*Brief Definitive Report*

# Recognition of Carbohydrate by Major Histocompatibility Complex Class I-restricted, Glycopeptide-specific Cytotoxic T Lymphocytes

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## Summary

Cytotoxic T cells (CTL) recognize short peptide epitopes presented by class I glycoproteins encoded by the major histocompatibility complex (MHC). It is not yet known whether peptides containing posttranslationally modified amino acids can also be recognized by CTL. To address this issue, we have studied the immunogenicity and recognition of a glycopeptide carrying an O-linked N-acetylglucosamine (GlcNAc) monosaccharide-substituted serine residue. This posttranslational modification is catalyzed by a recently described cytosolic glycosyltransferase. We show that glycosylation does not affect peptide binding to MHC class I and that glycopeptides can elicit a strong CTL response that is glycopeptide specific. Furthermore, glycopeptide recognition by cytotoxic T cells is dependent on the chemical structure of the glycan as well as its position within the peptide.

T lymphocytes recognize peptide antigens as they are presented on the cell surface by polymorphic proteins encoded in the MHC class I or II. Class I MHC presents peptide fragments of intracellular synthesized protein, whereas class II MHC predominantly presents peptide fragments of extracellular proteins that have been degraded in the endocytic compartment (1). The allelic specificity of peptide binding to MHC is governed by pockets in the MHC binding groove that confer the preferred binding of certain amino acid residues within an allele-specific motif (2).

All natural T cell antigens identified to date consist of peptides with unmodified amino acid side chains. However, it is not known whether some of the posttranslational modifications that occur on proteins in vivo contribute to the recognition of peptide antigens by T cells. Thus, it is not known whether sulfated, phosphorylated, carboxylated, or glycosylated peptides can be selected for presentation by MHC with the posttranslational modification intact.

Studies on the recognition by CTLs of peptides haptenated with trinitrophenyl have demonstrated that T cells will specifically recognize chemically modified peptide antigens (3). It is therefore possible that peptides carrying natural posttranslational modifications, such as glycosylation, might similarly be recognized by T cells.

Several different types of protein glycosylation are known including the N-linked glycosylation of asparagine and O-linked glycosylation of serine and threonine occurring in the endoplasmic reticulum (ER) and Golgi apparatus. In addition, a novel O-linked glycosylation, occurring almost exclusively on nuclear and cytosolic proteins, has been described (4). This glycosylation is characterized by substitution of serines or threonines with single O-$\beta$-linked N-acetylglucosamine (GlcNAc) residues catalyzed by a cytosolic N-acetylglucosaminyl transferase (reviewed in reference 5).

Since peptide fragments of cytosolic and nuclear proteins are the preferred substrates for antigen presentation by class I MHC, it is possible that glycopeptides derived from O-GlcNAc substituted cytosolic proteins could enter the class I presentation pathway. When bound to MHC class I, the O-$\beta$-GlcNAc modification on peptides could then be presented in such a way as to be specifically recognized by T cell receptors.

Here we report for the first time the efficient binding to class I MHC (H-2K$^b$ and H-2D$^b$) of a synthetic glycopeptide carrying this naturally occurring O-linked GlcNAc and its carbohydrate-specific recognition by CTL.

## Materials and Methods

*Reagents and Antibodies.* Fluorenylmethoxycarbonyl (Fmoc) amino acids and resins for the synthesis of peptides were purchased from Zinsser (Maidenhead, UK) and Novabiochem AG (Nottingham, UK). IMDM was from GIBCO, Ltd. (Glasgow, Scotland) and FCS from Seralab (Crawley Down, UK).

The cell line RMA-S is a Rauscher virus–transformed murine T cell line that has a defective TAP 2 gene encoding the peptide

transporter associated with antigen presentation (TAP) (6). A second, human TAP-defective cell line T2 (7), transfected with H2-$K^b$ and H2-$D^b$, was maintained as described previously. C57BL/6 mice were obtained from Harlan Olac, Ltd. (Bicester, UK). Antibodies were purified from culture supernatant by affinity chromatography on protein-A Sepharose (Sigma Chemical Co., Poole, UK). The mAb Y3 recognizes a conformational epitope on the $\alpha_1$ and $\alpha_2$ domains of $K^b$ (8) and mAb B22.249 recognizes a conformational epitope on the $\alpha_1$ domain of $D^b$ (9).

*Peptide Synthesis.* The Sendai virus nucleoprotein$_{324-332}$ wild-type (wt) peptide ([FAPGNYPAL]; amino acids are referred to by the single letter code) and the analogue K3 [FAPSNYPAL] were synthesized manually on Wang resin using conventional Fmoc chemistry. The solid phase synthesis, purification, and characterization of the glycopeptides K3-O-GlcNAc [FAPS (O-$\beta$-GlcNAc) NYPAL], K3-O-N-acetyl-D-galactosamine (GalNAc) [FAPS (O-$\alpha$-GalNAc) NYPAL], and K1-N-GlcNAc [FAPGN (N-$\beta$-GlcNAc) YSAL] will be reported elsewhere (Arsequell, G., A. C. Lellouch, S. Y. Wong, J. S. Haurum, T. Elliott, and R. A. Dwek, manuscript in preparation). Briefly, three glycosyl amino acids were synthesized as building blocks for solid-phase glycopeptide synthesis. FmocSer ($\beta$-pGlcNAc) OH was prepared by glycosylation of FmocSerOH with peracetylated (p)GlcNAc using boron trifluoride etherate catalysis. Glycosylation of Fmoc Serine pentafluorophenyl ester (FmocSerOpfp) with $\alpha$-pGalN$_3$Br (obtained from D-Galactal [10]) using silver triflate as a catalyst afforded FmocSer ($\alpha$-pGalN$_3$) Opfp. A coupling reaction between Fmoc aspartic *t*-butyl ester (FmocAspO *t*Bu) and the glycosylamine of GlcNAc, followed by acetylation with acetic anhydride and cleavage of the *t*Bu ester with TFA yielded FmocAsn ($\beta$-pGlcNAc) OH. The building blocks were purified and characterized before incorporation into the solid phase glycopeptide synthesis by a standard Fmoc protocol using FmocLeu derivatized Wang resin with benzotriazole-1-yl-oxy-tri-pyrrolidino-phosphonium hexa fluoroacetic acid (PyBOP) as an activating agent (except with the Opfp derivative which needed no further activation). In the case of FmocSer ($\alpha$-pGalN$_3$) Opfp, thioacetic acid was used to convert the azido group to the N-acetyl group while the glycopeptide was still resin bound (11). The peptides were cleaved from the resin using TFA/$H_2O$, purified by reverse phase HPLC to greater than 99% purity, and deacetylated using a catalytic amount of sodium methoxide in absolute methanol, followed by repurification by HPLC. The peptides were characterized by proton nuclear magnetic resonance, amino acid analysis, hexosamine analysis (in the case of glycopeptides), and laser desorption mass spectrometry. Amino acid and hexose contents were as expected, as were the parent ions of each compound. Sterile filtered stock solutions of peptides (1–4 mg/ml in PBS) were stored at $-70$°C.

*Assembly Assays for Peptide Binding to MHC.* Assembly assays for binding of the synthetic peptides to $K^b$ and $D^b$ molecules metabolically labeled with [$^{35}$S]methionine were carried out essentially as described previously (12). This assay is based on the peptide-dependent stabilization of $K^b$ and $D^b$ after lysis of the peptide transporter (TAP 2)–deficient cell line RMA-S and the detection by immunoprecipitation with the conformation-specific antibodies Y3 and B22, respectively. Peptide binding was quantified by densitometry of the heavy chain band of autoradiographs after SDS-PAGE. Optical density was plotted against peptide concentration.

*Establishment of CTL.* C57BL/6 mice were immunized inguinally with 100 $\mu$g s.c. of peptides K3 or K3-O-GlcNAc in incomplete Freunds adjuvant. Antipeptide CTL were stimulated in vitro on day 7 as described by Stauss et al. (13). RMA-S cells were cultured overnight at 25°C to induce high surface expression of (empty) $K^b$ and $D^b$ molecules (14) before pulsing for 2–4 h with 30 $\mu$M of the relevant peptide at 25°C and subsequent irradiation (7,000 rad). After washing, $2 \times 10^6$ peptide-pulsed RMA-S cells were used to stimulate $8 \times 10^6$ spleen cells from immunized animals per well in 24-well plates in basic medium (IMDM containing 10% FCS, with addition of penicillin, streptomycin, glutamine, and $5 \times 10^{-5}$ M 2-mercaptoethanol), and peptides were added to the cultures (final concentration 30 $\mu$M). After 7 d, cells were restimulated with $2 \times 10^6$ irradiated C57BL/6 spleen cells/well and 5 $\mu$M peptide at a stimulator/responder ratio of 3:1 in complete medium (basic medium with 10% Con A supernatant). Thereafter, the CTL lines were restimulated weekly as above.

*Cloning of T Cells.* CTL lines against K3 and K3-O-GlcNAc were cloned in 96-well plates by limiting dilution on irradiated peptide pulsed syngeneic spleen cells ($2.5 \times 10^5$/well) and temperature-induced peptide pulsed RMA-S cells ($10^5$/well) in 100 $\mu$l complete medium. Another 100 $\mu$l complete medium were added to each well on day 4 and good growing clones were transferred onto fresh feeder cells in 48-well plates on days 10–14.

*CTL Assays.* The peptide transporter–deficient cell line T2 transfected with $D^b$ or $K^b$ (T2-$D^b$ or T2-$K^b$, respectively) were used as target cells conventional CTL assays.

## Results and Discussion

The peptides used in this investigation (Fig. 1) were analogues of the CTL epitope FAPGNYPAL from Sendai virus nucleoprotein (15). This sequence was chosen because it contains both a $D^b$ (xxxxNxxxL) and a $K^b$ (xxxxxYxxL) binding motif (16, 17), allowing us to induce T cell responses to a single peptide presented by two different restriction elements. Also, a crystal structure of the peptide FAPGNYPAL in complex with $K^b$ is available (18), enabling us to choose positions for carbohydrate modifications most likely to point out of the groove and interact with the TCR.

The wt peptide as well as the analogue K3 and its O-glycosylated counterpart K3-O-GlcNAc were synthesized and tested for their ability to bind $K^b$ and $D^b$. Fig. 2 shows that K3-O-GlcNAc bound to both $K^b$ and $D^b$ as efficiently as the wt peptide, when tested in the class I MHC RMA-S assembly assay, with half maximal binding at peptide concentrations of 60 nM for $K^b$ and 12 nM for $D^b$ compared with 90 and 12 nM for wt, respectively. Similarly, the non-glycosylated K3 bound well to both $K^b$ and $D^b$ with half maximal binding at peptide concentrations of 160 and 22 nM, respectively. It is interesting to note, that in a previous study, N-glycosylation of a class II-binding peptide (19) led to greatly reduced binding, regardless of the position of the modification within the MHC binding region of the peptide.

Having confirmed the efficient binding to $K^b$ and $D^b$, K3 and K3-O-GlcNAc were used to generate CTL. After 2 in vitro stimulations, the peptide specificity and MHC restriction of CTL lines were tested in standard $^{51}$Cr release assays using peptide pulsed T2-$K^b$ and T2-$D^b$ as target cells. In addition, lines were cloned by limiting dilution in order to analyze the bulk response of each line at the clonal level. We derived nine CTL clones specific for K3 and 5 for K3-O-GlcNAc.

Immunization and restimulation with K3-O-GlcNAc gave rise to a CTL line with a high degree of specificity for the

wt FAPGNYPAL

K3 FAPSNYPAL

K3-*O*-GlcNAc FAPS($R_1$)NYPAL

K3-*O*-GalNAc FAPS($R_2$)NYPAL

K1-*N*-GlcNAc FAPGN($R_3$)YSAL

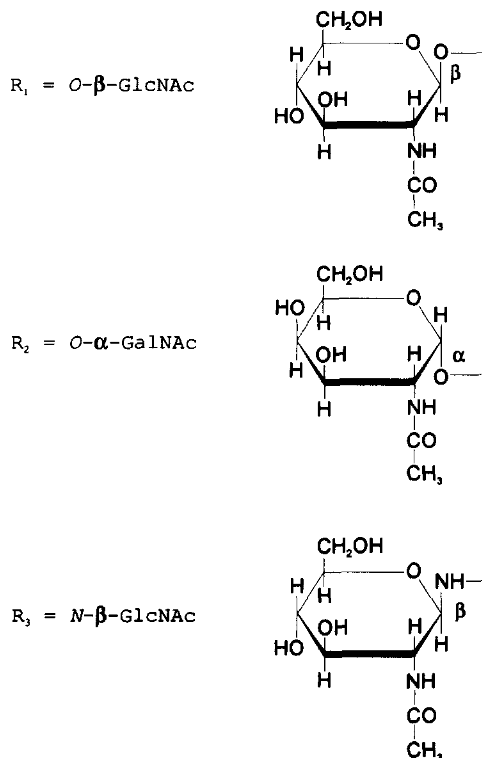


**Figure 1.** Peptide structure. The peptides used in this study were based on a $K^b$-restricted CTL peptide epitope (wt) from Sendai virus nucleoprotein. K3- O-GlcNAc carries *O*-β-linked GlcNAc ($R_1$) at position 4 of K3. K3-O-GalNAc has *O*-α-linked GalNAc ($R_2$) at position 4. K1-*N*-GlcNAc carries *N*-β-linked GlcNAc ($R_3$) at position 5 of wt as well as a P to S substitution at position 7, thus mimicking the natural *N*-glycosylation sequon.

glycosylated peptide with only marginal crossreactivity toward the nonglycosylated K3. Both $K^b$- and $D^b$-restricted responses were generated (Fig. 3). Analysis of CTL clones derived from this line showed that this was not due to crossreactivity between the restriction elements, but instead to the presence of both $K^b$- and $D^b$-restricted clones specific for K3-O-GlcNAc (Fig. 4, *a* and *b*). All of the five clones derived from CTL lines induced with K3-O-GlcNAc were specific for the glycopeptide, regardless of restriction element.

Similarly, immunization with the nonglycosylated counterpart K3 gave rise to lines specific for that peptide and with little crossreactivity with the glycosylated K3-O-GlcNAc.

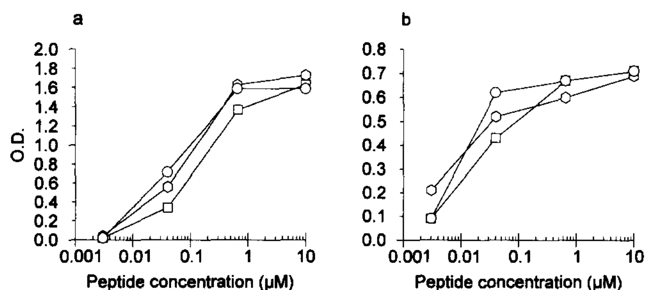


**Figure 2.** Peptide and glycopeptide binding to MHC. The binding of wt (*hexagon*), K3 (*box*), and K3-O-GlcNAc (*circle*) to $K^b$ (*a*) and $D^b$ (*b*) was measured using an assembly assay as described previously (12). Peptide binding was quantified by densitometry of the class I MHC heavy chain band on autoradiographs of SDS-PAGE gels.

Again both $K^b$- and $D^b$-restricted responses were observed that did not appear to be the result of crossreactivity when analyzed at the clonal level (Fig. 3 *b*). All of the nine clones derived against K3 were thus restricted through $K^b$ (Fig. 4 *c*). Only one clone derived from immunization with K3 crossreacted against K3-O-GlcNAc (data not shown).

These results clearly demonstrate the ability to generate specific class I MHC–restricted CTL responses to glycopeptides, and suggest that the glycan moiety is involved in a specific contact with TCR.

To investigate this issue in more detail, we synthesized two analogues of K3-O-GlcNAc. The glycopeptide K3-O-GalNAc (Fig. 1) has an *O*-α-linked GalNAc at position 4 instead of the *O*-β-linked GlcNAc in K3-O-GlcNAc. K3-O-GalNAc bound as well to both $K^b$ and $D^b$ as K3-O-GlcNAc (half maximal binding at 50 and 11 nM, respectively). This peptide was, however, not recognized by K3-O-GlcNAc–specific CTL lines (data not shown) or clones (Fig. 5), despite the fact that K3-O-GlcNAc only differs from K3-O-GalNAc by the anomeric glycosyl linkage to serine and the orientation of the hydroxyl group on C4 of the glycan ring (see Fig. 1). This result lends strong support to the interpretation, that the K3-O-GlcNAc–specific CTL recognition is partly due to specific interaction between the TCR and the solvent accessible surface area of the glycan moiety of K3-O-GlcNAc when complexed with $K^b$ or $D^b$.

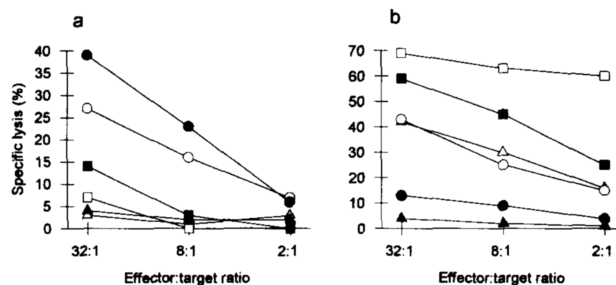


**Figure 3.** Peptide specificity and MHC restriction of CTL lines. CTL lines from mice immunized with K3-O-GlcNAc (*a*) or K3 (*b*) were tested in CTL assays using the cell lines T2-$K^b$ (*open*) or T2-$D^b$ (*closed*) as target cells prepulsed with 10 μM of the peptides K3-O-GlcNAc (*circle*), K3 (*box*), or without peptide (*triangle*).

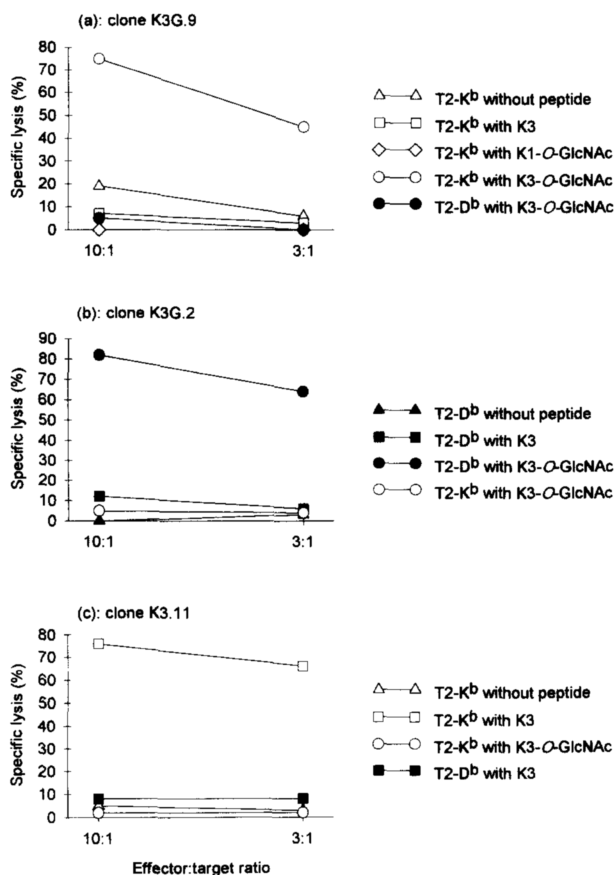


**Figure 4.** Peptide specificity and MHC restriction of CTL clones. T cell clones against peptides K3-O-GlcNAc (*a* and *b*) or K3 (*c*) obtained by limiting dilution were tested in CTL assays against T2-$K^b$ (*open*) or T2-$D^b$ (*closed*) sensitized with 10 $\mu$M peptide K3-O-GlcNAc (*circle*), K3 (*box*), or without peptide (*triangle*). The $K^b$-restricted clone K3G.9 (*a*) was also tested against K1-*N*-GlcNAc (*diamond*).

We also synthesized the glycopeptide K1-*N*-GlcNAc, which carries an *N*-linked GlcNAc residue at position 5 (as well as a P to S substitution at position 7). K1-*N*-GlcNAc was also shown to bind efficiently to $K^b$ with half maximal binding at 200 nM but was not recognized by any of the CTL lines (data not shown), nor by $K^b$-restricted CTL clones specific for K3-O-GlcNAc (Fig. 4 *a*). This result demonstrates that the K3-O-GlcNAc–specific CTL response was sensitive to the position of the glycan in the modified peptide.

The asparagine at position 5 (N5) of the wt peptide FAPGNYPAL is considered an anchor residue required for efficient binding of peptides to $D^b$ (17). Accordingly, we found that *N*-GlcNAc modification of N5 resulted in a dramatic decrease in the binding to $D^b$ (half maximal binding >10 $\mu$M) but not $K^b$ (half maximal binding 200 nM). This peptide was also not recognized by any K3-O-GlcNAc–specific $D^b$-restricted clones (data not shown).

In a separate set of experiments, CTL lines raised against K1-*N*-GlcNAc only displayed reactivity towards T2-$K^b$ prepulsed with K1-*N*-GlcNAc, but not when prepulsed with K3-O-GlcNAc, or a nonglycosylated analogue, or when using T2-$D^b$ as targets (data not shown).

Other groups have raised MHC class II–restricted T cells to glycopeptides (19, 20). These studies showed, however, that potent immunogenicity was only retained when peptides were glycosylated outside the MHC binding region of the peptide. In one of these studies T cells generated to glycopeptides of high immunogenicity crossreacted with the nonglycosylated peptide (19); whereas in another study T cell hybridomas were generated that reacted preferentially with glycosylated peptide but at the same time showed equal reactivity towards dramatically different $NH_2$-terminal glycosylations (20). Furthermore, T cells generated to the nonglycosylated peptide reacted equally well, or better, towards the $NH_2$-terminally substituted glycopeptide.

We have demonstrated that a class I MHC binding peptide can be modified by O-glycosylation within the MHC binding region without affecting its binding to MHC class I. The resulting peptides are highly immunogenic, and elicit carbohydrate-specific, MHC-restricted, antiglycopeptide CTL. Furthermore, the O-$\beta$-linked GlcNAc substitution used in the present study is the first example of T cell recognition of a naturally occurring type of glycosylation. Proteins carrying this glycosylation which have been identified to date include RNA polymerase transcription factors, cytoskeletal proteins, nuclear pore proteins, neurofilaments, and oncogene products (5). Also, cytosolic proteins from *Leishmania* (21) and viral proteins (22, 23) have been shown to carry O-linked GlcNAc.

The fact that O-GlcNAc monosubstituted serine and threonine residues are frequently found in cytosolic and nuclear proteins means that peptides derived from the cytosolic degradation of these proteins could enter the MHC class I presentation pathway (1). Recognition of glycosylated peptides (and other posttranslational modifications) could therefore be of significant importance in immunity towards malignant diseases and in viral infections. In addition, it is possible that the acquisition or loss of glycosylation might provide a novel strategy for viruses or malignantly transformed cells to escape from selection pressure by CTL (24, 25), which does not depend on a mutation at the gene level. Furthermore, the loss or acquisition of oligosaccharide residues in endogenously presented peptides could lead to the formation of neoantigens, loss of tolerance, and development of autoimmunity.

The relevance of studying T cell responses to posttranslational modifications such as glycosylation now depends on the identification of a naturally occurring epitope of this kind. This potentially new group of peptide epitope determinants

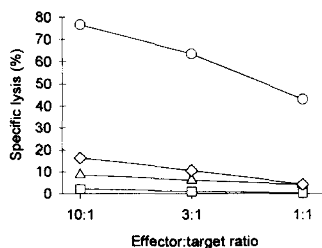


**Figure 5.** CTL clones recognize K3-O-GlcNAc but not K3-O-GalNAc. The CTL clone K3G.4 was tested against T2-$D^b$ target cells sensitized with K3-O-GlcNAc (*circle*), K3-O-GalNAc (*diamond*), K3 (*box*), or without peptide (*triangle*).

would so far have eluded discovery for technical reasons, since T cell epitope mapping conventionally has been achieved using synthetic peptides. The isolation of glycopeptides complexed with MHC class I molecules would be another approach to identifying this kind of epitope. The fact that no glycopeptides have so far been detected amongst the pool of peptides eluted from MHC class I could simply reflect the technical difficulties encountered in detecting O-GlcNAc modifications by Edman degradation and mass spectroscopy, even when they are specifically looked for (26). Indeed, using techniques specific for the detection of carbohydrate modifications, we have obtained preliminary data supporting the presence of O-GlcNAc modified peptides amongst the pool of peptides eluted from human spleen class I MHC (Haurum, J.S., unpublished observations).

Furthermore, glycopeptides might only comprise a small fraction of the peptides presented by MHC. This would not detract from their potential significance, since the relative abundance of individual peptides presented on the cell surface does not correlate with their importance as T cell antigens. Several immunodominant antigens have thus been extremely difficult to isolate from antigen-presenting cells in amounts sufficient for sequence analysis (27).

The subcellular location of the cytosolic glycosylation with O-linked GlcNAc could yield glycopeptides that are capable of entering the MHC class I presentation pathway. Our study shows that O-GlcNAc substitution is a promising candidate for a naturally occurring type of glycosylation capable of being recognized by CTL in an MHC-restricted way.

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