Introduction of a Glycosylation Site into a Secreted Protein Provides Evidence for an Alternative Antigen Processing Pathway: Transport of Precursors of Major Histocompatibility 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\textsuperscript{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\textsuperscript{T} cells (T\textsubscript{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)\textsuperscript{1}, the MHC-encoded peptide transporter (2). Newly synthesized \textalpha;-\textbeta;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\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2} is added to Asn residues present in the sequence AsnXSer/Thr (X, any residue except Pro). Under normal circumstances, glycosy-

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

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Glycosylation of cell 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<sub>CD4</sub> (17–19). Moreover, 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<sub>CD8</sub>, 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-translocon (PNGase); this results in the conversion of the oligosaccharide-modified residue from Asn to Asp. 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 (rVV's) expressing K<sup>d</sup>, TAP (1 and 2), and influenza virus nucleoprotein (NP) have been described (23, 24). A rVV expressing IC P47 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 and TAP (1 and 2), and influenza virus nucleoprotein (NP) have been described (23, 24). A rVV expressing IC P47 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. NP is synthesized on free ribosomes in the 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.

Results

Experimental Strategy and Characterization of Antigenic Peptides. As a model antigen we used influenza virus NP synthetized 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-2<sup>d</sup> mice, the T<sub>CD8</sub>-response to NP focuses on an immunodominant K<sup>d</sup>-restricted peptide located at residues 147–155 (TY Q T R A L V). Conversion of the Glu<sub>149</sub> to Asn creates a N-linked glycosyla-
Table I. rVVs Used in This Study

| Designation | Foreign gene product |
|-------------|---------------------|
| VV-N P      | Full-length NP      |
| VV-N P<sup>N</sup> | Full-length NP, Gln<sub>149</sub>→Asn<sub>149</sub> |
| VV-N P<sup>RA</sup> | Full-length NP, Gln<sub>149</sub>→Asn<sub>149</sub>, Thr<sub>151</sub>→Ala<sub>151</sub> |
| VV-SN P     | β1-IFN leader, full-length NP |
| VV-SN P<sup>N</sup> | β1-IFN leader, full-length NP, Gln<sub>149</sub>→Asn<sub>149</sub> |
| VV-SN P<sup>RA</sup> | β1-IFN leader, full-length NP, Gln<sub>149</sub>→Asn<sub>149</sub>, Thr<sub>151</sub>→Ala<sub>151</sub> |

Glycosylation site. To study the effects of glycosylation on antigen processing, we produced rVVs expressing NP with Gln<sub>149</sub> or Asn<sub>149</sub> directed to the ER by appendage of a NH<sub>2</sub>-terminal ER insertion sequence (termed secreted NP [SNP]; Table 1). We controlled for effects of the Gln<sub>149</sub>→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-containing peptides are related to glycosylation, we produced rVVs expressing cytosolic or ER-targeted NPs in which the glycosylation site created by the Gln<sub>149</sub>→Asn substitution is destroyed by converting Thr<sub>151</sub> 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<sup>d</sup>. This was examined in two ways. First, RMA/S K<sup>d</sup> 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<sup>d</sup> 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<sup>d</sup> molecules provides an approximation of peptide affinity for K<sup>d</sup>. This revealed that the Asn<sub>149</sub>- and Asn<sub>149</sub>Ala<sub>151</sub>-containing peptides have K<sub>s</sub>s approximately 2.5-3 fold higher than the wild-type peptide (K<sub>s</sub> = 1/ [peptide concentration], yielding values for variant and wild-type peptides, respectively, of 1-1.2 × 10<sup>7</sup> M<sup>-1</sup> versus 4.2 × 10<sup>6</sup> M<sup>-1</sup> (Fig. 1). By contrast, a peptide containing Asp<sub>149</sub> (which would result from PNGase-mediated removal of the N-linked oligosaccharide from Asn<sub>149</sub>) was bound with an estimated K<sub>s</sub> 230-fold lower than the wild-type peptide (1.8 × 10<sup>4</sup> M<sup>-1</sup>).

The relative affinities of the peptides were confirmed by examining the thermal stability of cell-surface K<sup>d</sup> molecules loaded with saturating concentrations 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<sup>d</sup> molecules were most efficiently stabilized by the N<sub>149</sub>-containing peptides, and were only marginally stabilized by the Asp<sub>149</sub>-containing peptide.

In the experiments that follow, we used T<sub>CD8</sub> 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<sub>CD8</sub> population or line used. In two of the experiments described below, the Asn<sub>149</sub>- and Asn<sub>149</sub>Ala<sub>151</sub>-containing peptides sensitized target cells for lysis by NP-specific T<sub>CD8</sub> with similar or slightly greater efficiencies than the wild-type peptide (see Figs. 3 and 5). The Asp<sub>149</sub>-containing peptide routinely required 100–,000 times the concentration of Gln<sub>149</sub>- or Asn<sub>149</sub>-containing peptides to achieve a similar level of lysis (not shown).

To confirm that attachment of an N-linked oligosaccharide to Asn<sub>149</sub> abolishes peptide antigenicity, we coupled Asn<sub>149</sub> in the TYN RTRALV synthetic peptide to the disaccharide maltobiose. This resulted in at least a 5 log<sub>10</sub>-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<sup>d</sup>. RMA/S K<sup>d</sup> 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<sup>d</sup> 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<sup>d</sup> 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. 3A were removed 90 min after infection and radiolabeled with [35S]methionine. Total cell extracts were analyzed by SDS-PAGE. Radioactivity in the fixed and dried gels was located using a Phosphorimaget 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 N, 2.16; SNP, 1.24; SNP N, 1.72; SNP N high, 2.23; and SNP N, 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 TCD8+ 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 A Sp149 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 [35S]methionine. As seen in Fig. 2A, the secreted forms of NP migrated more slowly than the cytosolic/nuclear forms. SNP and SNPNRA 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 non-targeted forms of NP (Fig. 2B). 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 chase at 37°C, all three forms of SNP became endl-H-resistant and were secreted into the medium with a t1/2 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). N on-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, SNPN, or SNPNRA ("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 Gln149→Asn Substitution on NP/SNP Antigenicity and Immunogenicity. The efficiency of generating the NP147-155 peptide from the six different forms of NP was determined using a NP-specific TCD8+ line that recognizes the wild type, Asn149- and Asn149Ala151-containing peptides with similar efficiency (Fig. 3, inset). The efficiency of peptide generation was estimated by adding BFA to L-K+ cells at increasing intervals after infection with rVV (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 Gln149→Asn substitution significantly reduced presentation of SNP. The similar levels of presentation of SNP N and SNP N indicates that glycosylation of Asp149 is responsible for decreased presentation of SNP relative to SNP.
stitution in SNP (Fig. 4). In fact, VV-SNP N–infected cells were recognized at slightly higher levels by K k-restricted TCD8 1 than cells expressing SNP, whereas the very same target cells gave the reverse hierarchy of lysis using K d-restricted TCD8 1. 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 SNPN 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 rVV s to primary TCD8 1 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 Gln149 fi 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 rVV s (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, N P N, SNP, and SN P 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 rVV s were able to sensitize T2-K d cells for lysis by NP-specific TCD8 1. 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 SNPN-expressing cells was at levels less than half that observed with SNP. This cannot be attributed to less efficient recognition of the Asn149-containing peptide by the TCD8 1 used since N P 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 SNPN 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 SNPN was expressed in similar

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 3H labeling, cells were incubated for 6 h at an E/T of 5:1 with a TCD8 1 line produced by PR8 in vitro secondary restimulation of splenocytes derived from mice inoculated with VV-N P. 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.

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 TCD8 1 lines specific for NP 147-155 or NP 50-57 at the indicated E/T.
Endoplasmic Reticulum to Cytosol Antigen-processing Pathway

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 (SNPN 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 NP147-155 determinant reflects a role for TAP in facilitating the association of Kd 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 Gln149 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 Kd or TCR recognition. Nor can it be attributed to diminished TAP-mediated transport or liberation by cellular proteases of the Asn149-containing peptide, since NPN 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 Thr151. Since the Asn149-containing peptide in cytosolic NP is presented normally, the effect of glycosylation on SNPN 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 Kd molecules via TAP.

A synthetic version of the Asn149-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 of NPN to indicate that the Asn149-containing peptide associates with Kd 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 Glu149->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 SNPN would be expected based on the high efficiency of glycosylation of Asn149.

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 coinoculation of VV-SNP-infected cells with 51Cr-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, Kd binding, or TCR recognition. Second, removal of the oligosaccharide by cytosolic PNGase would create an Asp149-substituted peptide with low affinity for Kd. 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<sub>CD8</sub> raised to NPN, and that these T<sub>CD8</sub> do not recognize cells expressing rVV encoded cytosolic or ER-targeted Asp149-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 SNPN is almost certainly due to incomplete glycosylation of Asn149. Although we cannot eliminate the possibility that a small amount of SNPN escapes glycosylation at Asn149, the degree of residual presentation of SNPN relative to SNP seems to be inconsistent with our failure to detect SNPN lacking one or both N-linked oligosaccharides by SDS-PAGE analysis of mAb-collected material. Instead, we believe that the residual presentation of SNPN reflects the generation of peptides from defective ribosomal products (DRiPs; 42), misfolded, possibly truncated forms of SNPN 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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