition

This study extends our previous observation that glycopeptides bind to class I major histocompatibility complex (MHC) molecules and elicit carbohydrate-specific CTL responses. The Sendai virus nucleoprotein wild-type (WT) peptide (FAPGNYPAL) binds H-2D^b using the P5-Asn as an anchor. The peptide K2 carrying a P5 serine substitution did not bind D^b. Surprisingly, glycosylation of the serine (K2-O-GlcNAc) with N-acetylglucosamine (GlcNAc), a novel cytosolic O-linked glycosylation, partially restored peptide binding to D^b. We argue that the N-acetyl group of GlcNAc may fulfil the hydrogen bonding requirements of the D^b pocket which normally accomodates P5-Asn. Glycosylation of the P5-Asn residue itself abrogated binding similar to K2, probably for steric reasons. The peptide K2-O-GlcNAc readily elicited D^b-restricted cytotoxic T lymphocytes (CTL), which did not cross-react with K2 or WT. However, all D^b-restricted CTL raised against K2-O-GlcNAc cross-reacted strongly with another glycopeptide, K3-O-GlcNAc, where the GlcNAc substitution is on a neighboring P4-Ser. Furthermore, D^b-restricted CTL clones raised against K2-O-GlcNAc or K3-O-GlcNAc displayed a striking TCR conservation. Our interpretation is that the carbohydrate of K2-O-GlcNAc not only mediates binding to D^b, but also interacts with the TCR in such a way as to mimic K3-O-GlcNAc. This unusual example of molecular mimicry extends the known effects of peptide glycosylation from what we and others have previously reported: glycosylation may create a T cell neo-epitope, or, conversely, abrogate recognition. Alternatively, glycosylation may block peptide binding to MHC class I and finally, as reported here, restore binding, presumably through direct interaction of the carbohydrate with the MHC molecule.

1 Introduction

Major histocompatibility complex (MHC) class I molecules present cytosolically derived peptide antigens for recognition by cytotoxic T lymphocytes (CTL). The repertoire of peptide antigens presented by MHC molecules [1, 2] has been analyzed indirectly by mapping the fine specificity of natural T lymphocyte responses against protein antigens using overlapping peptides. Furthermore, peptides presented by MHC have been analyzed directly by immunoaffinity purification of peptide-MHC complexes followed by separation and pooled or individual sequencing of the MHC-derived peptides [3]. The peptide selectivity of a given MHC molecule is governed by the structure of the MHC peptide-binding cleft. This selectivity has been forcefully illustrated by the X-ray crystallography of MHC molecules complexed with individual peptides, demonstrating how pockets in the MHC peptide binding cleft accommodate amino acid side chains at the so-called anchor positions of the peptide [4, 5]. Thus, it is firmly established that peptides are the natural ligands presented by MHC molecules, and that the preferred peptide length for stable binding to MHC class I molecules is between 8 and 11 amino acids [2, 6] whereas class II MHC presents slightly longer peptides [7].

To investigate whether class I MHC might also be able to present antigenic peptides carrying post-translational modifications, we recently designed a number of synthetic glycopeptide analogs of the immunodominant H-2K^b-restricted CTL epitope FAPGNYPAL from Sendai virus nucleoprotein$_{324-332}$ [8]. The wild-type peptide (WT) binds to H-2K^b using P6 (Tyr) and P9 (Leu) as anchor residues, and to H-2D^b using the residues Asn and Leu at P5 and P9 as anchors [9]. Based on the published crystal structure of the complex between WT and K^b [5], we designed glycopeptide analogs of the WT peptide containing glycosyl amino acid substitution at positions predicted to be neutral with respect to K^b binding. Our study focuses on a naturally occurring cytosolic O-linked monosaccharide substitution of serine or threonine residues with N-acetylglucosamine (GlcNAc) [10, 11]. In a previous study, we substituted position 4 of the WT peptide with serine-O-GlcNAc or non-glycosylated serine and have reported the efficient binding of the resulting glycopeptide K3-O-GlcNAc [FAPS(-O-GlcNAc)NYPAL] to both K^b and D^b. Also, we found that CTL raised against K3-O-GlcNAc were glycopeptide specific and did not recognize a glycopeptide carrying alpha-linked N-acetylgalactosamine instead of beta-linked GlcNAc at the same position [8].

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Present address: G. Aresquell, Unitat for Glycoconjugate Chemistry, C.I.D. – C.S.I.C., Jordi Girona 18-26, E-08034 Barcelona, Spain; A.C. Lellouch, CNRS-INSERM, Centre d'Immunologie, Parc Scientifique de Luminy, Marseille, France

Correspondence: John S. Haurum, Molecular Immunology Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, GB (Fax: +44 18 65 22 25 02; Email: john.haurum@imm.ox.ac.uk)

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Here, we have synthesized the O-glycosylated peptide K2-O-GlcNAc [FAPGS(-O-GlcNAc)YPAL], where the substitution is at peptide position 5, the main anchor residue for peptide binding to D^b. As expected, the non-glycosylated peptide K2 (FAPGSYPAL) did not bind well to D^b. We report the surprising finding that glycosylation of the Ser side chain with O-GlcNAc significantly restored peptide binding to D^b, and furthermore, K2-O-GlcNAc could readily elicit D^b-restricted CTL.

2 Materials and methods

2.1 Synthetic peptides

The peptides WT (FAPGNYPAL), K2 (FAPGSYPAL), K2-O-GlcNAc [FAPGS(-O-GlcNAc)YPAL], K1 (FAPGNYSAL), K1-N-GlcNAc [FAPGN(-*N*-GlcNAc)YSAL], and K3-O-GlcNAc [FAPS(-O-GlcNAc)NYPAL] used in this study were synthesized manually on Wang resin using conventional Fmoc chemistry as described [8, 13]. In the case of glycopeptide synthesis, protected glycosyl amino acid building blocks were prepared for use in the solid phase synthesis [13]. These peptides were purified by HPLC to greater than 99% purity and characterized by laser desorption mass spectrometry. The glycopeptides were further characterized by proton nuclear magnetic resonance, amino acid analysis, and hexosamine analysis. The peptides FAPGQYPAL, FAPGHYPAL, FAPGYYPAL, FAPGAYPAL and FAPGDYPAL were synthesized on an SMPS-350 automatic peptide synthesiser (Zinsser Analytical, Maidenhead, GB) and purity was verified by HPLC to be greater than 90%. The peptides were dissolved in PBS, sterilized by filtration and stored at −70 °C. Peptide concentrations were determined by measuring the absorbance of the peptide dissolved (1:50) in 6 M guanidinium hydrochloride (Aldrich, GB). The peptide concentrations could then be calculated using the molar extinction coefficient for tyrosine ($\varepsilon = 1475$) using the following formula $C_{Tyr} = A^{275}/\varepsilon$ [14].

2.2 Assembly assay for peptide binding to MHC

The assembly assay for peptide binding to H-2D^b was carried out essentially as described [8, 15]. The peptide transporter (TAP-2)-deficient cell line RMA-S (5×10^6 cells per sample) was metabolically labeled with [^{35}S]methionine (100 μCi per 10^7 cells; Amersham, GB) for 60 min in RPMI 1640 (Gibco) containing 10% FCS, solubilized in PBS containing 5 mM EDTA, 0.5% NP40 (Sigma, Poole, GB), 0.5% Mega-9 (Sigma), 0.02% sodium azide, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.5 mM PMSF, and 20 μg/ml iodoacetamide in the presence of serial dilutions of peptide and pre-cleared with Pansorbin (Calbiochem Novabiochem, GB) overnight. The peptide-dependent stabilization of D^b could then be quantified by immunoprecipitation with the conformation-sensitive antibody B22.249 [16] followed by SDS-PAGE, autoradiography and densitometry of the MHC heavy chain band.

2.3 Generation of CTL lines and clones

Peptide- and glycopeptide-specific CTL lines and clones were generated by immunization of C57BL/6 mice (Harlan Olac, Bicester, GB) with 100 μg peptide dissolved in IFA as reported [8, 17, 18]. Splenocyte suspensions were prepared on day 7, and 8×10^6 spleen cells per well in 24-well plates (Nunc, Kamstrup, Denmark) were incubated in the presence of peptide (30 μM) in Iscove's modified Dulbecco's medium (IMDM from Gibco, Glasgow, Scotland) with 10% FCS (Seralab, Crawley Down, GB), penicillin, streptomycin, glutamine, and 2-ME). RMA-S cells, which had been cultured overnight at 25 °C to induce high cell-surface expression of empty MHC class I molecules [9] were incubated with 30 μM peptide and irradiated (7000 rad), before addition to the spleen cells (2×10^6 per well). Thereafter, the cells were restimulated weekly with peptide-pulsed, irradiated, syngeneic spleen cells and RMA-S cells in complete IMDM (IMDM as above with 5–10% concanavalin A-stimulated rat splenocyte supernatant). CTL clones were established from peptide specific CTL lines by limiting dilution in 96-well plates using peptide pulsed, irradiated, temperature induced RMA-S and syngeneic spleen cells as feeder cells.

2.4 CTL assays

The peptide transporter-deficient cell line T2 transfected with H-2D^b (T2-D^b) or H-2K^b (T2-K^b) were used as target cells in conventional chromium-release assays for measuring the peptide-specific cytotoxicity mediated by the CTL clones. In some CTL assays, target cells were prepulsed with an excess of antigen (50 μM) and labeled with ^{51}Cr (Amersham) before incubation with CTL clones for 4 h at 37 °C in round-bottom 96-well plates. In other assays, serial dilutions of peptide (final concentration 50, 5, 0.5, or 0.05×10^{-9} M) were added directly to the CTL clones and ^{51}Cr-labeled target cells.

2.5 Cloning and sequencing of TCR from CTL clones

TCR transcripts were cloned using anchored polymerase chain reaction [19, 20]. Briefly, RNA was isolated using RNAzol® (Cinnex, TX) and cDNA was synthesized from 5 μg total RNA using AMV-reverse transcriptase. The cDNA was precipitated three times using 4 M ammonium acetate and a poly(dG) tail was added to the cDNA using terminal deoxytransferase (TdT). The cDNA was amplified using a primer complementary to the homopolymer tail [20] and one from the murine TCRA (5′-GATCAGATCTTTTAACTGGTACAC-3′) or TCRB (5′-GTCAGATCTGGCTCAAACAAGGAGACCT-3′) constant region. The PCR products were cloned into a modified M13mp18 vector and sequenced using T7 DNA polymerase. From each clone, 12 TCRA and 12 TCRB sequences were characterized.

3 Results

3.1 Anchor residue glycosylation restores peptide binding to H-2D^b

The Sendai virus nucleoprotein$_{324-332}$ WT peptide FAPGNYPAL binds to both D^b and K^b with high affinity [9]. Peptide binding to D^b is known to use the Asn at P5 and a hydrophobic residue at P9 as anchor residues, whereas

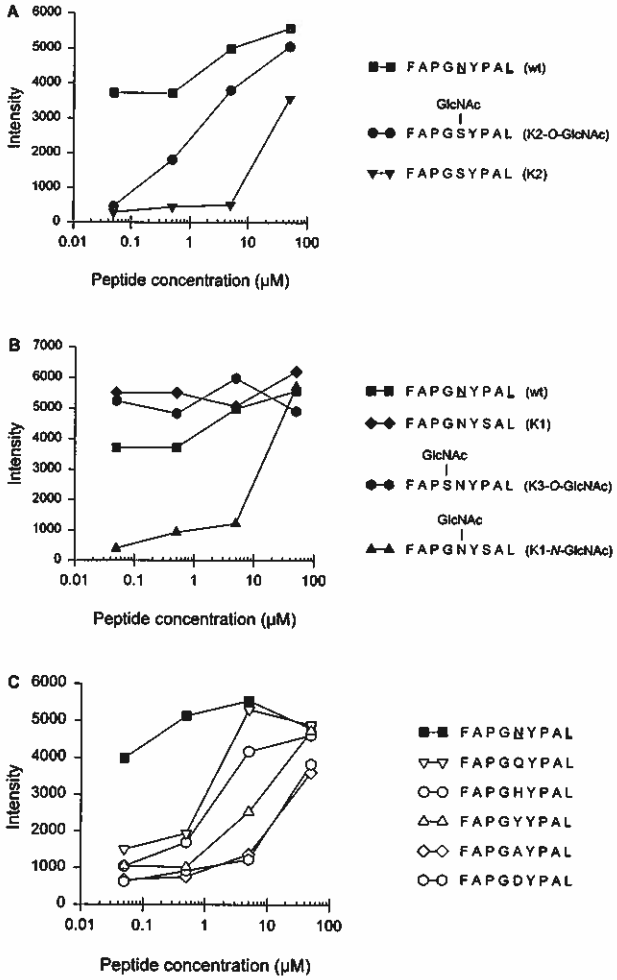

Figure 1. Peptide and glycopeptide binding to H-2D^b. The peptide transporter-deficient cell line RMA-S was metabolically labeled with [^{35}S]methionine, cells were lysed in the presence of 50 nM, 500 nM, 5 μM, or 50 μM peptide as indicated in the figure, followed by immunoprecipitation with the conformation-sensitive antibody B22.249, specific for H-2D^b. Quantification of peptide binding was performed by SDS-PAGE, autoradiography, and densitometric analysis of the D^b heavy-chain band. Anchor positions for WT peptide binding to D^b are underlined.

peptide binding to K^b uses Tyr at P6 and a hydrophobic residue at P9 [1, 2].

During the course of studying the class I MHC binding and immunogenicity of synthetic glycopeptide variants of WT, we analyzed the binding of the peptides K2 FAPGSYPAL, K2-O-GlcNAc [FAPGS(-O-GlcNAc)YPAL], K1 (FAPGNYSAL), and K1-N-GlcNAc [FAPGN(-N-GlcNAc)YSAL] to D^b. As shown in Fig. 1A, the WT peptide binds with high affinity to D^b. The binding of WT peptide to D^b titers out to a peptide concentration between 0.1 and 1 nM (not shown). The asparagine-to-serine substitution at P5 in K2 replaces the WT D^b anchor residue and, as expected, K2 did not bind significantly to D^b. Only at high peptide concentrations (in excess of 5 μM) did K2 stabilize the assembly of D^b (Fig. 1A). Substituting the non-anchor proline at P7 with serine (K1) did not affect binding to D^b, but subsequent N-GlcNAc glycosylation of anchor residue P5-Asn (K1-N-GlcNAc) resulted in dramatic reduction in binding (Fig. 1B), equivalent to the Asn → Ser substitution of K2 described above. Remarkably however, glycosylation of the P5-Ser side chain of K2 with O-linked GlcNAc (K2-O-GlcNAc), resulted in partial restoration of D^b binding activity (Fig. 1A). Glycosylation of the non-anchor P4 (K3-O-GlcNAc) did not affect binding (Fig. 1B), as we have shown previously [8].

Thus, glycosylation of WT peptide analogs resulted in three different outcomes with respect to the ability to bind to D^b. First, glycosylation of an amino acid side chain predicted to point towards the T cell receptor (K3-O-GlcNAc), had no effect on peptide binding to D^b, as we have previously reported [8]. Second, glycosylation of the asparagine anchor residue itself resulted in abrogation of binding (*cf.* K1-N-GlcNAc and K1). Third, glycosylation of the P5 side chain of a non-binding peptide resulted in restoration of binding (*cf.* K2-O-GlcNAc and K2).

The peptides WT and K3-O-GlcNAc [8], as well as K1, K2, K1-N-GlcNAc and K2-O-GlcNAc, all bind with similar high affinity to H-2K^b, in accordance with the presence of the K^b anchor residue Tyr at position 6, indicating that the indirect effect of glycosylation on peptide binding to class I MHC is minimal (data not shown).

3.2 Structural requirements for peptide binding to H-2D^b: implications for K2-O-GlcNAc binding

The crystallographic structure of H-2D^b complexed with the influenza virus nucleoprotein$_{366-374}$ peptide ASNENMETM revealed the physical and chemical properties of the D^b binding cleft which give rise to a preference for Asn at P5 [12]. The hydrophilic P5-Asn side chain is buried in the peptide binding cleft of H-2D^b, where it forms three hydrogen bonds with MHC residues lining the specificity pocket. The P5 peptide backbone amide hydrogen bonds to the side-chain oxygen of Gln-70 of the D^b α1 helix, whereas the carbonyl oxygen of the P5-Asn forms a hydrogen bond with the nitrogen atom of Gln-97 and the P5 side chain amino group hydrogen bonds with the hydroxyl group of Tyr-156. Our finding that the GlcNAc-glycosylation in K2-O-GlcNAc was able to restore some of the D^b binding activity lost by the P5-Ser substitution indicated that a part of the GlcNAc moiety may be able to restore appropriate hydrogen bonds and van der Waals interactions with the MHC residues lining the P5 specificity pocket of the D^b molecule.

Glycosylation of the longer asparagine side chain anchor in K1-N-GlcNAc resulted in loss of binding, most likely due to steric hindrance, whereas a serine residue alone at this position would conceivably be too small as well as being unable to make appropriate hydrogen bonds with pocket residues, leading to poor stability of the K2-D^b complex. The polar nature of both the GlcNAc moiety and the asparagine anchor residue prompted us to investigate the binding to D^b of a further set of WT peptide analogs. Fig. 1C illustrates that P5 substitution with alanine, aspartic acid, or tyrosine resulted in a marked loss of D^b binding activity, similar to the result of the serine substitution, whereas glutamine- and histidine-substituted peptides displayed intermediate binding activity comparable to the binding of K2-O-GlcNAc. Notably, the peptides which did not bind to H-2D^b carried negatively charged

(P5-Asp) or hydrophobic (P5-Ala and P5-Tyr) substitutions, whereas the peptides with substitutions with side chains able to act both as hydrogen bond donors and acceptors (P5-Gln and P5-His) bound with intermediate affinity. The P5-Gln and the P5-His side chain, as well as the N-acetyl group of the serine-linked GlcNAc, might therefore allow the formation of hydrogen bonds mimicking those between the side-chain nitrogen and carbonyl atoms of the canonical H-2D^b anchor residue asparagine and the D^b Tyr-156 and Gln-97, respectively [12].

3.3 The glycopeptide K2-O-GlcNAc elicits both H-2D^b- and H-2K^b-restricted, glycopeptide-specific CTL

The ability of serine glycosylation partially to restore the D^b binding activity of K2 prompted us to generate CTL against K2-O-GlcNAc to analyze the effect of anchor residue glycosylation on the nature of the class I MHC-restricted T cell response. Previously, we demonstrated that K3-O-GlcNAc elicited both D^b- and K^b-restricted, glycopeptide-specific CTL which did not cross-react with the non-glycosylated control peptide. If, however, the new glycopeptide K2-O-GlcNAc binds to D^b using the carbohydrate moiety as anchor residue, such that the GlcNAc moiety is partially buried in the peptide binding cleft, then D^b-restricted CTL raised against K2-O-GlcNAc might not discriminate between K2-O-GlcNAc, K2, and WT.

To test this hypothesis, we immunized C57BL/6 mice with K2-O-GlcNAc and established CTL lines by restimulation of spleen cells *in vitro* with peptide-pulsed antigen-presenting cells as described [8]. Mice immunized with K2-O-GlcNAc readily mounted both a D^b- and K^b-restricted CTL response against the glycopeptide, despite the fact that it binds several orders of magnitude better to K^b than to D^b (data not shown). Subsequent cloning of the CTL lines by limiting dilution generated a number of either D^b- or K^b-restricted CTL clones. As seen in Fig. 2A, the representative H-2D^b-restricted CTL clone K2G.6.9 lysed T2-D^b target cells in the presence of 10^{-9} M K2-O-GlcNAc. On the other hand, T2-D^b target cells were not killed in the presence of either WT or K2 peptide, demonstrating that K2-O-GlcNAc-specific CTL clones are glycopeptide specific, similar to the previously reported K3-O-GlcNAc-specific CTL clones raised by us [8]. Surprisingly, the D^b-restricted CTL clones raised against K2-O-GlcNAc exhibited a very strong reactivity towards K3-O-GlcNAc, which carries a serine-GlcNAc substitution at position 4 of the WT peptide instead of at position 5 in K2-O-GlcNAc. Thus, all six D^b-restricted clones raised against K2-O-GlcNAc lysed T2-D^b target cells in the presence of less than 50 pM K3-O-GlcNAc, whereas the concentration needed for equivalent K2-O-GlcNAc target sensitization was in the order of 5 nM.

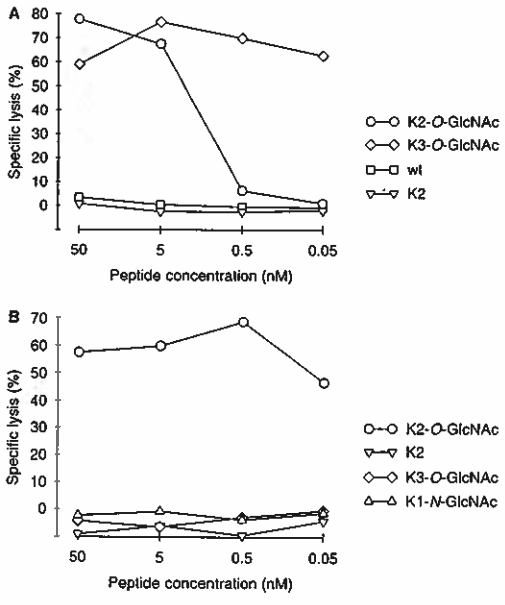

Figure 2. Peptide specificity of CTL clones raised against K2-O-GlcNAc. The specificity of the CTL clones K2G.6.9 (A) and K2G.6.19 were tested against ^{51}Cr-labeled target cells T2-D^b (A) or T2-K^b (B) in the presence of the peptide concentrations indicated in the figure.

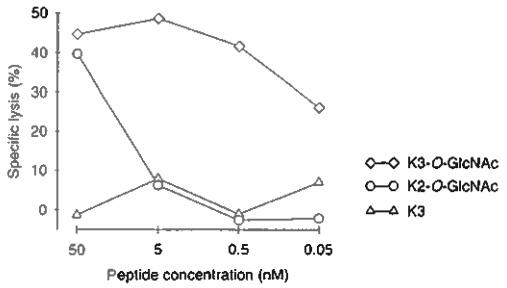

Figure 3. Peptide specificity of the CTL clone K3G.6.15 tested against ^{51}Cr-labeled T2-D^b in the presence of the indicated peptide concentrations.

This unexpected behavior of the CTL clones raised against K2-O-GlcNAc was specific to the D^b-restricted response, since the resulting K^b-restricted anti-K2-O-GlcNAc CTL clones, as exemplified by K2G.6.19, did not display cross-reactivity towards K3-O-GlcNAc, K1-N-GlcNAc, or the non-glycosylated K2 (Fig. 2B).

We also examined whether D^b-restricted CTL raised against K3-O-GlcNAc displayed a reciprocal cross-reactivity towards K2-O-GlcNAc. As seen in Fig. 3, the D^b-restricted CTL clone K3G.6.15 raised against K3-O-GlcNAc did indeed crossreact with T2-D^b in the presence of 50 nM K2-O-GlcNAc. The CTL clone K3G.5.8 did not lyse T2-D^b in the presence of 50 nM K2-O-GlcNAc (not shown), although it is likely that higher concentrations of K2-O-GlcNAc might also sensitize T2-D^b for lysis by this clone. In support of this contention was the observation that all D^b-restricted CTL clones so far tested that have been raised against K3-O-GlcNAc lysed T2-D^b prepulsed with 50 μM K2-O-GlcNAc (data not shown).

The avidity of MHC-restricted T cell activation is considered to be a function of both the number of MHC complexes on each cell presenting a particular peptide ligand as well as the affinity of the TCR for the individual MHC-peptide complex. The paradoxical strong recognition of K3-O-GlcNAc by D^b-restricted CTL clones raised against K2-O-GlcNAc as exemplified by the clone K2G.6.9 (Fig. 2A) might accordingly be explained by a combination of a high affinity towards K3-O-GlcNAc of the TCR selected by K2-O-GlcNAc/D^b complexes and the fact that

Table 1. Nucleotide and predicted amino acid sequence of TCRA and TCRB sequences from the H-2D^b-restricted CTL clones K2G.6.9 (raised against K2-O-GlcNAc) and K3G.5.8 and K3G.6.15 (raised against K3-O-GlcNAc), as well as the TCRB of the H-2K^b-restricted CTL clones K2G.6.1 and K3G.5.[a)]

Clone	Antigen	Restriction Element	V-Segment	Junctional region	J-Segment	Reference
K2G.6.9	K2-O-GlcNAc	H-2D^b	α5.3.18	A M R A Y G N E K I T gctatgagggcctatggaaatgagaaaataact	α40	[30]
K3G.5.8	K3-O-GlcNAc	H-2D^b	α5.3.18	A M R A Y G N E K I T gctatgagagcctatggaaatgagaaaataact	α40	[30]
K3G.6.15	K3-O-GlcNAc	H-2D^b	α4.4	A L G D Y G I T G S G G K L T gctctgggtgattacgggattactggcagtggtggaaaactcact	αTA46	[31,32]
K2G.6.9	K2-O-GlcNAc	H-2D^b	β11	L G A N E R L F ctaggggccaacgaaagattattt	β1.4	[33]
K3G.5.8	K3-O-GlcNAc	H-2D^b	β11	F P G Q S N E R L F ttcccgggacaatccaacgaaagattattt	β1.4	[33]
K3G.6.15	K3-O-GlcNAc	H-2D^b	β11	L E L S Q N T L Y ctagaacttagtcaaaacaccttgtac	β2.4	[33,34]
K2G.6.1	K2-O-GlcNAc	H-2K^b	β5.1	L I R D R S A E T L Y ctcatccgggacaggagtgcagaaacgctgtat	β2.3	[33,34]
K3G.5.1	K3-O-GlcNAc	H-2K^b	β8.2	G D R Q A Y E Q Y ggtgatcgacaggcctatgaacagtac	β2.6	[33,34]

a) The junctional region of TCRA was taken between the conserved cysteine of TCRAV and the conserved phenylalanine of TCRAJ. The junctional region of TCRB it shown between the two conserved serines of TCRBV and the conserved phenylalanine of TCRBJ. The single nucleotide difference between the TCRA sequences of K2G.6.9 and K3G.5.8 is highlighted.

K3-O-GlcNAc binds with a substantially higher affinity to D^b than K2-O-GlcNAc (compare Figs. 1A and 1B).

3.4 Conservation of TCR usage between D^b-restricted CTL raised against K2-O-GlcNAc and K3-O-GlcNAc

The strong cross-reactivity towards K3-O-GlcNAc displayed by CTL raised against K2-O-GlcNAc strongly suggests that the complex formed between K3-O-GlcNAc and D^b shares important structural features with the complex formed between D^b and K2-O-GlcNAc. This prompted us to compare the TCR usage of D^b-restricted CTL clones raised against K2-O-GlcNAc and K3-O-GlcNAc to establish whether the molecular mimicry displayed by the two different D^b-peptide complexes had resulted in the selection for similar TCR.

TCR sequences were cloned from three H-2D^b-restricted CTL clones, K2G.6.9 (raised against K2-O-GlcNAc), and K3G.5.8 and K3G.6.15 (raised against K3-O-GlcNAc). All expressed a common TCRBV segment, Vβ11 (Table 1), and TCRBV usage correlated with the H-2 restriction element since other CTL clones which recognize K2-O-GlcNAc (*i.e.* clone K2G.6.1) or K3-O-GlcNAc (*i.e.* K3G.5.1) in the context of H-2K^b, expressed different TCRBV segments (Table 1). Conservation of TCRBV segment usage is well documented in human CTL responses to single viral peptide-MHC complexes [20, 21].

Although clones K2G.6.9 and K3G.5.8 were raised against K2-O-GlcNAc and K3-O-GlcNAc, respectively, and differ in their patterns of fine specificity, both express TCRA chains which are identical at the amino acid level, although they differ by one nucleotide in the junctional region. This reveals strong selection for the TCRA sequence and implies that differences in fine specificity are mediated by the TCR β chain. Clone K3G.6.15 possesses a different TCR α chain with no clear homology to the TCRA sequence from K2G.6.9 or K3G.5.8.

The identity of the TCRA sequence of clones K2G.6.9 and K3G.5.8 together with the conserved TCRBV usage lends further support to the interpretation that the overall structure of the two peptide-MHC complexes is likely to be similar, and suggests that the TCR α chain may contact the MHC-peptide complex at a site distal to the glycosylated peptide residue, leaving the β chain to interact with the substituted serine.

4 Discussion

Since the first demonstration that synthetic peptides can prime carbohydrate-specific, MHC class II- [22, 23] and class I-restricted [8] T cell responses, several reports have appeared which demonstrate T cell reactivity to natural glycopeptide epitopes. These include a dominant autoantigen [24], an allergen [25], and a tumor-specific antigen [26]. The ability of T cells to recognize post-translational modifications is significant, as it would allow the creation of neo-epitopes without alteration at the genetic level, since the pattern of post-translational modifications displayed by proteins reflects the intracellular milieu in which they are expressed. Thus, alterations of the intracellular metabolism are affected by factors such as malignant transformation or environmental stress.

There are several potential outcomes of post-translational modification of a polypeptide with respect to the ensuing immune response. First, a neoepitope could be created by providing a novel structure for the T cell receptor to engage. Alternatively, in the event of complete modification of an epitope, an existing response to the unmodified peptide could be blocked at the level of T cell recognition. Third, an existing response could be blocked at the level of MHC binding by the modification of an anchor residue. Finally, a neo-epitope could be generated by providing a novel MHC anchor structure.

This study, along with previous reports [8, 22, 23], provides experimental examples of all four of these outcomes. Thus, T cell responses can be elicited to synthetic glycopeptides which do not cross-react with the non-glycosylated sequence; and T cells which recognize the non-glycosylated sequence are blocked by glycosylation of the peptide. In addition, we have shown that glycosylation of the side chain of the D^b anchor residue asparagine decreases the binding of K1 (FAPGNYSAL) by around four orders of magnitude. Most surprising, however, was the finding that glycosylation of the non-binding peptide K2 (FAPGSYPAL) at P5-Ser resulted in partial restoration of its binding to D^b.

The most likely explanation for this result is that the GlcNAc substitution of P5-Ser in K2-O-GlcNAc is able to mimic the specificity-determining contacts made by an asparagine side-chain in the pocket of the D^b peptide-binding cleft. In support of this is the finding that substitution with other amino acids at P5 can have a similar effect on binding as long as they contain a functional group which can act as both a hydrogen bond donor and acceptor. Therefore, this result provides the first example of a non-peptide structure interacting directly with a classical MHC molecule.

Despite the notion that the O-GlcNAc of K2-O-GlcNAc appears to interact directly with D^b, we found that it was still possible to generate CTL which recognized K2-O-GlcNAc, but not K2. One possible explanation of this finding is that glycosylation has an indirect effect on CTL recognition, similar to that described for a glycopeptide-class II MHC interaction [23], where it was suggested that the N-terminal glycosylation altered the conformation of bound peptide such that T cells recognizing it did not cross-react with non-glycosylated peptide. This gave rise to glycopeptide-specific T cells even though it was thought unlikely that the T cell receptor actually made contact with the carbohydrate structure. However, we believe this to be an unlikely explanation in the present case, since all our D^b-restricted, K2-O-GlcNAc-specific CTL cross-reacted strongly with a second glycopeptide, K3-O-GlcNAc, where the GlcNAc substitution almost certainly points out of the peptide-binding groove towards the T cell receptor. We believe that this cross-reactivity, along with the reciprocal cross-reactivity of D^b-restricted, K3-O-GlcNAc-specific clones, indicates that the complex formed between K2-O-GlcNAc and D^b shares many structural features with the complex formed between K3-O-GlcNAc and D^b. Indeed, if K2-O-GlcNAc is anchored at P5 via the N-acetyl group of O-GlcNAc and the ring structure of GlcNAc is too bulky to fit into the pocket, then the ring structure of GlcNAc could be forced outwards, away from the groove, facilitating its access to the T cell receptor. A characteristic feature of the structure of H-$2D^b$ is the presence of a marked ridge just C terminal to the P5 specificity pocket across the D^b binding cleft caused by the residues Trp-73, Tyr-156, and Trp-147 [12]. This causes the backbone of bound peptide ligands to arch upwards between P5 and P9. This ridge might force the ring structure of GlcNAc to be orientated not only upwards but also backwards towards the N terminus of the peptide and the P4 side chain, thus mimicking the structure of the complex formed between K3-O-GlcNAc and D^b.

In support of this idea of molecular mimicry is our finding that cross-reactive CTL raised to K2-O-GlcNAc and K3-O-GlcNAc select T cell receptors, which are remarkably similar in structure. Two of the cross-reactive clones we analyzed (K2G.6.9 raised against K2-O-GlcNAc and K3G.5.8 raised against K3-O-GlcNAc) selected the same Vα, Jα, Vβ and Jβ gene segments, and differed only in the junctional regions which are thought to make contact with bound peptide in the peptide/MHC complex [27–29]. Thus, the only differences between these two clones were a conservative nucleotide difference in the α junctional region and five amino acid differences in the β junctional region. The difference in fine specificity between these two clones is therefore probably controlled by differences in the CDR3β. Indeed, it is tempting to speculate that the greater cross-reactivity of clone K2G.6.9 is due to a slightly larger and more promiscuous pocket in the antigen combining site of its T cell receptor, resulting from the shorter CDR3β loop (LGANERLF compared to FPGQSNERLF in clone K3G.5.8).

Other conserved features were observed in the junctional regions of cross-reactive clones. In the alpha chains, we observed a conserved Tyr-Gly motif, appearing in the general sequence A-M/L-x-x-Y-G-x-x-(x-x-x-x)-K-I/L-T, where the (x-x-x-x) seen in clone K3G.6.15 is glycine rich and therefore flexible. In the β chains, we saw a conserved Asn in the general sequence x-x-x-(x-x)-N-x-(x)-L-F/Y. Both of these conserved side chains would be appropriate for making hydrogen bonds with the hydroxyl groups of the GlcNAc ring structure.

In summary, we provide evidence that post-translational modifications can modulate CTL responses at the level of both MHC binding and TCR recognition. We have also presented data suggesting that a single monosaccharide-substituted side chain can interact directly with both MHC and TCR, and describe the possible interactions at the molecular level.

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