Brief Definitive Report

Glycan-regulated Antigen Processing of a Protein in the Endoplasmic Reticulum Can Uncover Cryptic Cytotoxic T Cell Epitopes

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Summary

We and others have shown that influenza A nucleoprotein (NP) targeted to the secretory pathway cannot be processed to yield several cytotoxic T lymphocyte (CTL) epitopes in cell lines that lack the transporter associated with antigen processing (TAP). However, a large COOH-terminal fragment of NP is processed and presented in these cells. Full-length NP is cotranslationally glycosylated in the lumen of the endoplasmic reticulum at two sites distal to the major H2-K^k and H2-D^b restricted CTL epitopes, and we show here that pharmacological or genetic inhibition of N-linked glycosylation, leads to the processing and presentation of both these epitopes in a TAP-independent way.

Key words: cytotoxic T lymphocyte • antigen processing • endoplasmic reticulum • transporter associated with antigen processing • class I major histocompatibility complex

Cytotoxic T lymphocytes (CTLs) recognize complexes formed between MHC class I molecules and peptides generally derived from the breakdown of intracellular proteins. There is a large body of evidence indicating that proteolysis of these proteins begins in the cytosol, probably mediated by a multicatalytic cytosolic protease known as the proteasome (1), and that after these initial events, peptide fragments are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP; for review see reference 2). The continued processing or trimming of peptide epitope precursors in the ER has been suggested by studies in which polypeptides of up to 150 amino acids have been shown to yield nonamer peptides in the ER of TAP-negative cell lines (3, 4). The release of epitopes from the COOH terminus of such precursors is more efficient than from their NH_2 termini, suggesting the involvement of N-aminopeptidases (4, 5). Other studies in which peptide epitopes were liberated from the interior of a long polypeptide or from a protein with an inaccessible NH_2 terminus also suggests the involvement of endoproteases in the ER (5). These activities are insensitive to inhibitors of the proteasome, lysosomal enzymes and calpains (3, 5).

The extent to which these proteases can act on secretory and membrane proteins that are co-translationally inserted into the ER is unclear. Peptides derived from glycoproteins can be presented by class I MHC, but several of these have been shown to be processed in the cytosol by a process of either mistranslation (6, 7) or dislocation (8, 9), which involves the retrograde translocation of glycoproteins from the ER to the cytosol (for example see reference 10). However, there are examples of MHC class I binding peptides derived both from signal sequences and from within secretory and membrane proteins found in association with MHC class I molecules in TAP-negative cells (11, 12), suggesting that these may be generated in the ER itself. Also, the demonstration that other ER-targeted proteins can be processed in a TAP-independent manner (3, 4, 13–15) suggests the involvement of an alternative degradation pathway in the ER.

One issue that has not been addressed by any of the studies looking at the processing of full-length secretory and membrane proteins is the relationship between N-linked glycosylation and susceptibility to antigen processing in the ER. All the full length proteins studied thus far contained consensus sites for attachment of N-linked glycans, and regardless of other factors this could prevent their processing and presentation from within the ER.

As a way of investigating the role of glycosylation in the ER, we have used the observation that ER-targeted NP, which is cotranslationally glycosylated, is not processed in the ER to yield CTL epitopes (3, 16). Here we show that N-glycosylations at sites distal to CTL epitopes can protect them from processing in the ER and that the removal of both sites is necessary before the polypeptide is susceptible to ER processing.

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Materials and Methods

Cell Lines. Simian COS-1 cells were grown in DMEM. Murine L cells transfected with the H-2D^b gene were a gift from Dr. A. Townsend (Institute of Molecular Medicine, Oxford, UK) and were maintained in RPMI 1640 medium. The human TAP-negative T2 cell lines, transfected with the genes encoding H-2D^b (T2-D^b) or H-2K^k (T2-K^k), were gifts from Dr. P. Cresswell (Yale University, New Haven, CT) and were maintained in RPMI 1640. LBL.174 was a gift from Dr. R. DeMars (University of Wisconsin, Madison, WI) and was transfected with the murine H-2 gene H-2D^b (0.174D^b) by Dr. V. Cerundolo (University of Oxford, Oxford, UK). The thymidine kinase–negative human fibroblast line 143 was maintained in DMEM. All media were supplemented with 10% FCS (Meldrum Ltd., Bourne End, UK), 50 U/ml penicillin, 50 mg/ml streptomycin, and 2 mM l-glutamine.

Antibodies. The mAb 4.7.18 recognizes a region near the NH_2 terminus of influenza A nucleoprotein (NP) and was a gift from J. Skehel (National Institute of Medical Research, Mill Hill, UK). For immunoprecipitation studies, it was used at a final dilution of 1:100. The mAb B22.249 is specific for a conformational epitope on the α1 domain of H-2D^b (17) and was used for immunoprecipitation studies B22.949 at a final concentration of 15 μg/ml.

Peptides. The nonamer ASNENMDAM corresponds to residues 366–374 of NP from strain A/NT/60/68 and is the naturally occurring epitope presented to CTL by H-2D^b (18). SDYERGLI, corresponding to residues 50–57 of NP, is presented by H-2K^k (19). ILKEPVHGV corresponds to residues 476–484 of HIV pol, binds to HLA-A2, and is the naturally processed epitope (20). All peptides were synthesized by Research Genetics, Inc. (Huntsville, AL).

Site-directed Mutagenesis. Oligonucleotide-directed mutagenesis was performed using the Muta-Gene Phagemid In Vitro Mutagenesis kit (Bio-Rad Labs. Ltd., Hemel Hempstead, Hertfordshire, UK) and single stranded pcDNA3 containing the cDNA for wild-type NP tagged with the hemagglutinin leader sequence (3) as a template. Single-site mutants for position 21 (NAT to DAT) and 144 (NDT to NDA) were generated, and a further round of mutagenesis with the reciprocal oligonucleotide was performed to generate a gene in which both glycosylation sequons had been deleted. Wild-type NP (L$^+$NP$^{+/+}$) contains both sites. The position 21 mutant is designated L$^+$NP$^{-/+}$, the position 144 mutant is designated L$^+$NP$^{+/-}$, and the nonglycosylated mutant is designated L$^+$NP$^{-/-}$.

Recombinant Vaccinia Viruses. Recombinant vaccinias were made by homologous recombination into the thymidine kinase gene using the shuttle vector pSC11.30R.2 as previously described (3). Vaccinias encoding wild-type L$^+$NP (L$^+$NP$^{+/+}$vacc), a nonglycosylated COOH-terminal fragment of NP containing residues 368–498 tagged with the hemagglutinin leader sequence (L$^+$IMPvacc) and influenza matrix protein (M1vacc) had been constructed previously (3, 18, 21, 22). All constructs were confirmed by dideoxy sequencing.

Expression of NP Glycoforms in Mammalian Cells. COS-1 cells were transfected with 10 μg of sterile plasmid DNA by culturing in 500 μg/ml DEAE-dextran in RPMI, followed immediately by 20 μl of 100-mM chloroquine. Cells were incubated at 37°C for 4 h, after which the transfection mix was removed and replaced with 10% DMSO in RPMI for 3 min. This was replaced with 20 ml of medium. Assays were performed after 48 h. For expression from recombinant vaccinia, cells were infected at a multiplicity of 10 for 90 min at 37°C, after which they were washed twice, resuspended at 5×10^5/ml and incubated for a total of 3 h before further manipulation. 721.174D^b at 10^7/ml were transfected by electroporation with 50 μg of DNA (400 V/cm, 960 μF) using a Cell Electroporator (Bio-Rad Labs., Ltd.). After 1 wk in culture, G418-resistant cells were examined for L$^+$NP expression by immunoprecipitation.

CTL Production. 6-wk-old female C57Bl/6 (H-2^b) and CBA (H-2^k) mice (Harlan Olac, Bicester, Oxfordshire, UK) were immunized by intraperitoneal injection of 10^7 PFU of NP-vacc in 200 μl of sterile PBS. Splenocytes were prepared at day 8. Autologous, irradiated splenocytes pulsed for 1 h with 5 mM peptide were used as stimulators. Recombinant human IL-2 was added to the cultures at a final 10 U/ml after the third restimulation. CTL clone F5 was a gift from A. Townsend.

CTL Assays. Standard 4-h ^{51}Cr-release assays were performed using infected or peptide-pulsed target cells. Targets were labeled with ^{51}Cr at the time of infection for 1 h. Virus was then removed and the cells were incubated for a further 2 h before their exposure to CTL. Where appropriate, cells were treated with 10 μg/ml tunicamycin 2 h after the start of virus infection for a further 2 h before the 4-h killing assay. This approach was taken to ensure that all H-2D^b molecules at the cell surface at the start of the killing assay had been synthesized in the presence of tunicamycin.

Metabolic Labeling and Immunoprecipitation. This was performed as previously described (23). 10^7 cells labeled with 100 μCi per 10^7 cells of ^{35}S Promix (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) for 30 min at 37°C were used for each experimental point. Endoglycosidase H (Endo-H) treatment of immunoprecipitates was with 1 U overnight at 37°C. Immunoprecipitates were resolved on a 10% SDS-polyacrylamide gel and analyzed by autoradiography. Comparative experiments were exposed for the same time period. Autoradiographs were quantitated using the BioImage Analyzer and software (Millipore Corp., Bedford, MA) where, for pulse–chase analyses, optical density readings were converted into a percentage value based on the reading at the start of the chase being 100%. Where the effects of tunicamycin were studied, the drug was added (from a stock of 1 mg/ml in DMSO) 1 h before labeling and was present at the indicated concentration throughout the experiment. An equivalent volume of DMSO was added to controls.

Results

We have previously shown that full-length NP expressed in the ER is not processed to reveal two immunodominant CTL epitopes in TAP-deficient cells (3, 16). One possible reason for this is that its cotranslational glycosylation at residues 21 and 144 somehow blocks antigen processing in this compartment.

Blocking the Glycosylation of L$^+$NP Promotes the Release of CTL Epitopes after Processing in the ER. To test this hypothesis, we assessed the effect of the glycosylation inhibitor tunicamycin on the ER processing of L$^+$NP in T2-D^b. At a concentration of 10 μg/ml of tunicamycin, L$^+$NP but not H-2D^b glycosylation is substantially inhibited (data not shown). Fig. 1 shows that, under these conditions, T2-D^b are able to present both the D^b-restricted epitope 366–374 (Fig. 1 *A*) and T2-K^k the K^k-restricted epitope SDYEGRLI (50-57, Fig. 1 *B*) to CTLs. In the absence of tunicamycin treatment, no presentation occurs. Presentation of the NP fragment L$^+$IMP (which contains the D^b-restricted but not

the K^k-restricted CTL epitope) is unaffected by tunicamycin treatment. In contrast to the cell lines T2-D^b and T2-K^k, the TAP-competent cell line L-D^b is able to present these epitopes independently of the glycosylation status of L^+NP, indicating that tunicamycin exerts its effect on the alternative, TAP-independent antigen processing pathway. Tunicamycin treatment restores the presentation of NP366–374 from L^+NP in a dose-dependent manner (Fig. 1 *C*). Thus, the presentation of NP366–374 by T2-D^b, induced using doses of tunicamycin that inhibit the glycosylation of NP, suggests that processing of proteins within the ER for presentation through MHC class I is directly linked to the absence of N-linked glycans on the antigen. The decrease in the efficiency of presentation of L^+IMP-derived NP366–374 by T2-D^b in the presence of tunicamycin is probably due to its effect on the level of H-2D^b, and serves to indicate that the restoration of presentation of NP366–374 from $L^+NP^{+/+}$ that was seen in the presence of tunicamycin is significant.

To observe the effect of deglycosylation on the ER processing of L^+NP in isolation of vaccinia virus infection, presentation of the H2-D^b–restricted epitope was investigated in the TAP-negative cell line LBL721.174 transfected with the L^+NP gene. Fig. 2 shows that treatment of the transfected cells with 5 μg/ml tunicamycin restored the presentation of NP366–374 to CTLs to ~70% of that seen when tunicamycin-treated T2-D^b were pulsed with 10 nM of peptide.

To eliminate the possibility that tunicamycin restored presentation of class I epitopes from within L^+NP by a mechanism separate from inhibition of antigen glycosylation, we constructed a series of mutants of L^+NP by site-directed mutagenesis. The asparagine at position 21 is found in the nucleoproteins of the majority of known strains of influenza A virus, but aspartic acid is present in some strains. Similarly, threonine 146 is found in ~50% of the known strains, with alanine present in the majority of the remainder. We used these two naturally occurring amino acid substitutions to remove one or both of the glycosylation sequons from $L^+NP^{+/+}$. Recombinant vaccinias encoding the three possible glycosylation variants were then constructed. Fig. 3 shows that in contrast to infection with $L^+NP^{+/+}$, T2-D^b infected with $L^+NP^{-/-}$vacc is able to process and present the H2-D^b–restricted epitope NP366–374 to CTLs as efficiently as from L^+IMPvacc. This demonstrates a direct correlation between the absence of N-linked glycans and the ability to process L^+NP within the ER to yield CTL epitopes. The presence of either of the two glycosylation sites alone also prevents the presentation of NP366–374 from L^+NP when expressed in T2-D^b (Fig. 3). In contrast, like $L^+NP^{+/+}$, both are readily processed in the cytosol of a TAP-competent cell. These results indicate that in addition to the cytosolic processing of secretory glycoproteins, deglycosylated glycoproteins can also be exposed to TAP-independent processing pathways that are located in a distinct compartment. The possibility that pro-

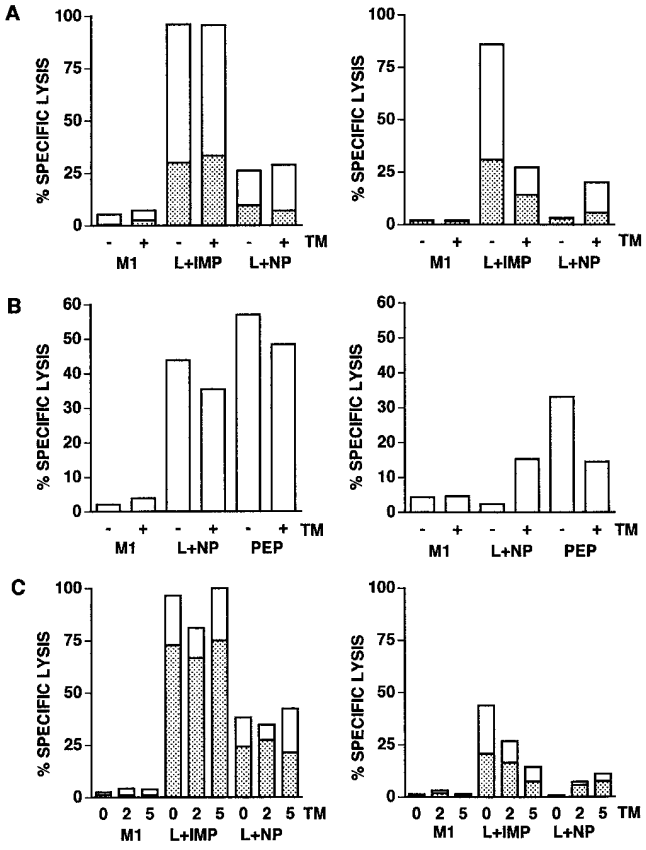

Figure 1. Tunicamycin restores presentation of two MHC class I epitopes from NP by T2 infected with recombinant vaccinia. (*A*) T2-D^b (*right*) and L-D^b (*left*) were infected with recombinant vaccinias as indicated and incubated in the presence or absence of tunicamycin (*TM*) at 10 μg/ml before testing their ability to present the H2-D^b–restricted epitope ASNENMDAM to CTL clone F5. Two E/T ratios are shown: 5:1 (*white bars*) and 1:1 (*stippled bars*). (*B*) The experiment was repeated with T2-K^k (*right*) and L-D^b (*left*) to test the presentation of the H2-K^k–restricted epitope SDYERGLI (NP50–57) to a polyclonal CTL population used at an E/T ratio of 25:1. (*C*) T2-D^b (*right*) or L-D^b (*left*) were infected with recombinant vaccinias as indicated and incubated in the presence or absence of tunicamycin (*TM*) at the doses indicated before testing their ability to present the H2-D^b–restricted epitope ASNENMDAM to CTL clone F5. Two E/T ratios are shown: 5:1 (*white bars*) and 1:1 (*stippled bars*).

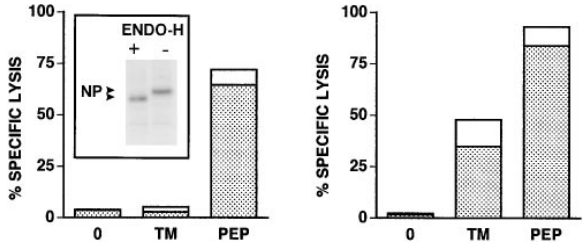

Figure 2. Tunicamycin restores presentation of NP366–374 by transfected 721.174D^b expressing $L^+NP^{+/+}$. .174D^b expressing $L^+NP^{+/+}$ (*right*) (*inset*: expression of the transgene after pulse-labeling and immunoprecipitation after 3-h chase) or untransfected .174D^b (*left*) were left untreated (*0*), incubated in the presence of 5 μg/ml of tunicamycin (*TM*), or pulsed with 10 nM ASNENMDAM (*PEP*) before testing their ability to present the H2-D^b–restricted epitope ASNENMDAM to CTL clone F5. Two E/T ratios are shown: 5:1 (*white bars*) and 1:1 (*stippled bars*).

cessing of the nonglycosylated form of NP also occurs in the cytosol with TAP-independent transfer of the epitope into the ER is not supported by the observation that presentation of the epitope by TAP-negative cells occurs only if it is preceded by an ER targeting signal (3).

The Rate of L^+NP Turnover Is Glycan Regulated but Does Not Correlate with Antigen Presentation. For cytosolic processing, there is a correlation between the rate of antigen degradation and the efficacy with which it is presented to CTLs. To determine whether the same correlation was true for glycoproteins processed in the ER, we measured the rate of degradation of all four L^+NP constructs in COS-1 cells. Fig. 4 *a* shows that $L^+NP^{+/+}$ was long-lived with a half-life of >4 h. It remained sensitive to Endo-H during this time (Fig. 2 and data not shown), suggesting that it was retained in the early part of the secretory pathway. We could not detect any secreted NP even after prolonged (24 h) expression (data not shown). The half-life of NP when expressed in the ER is thus comparable to that of NP expressed in the cytosol (21). Treatment of $L^+NP^{+/+}$ expressing COS-1 cells with 10 μg/ml tunicamycin (a dose that completely inhibits glycosylation of the protein) causes it to be degraded more rapidly with only 40% remaining after a 4-h chase, as compared with 75% of untreated NP. This observation was not due to a nonspecific effect of tunicamycin on the cells, because $L^+NP^{-/-}$ is degraded with similar kinetics to L^+NP after tunicamycin treatment and is unaffected by tunicamycin treatment. The increased rate of degradation of deglycosylated NP appeared to be controlled by the presence or absence of an N-linked glycan at position 21. Thus, Fig. 4 *b* shows that, although $L^+NP^{+/-}$ was degraded at the same rate as $L^+NP^{+/+}$, $L^+NP^{-/+}$ was degraded at the same rate as $L^+NP^{-/-}$. The slow degradation of $L^+NP^{+/-}$ was accelerated when it was synthesized in the presence of tunicamycin. Since neither of the monoglycosylated forms are processed and presented by T2-D^b, we conclude that, unlike in the cytosol, where induction of rapid degradation is sufficient to potentiate presentation of class I epitopes (21), antigen processing in the ER is limited by the presence of N-linked glycans on a protein and this effect is independent of the degradation rate of the protein. It is important to note that, in these experiments, the term 'degradation' is used operationally, since the failure to detect NP by immunoprecipitation does not necessarily reflect its hydrolysis but could reflect other events leading to the loss of the anti-NP mAb epitope.

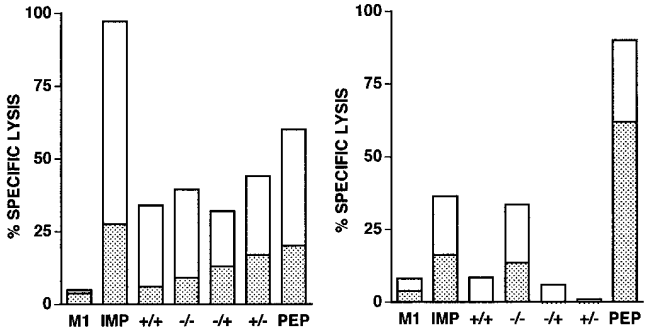

Figure 3. Presentation of NP366–374 to CTL clone F5 by T2-D^b infected with recombinant vaccinia is blocked by the presence of N-linked glycans. T2-D^b (*right*) and L-D^b (*left*) were infected with recombinant vaccinias encoding influenza matrix protein (*M1*), L^+IMP (*IMP*), $L^+NP^{+/+}$ (+/+), $L^+NP^{-/-}$ (−/−), $L^+NP^{-/+}$ (−/+), or $L^+NP^{+/-}$ (+/−), or were pulsed with 10 nM NP366–374 (*PEP*) and tested for their ability to present NP366–374 to CTL clone F5 at an E/T ratio of 5:1 (*white bars*) or 1:1 (*stippled bars*).

Discussion

Many secretory and membrane proteins have been shown to yield class I–restricted CTL epitopes. With respect to cytosolic antigen processing, this raises a topological paradox because these proteins are cotranslationally transported into the ER. However, they can be exposed to cytosolic proteases by either their mistranslation (6, 7), or dislocation to the cytosol (8, 9). Alternatively, they can be processed in the ER itself (3, 4, 13–15, 24).

The majority of glycoprotein antigens studied to date are processed and presented in a TAP-dependent manner, as is $L^+NP^{+/+}$ described here and elsewhere (8). Using L^+NP as a model antigen, we have shown that a controlling factor in determining whether ER-targeted proteins can be processed in the ER is N-glycosylation. Like the diglycosylated form of the antigen ($L^+NP^{+/+}$), neither of the two monoglycosylated proteins ($L^+NP^{-/+}$ and $L^+NP^{+/-}$) yielded CTL epitopes, although they were degraded with different kinetics. This suggested that the glycan-regulated rate of degradation was not the controlling factor in determining whether the protein was processed for presentation to CTLs. Our results are consistent with the idea that a failure to glycosylate might prohibit a nascent glycoprotein from entering the quality control procedure that normally culminates in export from the ER into either the secretory pathway or the cytosol (where they are degraded), allowing it to become exposed to proteases in the ER itself. The glycosylation of proteins targeted to the secretory pathway would therefore have the effect of preventing the generation of large numbers of class I binding peptides within the ER by enzymes that normally function to trim peptides delivered by TAP. Our results show that N-glycosylation can

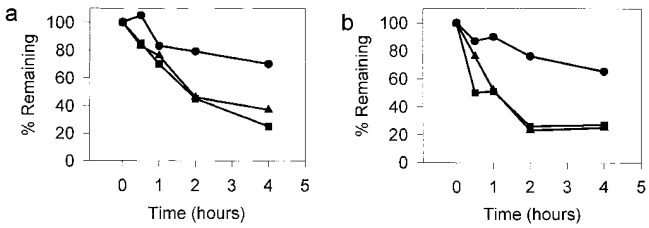

Figure 4. Stability of L^+NP glycoforms expressed in the ER of COS-1. The intracellular stability of (*a*) untreated $L^+NP^{+/+}$ (●), $L^+NP^{+/+}$ after tunicamycin treatment (■), and $L^+NP^{-/-}$(▲); and (*b*) untreated $L^+NP^{+/-}$ (●), $L^+NP^{+/-}$ after tunicamycin treatment (■), and $L^+NP^{-/+}$ (▲) are shown.

inhibit the generation of epitopes distal to the site of glycosylation, presumably by affecting the general pathway for degradation as outlined above. By ensuring that proteins targeted for degradation are disposed of at a site that is remote from peptide delivery and trimming, the system can ensure that under normal conditions the favored route by which peptides interact with class I MHC molecules is via TAP. This process may not be totally efficient since some epitopes (25), including one in L^+NP itself (16), are apparently generated from glycoproteins in a TAP-independent manner. Alternatively, some epitopes may be able to gain entry to the ER in a TAP-independent manner, although this is not the case for the epitopes studied here.

There are other possible immunological consequences of this glycan-regulated antigen presentation. For example, it is possible that some glycoprotein-derived epitopes may be hidden from CTL surveillance through a combination of their failure to be generated in the cytosol and the protection afforded by N-linked oligosaccharides. The possible existence of "hidden" epitopes within glycoproteins has implications for the maintenance of tolerance; exposure of these epitopes by affecting the glycosylation of the source antigen provides a potential route for the initiation of CTL responses to novel epitopes. This could also open up the possibility of pharmacological intervention to modulate presentation of viral glycoproteins in vivo.

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