Introduction of a Glycosylation Site into a Secreted Protein Provides Evidence for an Alternative Antigen Processing Pathway: Transport of Precursors of Major Histocompatability Complex Class I–Restricted Peptides from the Endoplasmic Reticulum to the Cytosol

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Summary

We found that the presentation of a H-2K^d-restricted determinant from influenza virus nucleoprotein (NP) to T cells is strictly dependent on expression of the transporter associated with antigen presentation (TAP), regardless of whether NP is expressed as a cytosolic or secreted NP (SNP). Introducing an N-linked glycosylation site into the determinant selectively reduced presentation of SNP. This indicates that glycosylation does not interfere with TAP-transported peptides, and therefore that cytosolic peptides derived from SNP must have been exposed to the glycosylation machinery of the endoplasmic reticulum (ER) before their existence in the cytosol. Based on these findings, we propose that TAP-dependent processing of at least some ER-targeted proteins entails the reimportation of protein from the secretory pathway to the cytosol, where the protein is processed via the classical pathway.

Class I molecules of the MHC function to display peptides from intracellular proteins on the cell surface for inspection by CD8$^+$ T cells (T_{CD8+}). In most cases, peptides derive from a cytosolic/nuclear pool of proteins through the action of proteasomes or other cytosolic proteases (1), and are delivered to class I molecules by transporter associated with antigen presentation (TAP)[1], the MHC-encoded peptide transporter (2). Newly synthesized α-$\beta_2$microglobulin heterodimers associate with TAP and remain tethered until released by a conformational alteration induced by peptide binding (3, 4). These events occur in the endoplasmic reticulum (ER) (5).

Although many class I binding peptides derive from cytosolic or nuclear proteins, proteins targeted to the ER (perhaps a third of all cellular proteins, and important antigens for membrane viruses) also represent a major source of peptides. Most peptides from ER-targeted proteins are presented in a TAP-dependent manner. This, in conjunction with earlier findings that removal of the ER-insertion sequence from integral membrane proteins increases their antigenicity (6, 7), indicates that antigenic peptides from ER-targeted proteins are often produced by cytosolic proteases. It remains unresolved however, to what extent this reflects generation of peptides from defective cytosolic forms of the protein that are not exported to the ER, as originally proposed (8), versus reimportation of exported proteins to the cytosol where they are processed as cytosolic antigens. The latter possibility has gained credence with recent findings from several systems that membrane-anchored and soluble proteins and peptides can be returned to the cytosol where they are degraded in a proteasome dependent manner (9–12).

In the present study we examine this question using Asn-linked glycosylation (commonly termed N-linked glycosylation) as a means of determining whether the source protein has visited the ER. N-linked glycosylation occurs exclusively in the ER, where $Glc_3Man_9GlcNac_2$ is added to Asn residues present in the sequence Asn X Ser/Thr (*X*, any residue except Pro). Under normal circumstances, glycosy-

[1] *Abbreviations used in this paper:* BFA, brefeldin A; endo H, endo-β-*N*-acetylglucosaminidase; ER, endoplasmic reticulum; NP, nucleoprotein; PNGase, peptide *N* glycanase; rVV, recombinant VV; TAP, transporter associated with antigen presentation; VV, vaccinia virus.

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lation is believed to occur cotranslationally as proteins are threaded through the translocon. Extrusion through the translocon is not an absolute requirement, however, since synthetic peptides can also be glycosylated when delivered to the ER via TAP (13–16). Protein conformation can influence glycosylation, since potential glycosylation sites present on exported proteins are not always used.

Glycosylation of class I binding peptides is likely to modify or destroy peptide antigenicity. Depending on the orientation of the glycosylated Asn in the class I binding groove, addition of the oligosaccharide (which is bulkier than the typical class I binding peptide) would be expected to prevent either class I binding or T cell recognition. Indeed, the addition of N-linked oligosaccharides to MHC class II–associated peptides has been found to abolish presentation to T_{CD4+} (17–19). More directly, addition of O- or N-linked oligosaccharides to MHC class I–associated peptides either greatly reduces binding to class I molecules or interferes with recognition by T_{CD8+}, depending on the orientation of the modified residue (20). N-linked oligosaccharides can be removed by peptide *N* glycanase (PNGase); this results in the conversion of the oligosaccharide modified residue from Asn to Asp. Skipper et al. found just such a conversion in a peptide derived from an ER-targeted protein (21). Although the TAP dependence of peptide presentation was not reported in this study, it is thought that PNGase is located exclusively in the cytosol/nucleus (22), which, if true, would mean that peptides were derived from glycosylated protein returned to the cytosol. In the present study, we provide evidence that this ER to cytosol pathway does indeed contribute to the generation of antigenic peptides from proteins cotranslationally targeted to the secretory pathway.

Materials and Methods

Recombinant Vaccinia Viruses. Recombinant vaccinia viruses (rVVs) expressing K^d, TAP (1 and 2), and influenza virus nucleoprotein (NP) have been described (23, 24). A rVV expressing ICP47 (25) was provided by B. Rouse (University of Tennessee, Knoxville, TN). The full-length NP gene modified by standard molecular genetic methods to encode molecules listed in Table 1 was inserted into the thymidine kinase locus of vaccinia viruses (VVs) by homologous recombination as described using the pSC11 plasmid to express foreign proteins under the control of the VV p7.5 early/late promoter (26). NP was directed to the secretory pathway using the signal sequence from β interferon (27). The NP-coding sequences of all of the rVVs were verified by sequencing PCR-amplified copies of full-length NP genes isolated from the rVV.

Mice and Cytotoxicity Assays. 6–8-wk-old BALB/cByJ ($H\text{-}2^d$) and CBA/J ($H\text{-}2^k$) mice were obtained from Jackson Labs (Bar Harbor, ME). Mice were immunized with PR8 by intraperitoneal injection, and with rVV by intravenous injection. Target cells were infected with viruses as described previously (28). To generate T_{CD8+}, splenocytes derived from animals immunized with viruses 2–6 wk previously were stimulated in vitro for 7 d with either antigenic peptides at 1 μg/ml or virus-infected autologous splenocytes as described (29). In some cases, T_{CD8+} lines were used in place of secondary cultures. Lines were propagated by weekly stimulation with L929-K^d cells that had been incubated for 3 h at 37°C with IMDM supplemented with 20 μg/ml human β_2-microglobulin (Sigma Chemical Co., St. Louis, MO) and 1.2 μg/ml synthetic peptide, fixed for 10 min at room temperature with 0.5% paraformaldehyde in PBS, and washed by three consecutive 30-min incubations with IMDM at 37°C. 1–2 d after stimulation with cells, fresh IMDM supplemented with 10% (vol/vol) supernatant from rat splenocytes stimulated with Con A was added. This stimulation protocol was repeated weekly. Microcytotoxicity assays were performed as previously described (28). Where indicated, brefeldin A (BFA; Sigma Chemical Co.) was added to cells at a concentration of 5 μg/ml before assay and at a concentration of 2.5 μg/ml during the cytotoxicity assay to block further antigen presentation (29). Data are expressed as percent-specific release defined as:

$$\text{Experimental release} - \text{medium release} / \text{total release} - \text{medium release} \times 100.$$

Synthetic Peptides. Synthetic peptides were provided by the Biological Resources Branch (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Glycopeptides were produced as described (18). All peptides were purified by HPLC and analyzed by mass spectroscopy to confirm purity and mass.

Biochemical Procedures. 2×10^7 L929 cells infected for 1 h with VV were incubated in 2 ml Met-free DMEM for 30 min at 37°C. After adding 200 μCi [^{35}S]methionine in 200 μl Met-free DMEM, cells were incubated for an additional 10 min at 37°C. After washing with PBS, cells were incubated for 20 min at 0°C with 0.14 M NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 10 mM Tris HCl, pH 7.4, and the nuclei were pelleted by centrifugation at 15,000 *g*. Supernatants were incubated with protein A–Sepharose preloaded with the anti-NP mAb H16-L10. After extensive washing, beads were boiled in sample buffer according to Laemmli (30), or in 0.1 M sodium acetate, 3 mM EDTA, and 0.25% SDS, pH 6. In the latter case, samples were split in half, incubated overnight at 37°C with or without 2 mU endo-β-*N*-acetylglucosaminidase (endo H). All samples were analyzed by SDS-PAGE according to Laemmli. After fixing, gels were dried under vacuum and exposed to Phosphorimager screens (Molecular Dynamics, Sunnyvale, CA), which were imaged using the associated hardware and software. For quantitation of viral gene expression in cells used for the microcytotoxicity assay, an aliquot of 10^6 cells was removed, incubated in methionine-deficient medium for 15 min, and labeled with [^{35}S]-methionine for 45 s. Labeling was terminated by the addition of Laemmli sample buffer heated to 100°C. Samples were analyzed by SDS-PAGE, and the amount of protein in fixed gels determined using a Phosphorimager (Molecular Dynamics). Images of gels were arranged and labeled with Adobe Photoshop (Adobe Systems, Mountain View, CA) and printed with a Fujix 3000 pictography digital printer (Fuji Photo Film USA, Elmsford, NY).

Results

Experimental Strategy and Characterization of Antigenic Peptides. As a model antigen we used influenza virus NP synthesized by rVV. NP is synthesized on free ribosomes in the cytosol and efficiently translocated to the nucleus due to a nuclear localization sequence. In $H\text{-}2^d$ mice, the T_{CD8+} response to NP focuses on an immunodominant K^d-restricted peptide located at residues 147–155 (TYQRTRALV). Conversion of the Gln_{149} to Asn creates a N-linked glycosyla-

Table I. *rVVs Used in This Study*

Designation	Foreign gene product
VV-NP	Full-length NP
VV-NP^{N}	Full-length NP, $Gln_{149} \rightarrow Asn_{149}$
VV-NP^{NRA}	Full-length NP, $Gln_{149} \rightarrow Asn_{149}$, $Thr_{151} \rightarrow Ala_{151}$
VV-SNP	β-IFN leader, full-length NP
VV-SNP^{N}	β-IFN leader, full-length NP, $Gln_{149} \rightarrow Asn_{149}$
VV-SNP^{NRA}	β-IFN leader, full-length NP, $Gln_{149} \rightarrow Asn_{149}$, $Thr_{151} \rightarrow Ala_{151}$

tion site. To study the effects of glycosylation on antigen processing, we produced rVVs expressing NP with Gln_{149} or Asn_{149} directed to the ER by appendage of a NH_2-terminal ER insertion sequence (termed secreted NP [SNP]; Table 1). We controlled for effects of the $Gln_{149} \rightarrow$Asn substitution on peptide liberation, class I binding, and TCR recognition by using rVVs expressing the same proteins in the cytosol. To examine whether any alterations in presentation of SNP^{N} are related to glycosylation, we produced rVVs expressing cytosolic or ER-targeted NPs in which the glycosylation site created by the $Gln_{149} \rightarrow$Asn substitution is destroyed by converting Thr_{151} to Ala (Table 1).

Obviously, for this to be a useful strategy, the amino acid substitution alone cannot adversely affect the binding of the peptides to K^{d}. This was examined in two ways. First, RMA/S K^{d} cells incubated at 26°C to accumulate cell-surface peptide–receptive molecules were exposed to decreasing amounts of peptides for an hour at 26°C, and then for 2 h at 37°C to denature class I molecules lacking peptides. BFA was included with peptides to prevent the transport of additional K^{d} molecules to the cell surface. Conformed class I molecules were detected using the mAb SF1.1.1 conjugated to FITC. The peptide concentration required to preserve half of the cell-surface K^{d} molecules provides an approximation of peptide affinity for K^{d}. This revealed that the Asn_{149}- and $Asn_{149}Ala_{151}$-containing peptides have K_as approximately 2.5–3-fold higher than the wild-type peptide ($K_a = 1/$[peptide concentration], yielding values for variant and wild-type peptides, respectively, of $1–1.2 \times 10^{7}$ M^{-1} versus 4.2×10^{6} M^{-1}) (Fig. 1). By contrast, a peptide containing Asp_{149} (which would result from PNGase-mediated removal of the N-linked oligosaccharide from Asn_{149}) was bound with an estimated K_a 230-fold lower than the wild-type peptide (1.8×10^{4} M^{-1}).

The relative affinities of the peptides were confirmed by examining the thermal stability of cell-surface K^{d} molecules loaded with a saturating concentration of peptide at 26°C and incubated at 37°C for up to 8 h in BFA in the absence of peptides. This provides a measure of the off-rate for peptide binding, which is the major factor in peptide affinity for class I molecules. K^{d} molecules were most efficiently stabilized by the N_{149}-containing peptides, and were only marginally stabilized by the Asp_{149}-containing peptide.

In the experiments that follow, we used T_{CD8+} populations or lines raised to the wild-type sequence as a measure of presentation of the 147–155 determinant from the different cytosolic or ER-targeted NPs. The effects of the amino acid substitutions on peptide antigenicity varied somewhat with the T_{CD8+} population or line used. In two of the experiments described below, the Asn_{149}- and $Asn_{149}Ala_{151}$-containing peptides sensitized target cells for lysis by NP-specific T_{CD8+} with similar or slightly greater efficiencies than the wild-type peptide (see Figs. 3 and 5). The Asp_{149}-containing peptide routinely required 100–1,000 times the concentration of Gln_{149}- or Asn_{149}-containing peptides to achieve a similar level of lysis (not shown).

To confirm that attachment of an N-linked oligosaccharide to Asn_{149} abolishes peptide antigenicity, we coupled Asn_{149} in the TYNRTRALV synthetic peptide to the disaccharide maltobiose. This resulted in at least a 5 log_{10}-fold reduction in the capacity of the peptide to sensitize cells for lysis (not shown). Oligosaccharides added in the ER are

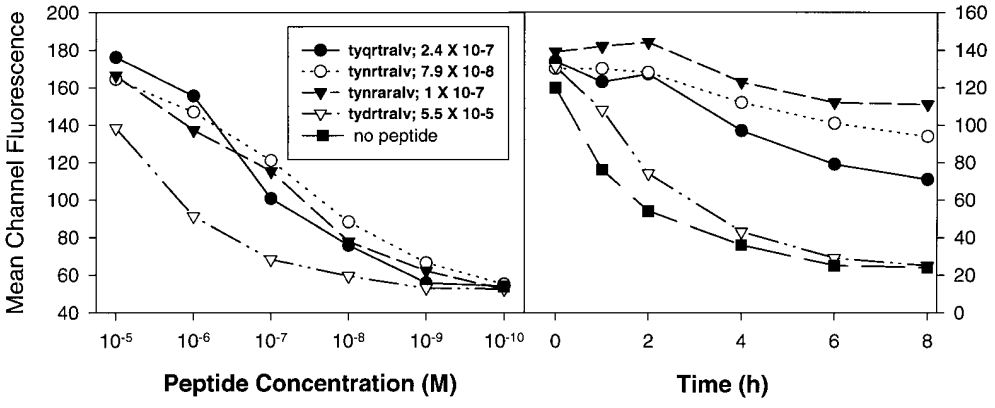

Figure 1. Peptide binding to K^{d}. RMA/S K^{d} cells incubated overnight at 26°C were incubated for an additional hour with the indicated concentration of peptide and BFA (5 μg/ml), and then at 37°C for 2 h (*left*). Alternatively, cells were incubated with 10 μM peptide and BFA, extensively washed, and then incubated at 37°C for the indicated time in the absence of peptide (*right*). The amount of cell-surface K^{d} was determined using the fluorescein-conjugated mAb SF1.1.1 (PharMingen, San Diego, CA). The concentration of peptide required for protection of one half of the K^{d} molecules was determined by linear extrapolation of the mean channel fluorescence values.

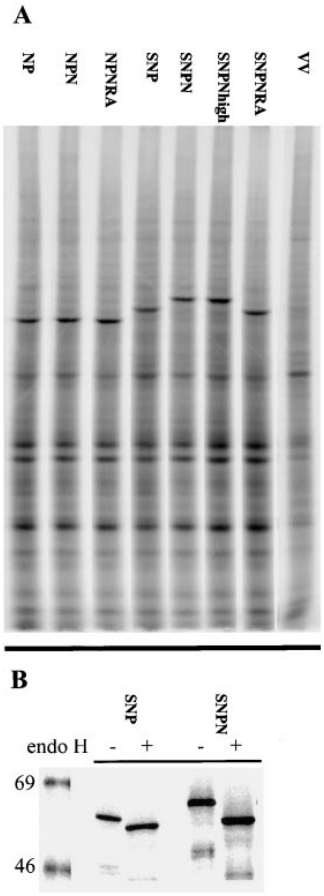

Figure 2. Characterization of SNP. (*A*) Aliquots of the target cells used in Fig. 3 *A* were removed 90 min after infection and radiolabeled with [^{35}S]methionine. Total cell extracts were analyzed by SDS-PAGE. Radioactivity in the fixed and dried gels was located using a Phosphorimager that was used to quantitate the radioactivity in the NP and SNP band, which are clearly evident. The total amounts of counts per lane were normalized to allow for differences in sample preparation, and the corrected amount of radioactivity present in the same region of an extract prepared from cells infected with a control VV was subtracted from each value. The adjusted amounts of various forms of NP synthesized relative to NP (= 1.00) are: NP^{N}, 1.65; NP^{NRA}, 2.16; SNP, 1.24; SNP^{N}, 1.72; SNP^{N} high, 2.23; and SNP^{NRA}, 1.82. (*B*) NP present in detergent lysates from cells infected with rVV expressing various forms of NP was collected using protein A agarose preloaded with the NP-specific mAb H16-L10, digested with endo H and analyzed by SDS-PAGE.

much larger than this disaccharide, consisting of between 10 and 14 saccharide subunits, depending on the degree of modification by ER glucosidases and mannosidases (31), and would be expected to interfere to an even greater extent than maltobiose with peptide binding to class I molecules or interaction with the TCR. These findings demonstrate that (*a*) we can meaningfully compare presentation of variant and wild-type peptides using T_{CD8+} effectors raised to the wild-type sequence, and, (*b*) glycosylation will destroy peptide antigenicity due either to the presence of the N-linked oligosaccharide, or after its removal by PNGase, the poor antigenicity of the Asp_{149} containing peptide.

Characterization of SNP. The function of the ER-targeting sequence was confirmed by SDS-PAGE analysis of total cell extracts of rVV-infected cells metabolically labeled with [^{35}S]methionine. As seen in Fig. 2 *A*, the secreted forms of NP migrated more slowly than the cytosolic/nuclear forms. SNP and SNP^{NRA} comigrated, whereas SNP^{N} migrated more slowly, which is consistent with the existence of one site for N-linked glycosylation in SNP and SNP^{NRA} (NP has a natural glycosylation site at position 21) and two sites in SNP^{N}. This was confirmed by digestion of immunocollected SNP and SNP^{N} with endo H, which eradicated the difference in mobility between the ER-targeted and nontargeted forms of NP (Fig. 2 *B*). It is important to note that all three forms of SNP were efficiently delivered to the ER and glycosylated at all potential sites, since we did not detect, by either immunoprecipitation or by Western blotting (not shown), full-length forms that were nonglycosylated or partially glycosylated. Upon prolonged chasing at 37°C, all three forms of SNP became endo H–resistant and were secreted into the medium with a $t_{1/2}$ of ~3 h (not shown).

These biochemical findings were confirmed using anti-NP mAbs in indirect immunofluorescence of fixed and permeabilized rVV-infected cells (not shown). Non-ER–targeted forms were largely localized to the nucleus as expected, whereas secreted forms were located in the ER and Golgi complex. Upon prolonged infection (16 h), weak NP-specific staining was detected in the nuclei of cells expressing SNP, SNP^{N}, or SNP^{NRA} ("SNPs").

Together, these findings demonstrate that (*a*) SNPs are efficiently targeted to the ER, (*b*) all potential glycosylation sites are used in SNPs to within the limits of detection, and (*c*) a minor portion of SNPs make their way to the nucleus.

Effect of Gln_{149}→Asn Substitution on NP/SNP Antigenicity and Immunogenicity. The efficiency of generating the $NP_{147-155}$ peptide from the six different forms of NP was determined using a NP-specific T_{CD8+} line that recognizes the wild type, Asn_{149}- and $Asn_{149}Ala_{151}$-containing peptides with similar efficiency (Fig. 3, inset). The efficiency of peptide generation was estimated by adding BFA to L-K^{d} cells at increasing intervals after infection with rVVs (Fig. 3; 32). As with other proteins, targeting NP to the ER reduced the efficiency of peptide generation. Modification of amino acids 149 or 151 had little effect on presentation of cytosolic NP. The same alterations, however, had clear effects on presentation of SNP. The Gln_{149}→Asn substitution significantly reduced presentation of SNP. The similar levels of presentation of SNP^{NRA} and SNP indicates that glycosylation of Asn_{149} is responsible for decreased presentation of SNP^{N} relative to SNP.

To eliminate the possibility that the differences in antigen presentation reflected differences in levels of expression of the various antigens (and not real differences in antigen processing), we quantitated the amounts of NP produced by the target cells used in this experiment. Cells were radiolabeled with [^{35}S]methionine for 45 s and immediately immersed in boiling SDS-PAGE sample buffer. This method allows recovery of virtually all of the biosynthesized proteins, and not just those extracted into relatively mild detergents that are capable of binding antibody (the technique most commonly used to quantitate antigen expression). SDS-PAGE of extracts revealed that newly synthesized NP could be easily detected due to the strength of the VV p7.5 promoter combined with the ability of VV to shut down host cell protein synthesis. As seen in Fig. 2 *A*, the difference in presentation between the secreted forms of NP cannot be attributed to differences in levels of expression. Indeed, increasing the amount of SNP^{N} synthesized by increasing the virus dose by threefold (SNP$^{N}_{High}$) did not increase presentation to T_{CD8+} (Fig. 3). NP-specific T_{CD8+} from H-2^{k} mice recognize residues 50–57 in association with K^{k} (33). Unlike the K^{d}-restricted determinant, the presentation of NP_{50-57} was not adversely affected by the Gln_{149}→Asn sub-

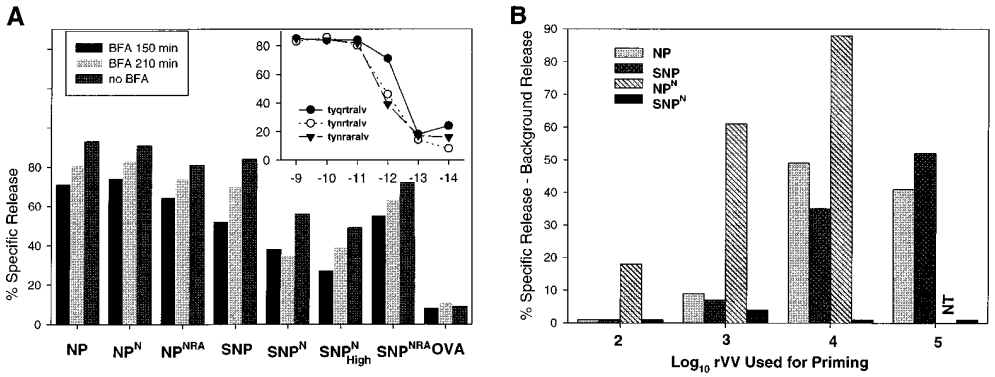

Figure 3. Antigenicity and immunogenicity of rVVs expressing various forms of NP. (*A*) L-K^d cells were infected for the indicated times with the rVVs indicated before BFA was added and then maintained continuously. After ^{51}Cr labeling, cells were incubated for 6 h at an E/T of 5:1 with a T_{CD8+} line produced by PR8 in vitro secondary restimulation of splenocytes derived from mice inoculated with VV-NP. The recognition of synthetic peptides incubated with cells at the indicated concentration is shown in the inset. (*B*) Splenocytes from BALB/c mice immunized with the indicated rVV 3 wk previously were restimulated in vitro for 7 d with the homologous synthetic peptide (TYQRTRALV or TYNRTRALV), and tested for their ability to lyse P815 cells sensitized with the same peptide. Cultures were used at the same dilution. All values have been corrected for background lysis values obtained with cells in the absence of added synthetic peptides.

stitution in SNP (Fig. 4). In fact, VV-SNP^N–infected cells were recognized at slightly higher levels by K^k-restricted T_{CD8+} than cells expressing SNP, whereas the very same target cells gave the reverse hierarchy of lysis using K^d-restricted T_{CD8+}. This provides functional evidence that the diminished recognition of VV-SNP^N–infected cells relative to VV-SNP–infected cells is not related to diminished levels of SNP^N expression. Moreover, this experiment indicates that the inhibitory effect of glycosylation on SNP antigen processing is limited to the determinant containing the glycosylation site.

We next compared the presentation of the various rVVs to primary T_{CD8+} in vivo by determining the amount of virus required to prime for secondary in vitro responses to synthetic peptides (Fig. 3 *B*). Targeting NP to the secretory pathway did not significantly alter its immunogenicity. Remarkably, introducing the Gln_{149}→Asn substitution into SNP decreased the immunogenicity of the rVV 100-fold relative to the other viruses (in additional experiments we detected priming after inoculation with 10^6 plaque-forming units/mouse [not shown]). This effect was specific for the secreted form, since the same substitution actually increased the immunogenicity of cytosolic NP 10-fold. The low immunogenicity of VV-SNP^N cannot be attributed to poor infectivity in vivo since it primed for VV-specific responses with similar efficiency as the other rVVs (not shown).

These findings demonstrate that the presence of an N-linked glycosylation site in a secreted protein specifically affects its antigenicity and immunogenicity, presumably due to the attachment of an N-linked oligosaccharide in the ER. Creation of the same site in a cytosolic version of the protein has no significant effect on antigenicity.

TAP Dependence of Antigen Presentation. The involvement of TAP in the presentation of NP, NP^N, SNP, and SNP^N was examined using T2-K^d cells, a TAP-deficient human lymphoblastoid cell line expressing K^d from a transfected gene (34; Fig. 5 *A*). None of the corresponding rVVs were able to sensitize T2-K^d cells for lysis by NP-specific T_{CD8+}. The precise hierarchy of presentation of the various gene products observed in L-K^d cells was faithfully restored, however, by coinfection with VV-TAP (1 and 2), which encodes both human TAP subunits (35). Lysis of SNP^N-expressing cells was at levels less than half that observed with SNP. This cannot be attributed to less efficient recognition of the Asn_{149}-containing peptide by the T_{CD8+} line used since NP^N was presented slightly more efficiently than NP and the synthetic peptides were recognized with similar efficiency. Nor can it be attributed to decreased expression of SNP^N relative to SNP. As in the previous experiment, cells were briefly radiolabeled and the expression of NPs quantitated after SDS-PAGE of whole cell lysates. This demonstrated that SNP^N was expressed in similar

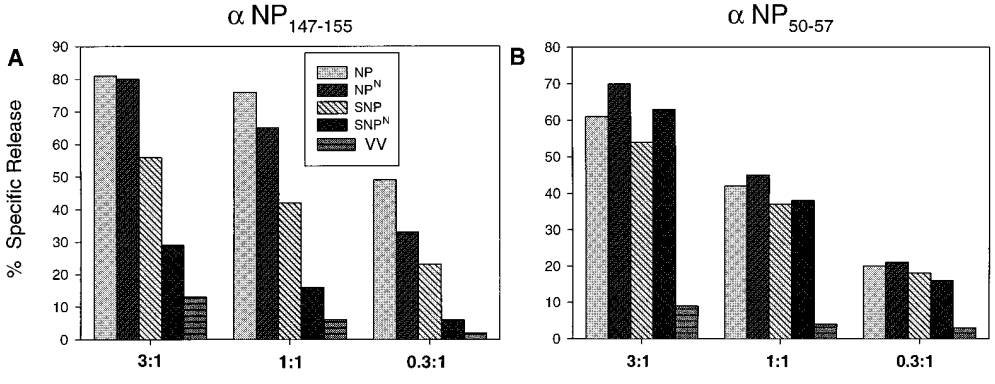

Figure 4. Comparison of presentation of $NP_{147-155}$ to NP_{50-57}. L-K^d cells infected with the rVV indicated were tested for lysis by T_{CD8+} lines specific for $NP_{147-155}$ or NP_{50-57} at the indicated E/T.

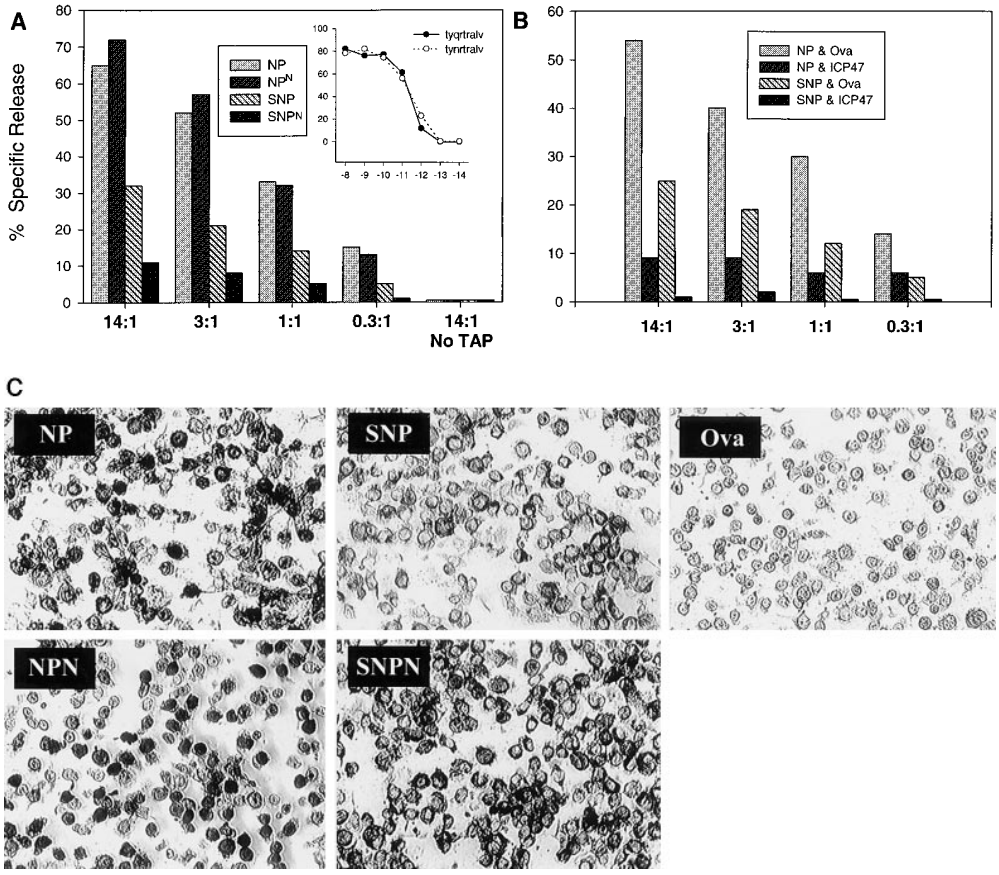

Figure 5. TAP dependence of antigen presentation. (*A*) T2 K^d cells coinfected with VV-TAP (1 and 2) and the rVVs indicated, or just with the rVV indicated ("No TAP") were tested for lysis by a NP-specific T_{CD8+} line at the E/T ratios indicated. The recognition of synthetic peptides incubated with cells at the indicated concentration by the same T_{CD8+} line is shown in the inset. The amount of antigen expressed by the target cells 4 h after infection was determined by quantitating bands detected in SDS-PAGE of whole cell extracts as described in Fig. 2. The adjusted amounts of various forms of NP synthesized relative to NP (= 1.00) are: NP^N, 1.49; SNP, 1.01; and SNP^N, 1.01. (*B*) Effect of ICP47 on antigen presentation. T2 K^d cells infected with the rVVs indicated were tested for lysis by the T_{CD8+} line used in *A* at the indicated E/T ratio. (*C*) The target cells used in *A* were incubated in flat-bottom 96-well plates coated with Con A to adhere the cells. At the conclusion of the ^{51}Cr release assay, cells were fixed by 2-min incubation with acetone/methanol (1:1) and indirectly immunoperoxidase stained using the H16-L10 mAb. Video images of the stained cells were printed using a printer (UP5500; Sony, San Jose, CA), digitized by a flat bed scanner, assembled using Adobe Photoshop software, and, printed with a digital printer (3000; Fuji Photo Film USA).

amounts to SNP (Fig. 5 legend). To eliminate the possibility that a minor population of cells synthesized most of the antigen, NP expression was examined by immunoperoxidase staining of fixed and permeabilized target cells. As seen in Fig. 5, a similar percentage of cells expressed the various forms of NP (SNP^N was actually detected in a greater percentage of cells than SNP).

These findings demonstrate that the TAP-dependent presentation of the 147-155 peptide derived from SNP is blocked by glycosylation. TAP has recently been shown to directly bind to class I molecules (3, 4), and its potential as a molecular chaperone in class I folding has been suggested (36). Thus, it is possible that the TAP dependence of presentation of the $NP_{147-155}$ determinant reflects a role for TAP in facilitating the association of K^d with the free peptide in the ER lumen, and not the transport of 147-155 peptide from the cytosol. To examine this possibility, we used an rVV expressing the herpes simplex virus protein ICP47 to specifically inhibit the transport function of TAP (23, 37–40). Coexpression of TAP with ICP47 blocked the TAP-mediated rescue of both NP and SNP (Fig. 5 *B*).

These findings demonstrate that the 147-155 determinant produced from SNP is delivered to class I molecules by TAP from the cytosol, and that this presentation is partially blocked by glycosylation.

Discussion

Based on its dependence on TAP-mediated peptide transport, we conclude that antigenic peptides from SNP are produced in the cytosol. Substituting Asn for Gln_{149} in SNP specifically decreases the immunogenicity and antigenicity of the 147-155 determinant. This cannot be attributed to negative effects of the substitution on peptide affinity for K^d or TCR recognition. Nor can it be attributed to diminished TAP-mediated transport or liberation by cellular proteases of the Asn_{149}-containing peptide, since NP^N is presented with similar or even greater efficiency than wild-type NP. Rather, these findings implicate glycosylation as the cause of reduced antigenicity, which is confirmed by the enhanced presentation observed when the glycosylation site is destroyed by substituting Ala for Thr_{151}. Since the Asn_{149}-containing peptide in cytosolic NP is presented normally, the effect of glycosylation on SNP^N must occur before TAP-mediated transport. This indicates that most antigenic peptides from SNP are derived from protein that is delivered to the ER and then routed back to the cytosol where it is processed by cytosolic proteases into peptides that are delivered to K^d molecules via TAP.

A synthetic version of the Asn_{149}-substituted 147-155 peptide is glycosylated in a TAP-dependent manner when added to cells permeabilized by streptolysin O (our unpub-

lished results). Thus, we interpret the efficient presentation NP^N to indicate that the Asn_{149}-containing peptide associates with K^d before the peptide can be glycosylated. This is consistent with the idea that TAP delivers peptides directly to the class I molecules they bind (3, 4). The practical importance of this is that the antigenicity and immunogenicity of peptides in cytosolic/nuclear proteins are probably not affected by the presence of a consensus site for N-linked glycosylation.

By contrast, the antigenicity, and particularly the immunogenicity of SNP, were greatly affected by the presence of a glycosylation site, indicating that glycosylation can have a major negative effect on the antigenicity of peptides in ER-targeted proteins. The disproportionate effect of the $Gln_{149} \Rightarrow Asn$ substitution on SNP immunogenicity relative to its effect on antigenicity indicates that presentation of antigen to primary T cells in vivo is fundamentally different from in vitro presentation. This may reflect differences in antigen processing in the APCs used in vivo versus the various tumor cells we have used. An intriguing possibility that we favor is that in vivo presentation of SNP relies largely on processing of exogenous antigen scavenged from infected cells by professional APCs (41). Exogenous antigens would consist of the metabolically stable proteins produced by cells, and in this case, the very large difference between presentation of SNP and SNP^N would be expected based on the high efficiency of glycosylation of Asn_{149}.

The present results do not distinguish in which secretory organelle egress of SNP to the cytosol occurs (anywhere from ER to lysosomes is possible). Cells are able to present exogenous antigens in a TAP-dependent manner, due to release of pinocytosed material to the cytosol. This pathway is largely limited to phagocytosing cells, however. Moreover, in additional experiments (not shown), we found that presentation of SNP could not be attributed to uptake of secreted material, since coincubation of VV-SNP–infected cells with ^{51}Cr-labeled uninfected cells during the course of a 6-h cytotoxicity assay did not result in the lysis of uninfected cells (not shown). It was recently shown that two cytomegalovirus proteins, US2 and US11, have the remarkable ability to transport newly synthesized class I molecules from the ER to the cytosol, probably by inducing reverse translocation through the translocon (10, 11). The authors proposed that cytomegalovirus takes advantage of a constitutive operating cellular pathway that underlies the phenomenon of ER-associated degradation of defective or unassembled proteins. We propose that SNP is reimported from the ER to the cytosol via the same pathway.

The negative effect of glycosylation on peptide antigenicity could have several causes. First, if the oligosaccharide is not removed, peptide antigenicity would be eradicated by interference with TAP-mediated transport, K^d binding, or TCR recognition. Second, removal of the oligosaccharide by cytosolic PNGase would create an Asp_{149}-substituted peptide with low affinity for K^d. In additional experiments, we found that this peptide sensitizes cells 1,000-fold less efficiently than the wild-type peptide for lysis mediated by T_{CD8+} raised to NP, and that these T_{CD8+} do not recognize cells expressing rVV encoded cytosolic or ER-targeted Asp_{149}-containing peptides (our unpublished results). Thus, removal of the glycan would destroy peptide antigenicity as surely as its nonremoval.

The inhibitory effect of glycosylation on in vitro or in vivo presentation of SNP was incomplete. Based on the argument presented in the preceding paragraph, the residual presentation of SNP^N is almost certainly due to incomplete glycosylation of Asn_{149}. Although we cannot eliminate the possibility that a small amount of SNP^N escapes glycosylation at Asn_{149}, the degree of residual presentation of SNP^N relative to SNP seems to be inconsistent with our failure to detect SNP^N lacking one or both N-linked oligosaccharides by SDS-PAGE analysis of mAb-collected material. Instead, we believe that the residual presentation of SNP^N reflects the generation of peptides from defective ribosomal products (DRiPs; 42), misfolded, possibly truncated forms of SNP^N that are not inserted into the ER and are processed by the standard cytosolic route.

In conclusion, the present findings expand the possible mechanisms underlying the presentation of secreted and membrane-bound proteins. It will be important to test in future experiments whether the results we describe are limited to cytosolic proteins artificially routed to the secretory pathway, or also apply to naturally exported proteins that have evolved to be resistant to secretory proteases. The major mode of peptide generation from any given exported protein must be determined on a case by case basis until general principles have been more firmly established.

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