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Efficient and Qualitatively Distinct MHC Class I-Restricted Presentation of Antigen Targeted to the Endoplasmic Reticulum

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For most nascent glycoprotein Ags, the MHC class I-restricted processing pathway begins in the endoplasmic reticulum (ER). From this location, they are translocated to the cytosol for degradation by the proteasome. A reasonable assumption is that processing of exocytic Ags is less efficient than that of cytosolic Ags, due to the requirement for additional handling, but that the processing pathways for the two types of proteins are otherwise similar. To test this, we compared the presentation of three epitopes within influenza nucleoprotein (NP) when this Ag is targeted to the cytosol or the ER. Surprisingly, under conditions of limited Ag expression, presentation of two proteasome-dependent epitopes is comparable when NP is targeted to the ER while presentation of a third is negatively impacted. Furthermore, presentation of the third epitope is unaffected by the addition of proteasome inhibitor when cytosolic NP is expressed but is significantly enhanced when exocytic NP is expressed. These results indicate that delivery of Ag to the ER need not preclude efficient presentation and that processing of cytosolic and ER-targeted Ag is qualitatively distinct.


Two decades ago it was widely accepted that CD8+ T lymphocytes (TCD8+) recognize Ags in their native form associated at the cell surface with MHC class I molecules (1–3). The most feasible activators of TCD8+ responses, therefore, appeared to be membrane-bound glycoproteins (4, 5), such as the influenza hemagglutinin (HA) molecule. This general view remained valid even when it was determined that TCD8+ can be specific for internal proteins (6), such as influenza nucleoprotein (NP) (7) and matrix protein (8), because low levels of these Ags in their native form can be detected at the cell surface of influenza-infected cells (9, 10).

Within the past 15 years, several key findings have led to a dramatic revision of the model. First, it was deduced that fragments of Ag rather than full-length protein are recognized by TCD8+ (11, 12). Soon thereafter it was demonstrated that delivery to the cytosol was a critical step in the presentation of exogenously provided native Ags (13, 14). Subsequently, crucial roles in most cases of peptide presentation were demonstrated for TAP, a heterodimeric member of the ATP transporter family that efficiently transfers short peptides from the cytosol to the lumen of the endoplasmic reticulum (ER) (15) and the proteasome, a macromolecular multicatalytic protease located in the cytosol and nucleus (16).

These advances firmly established a need for Ag processing in the cytosol (for reviews, see Refs. 16–18) and, whereas the original model predicts that exocytic rather than cytosolic proteins are the primary stimulators of TCD8+ responses, the revised model predicts the opposite. Compatible with the latter prediction are the observations that relocation of HA and HIV envelope protein to the cytosol by genetic ablation of their signal sequences can significantly enhance presentation (19, 20). The reverse approach, redirection of influenza NP to the ER, was observed to reduce the presentation of an H-2Kd-restricted epitope (21). The true magnitude of these differences is difficult to gauge given the semiquantitative nature of the standard cytolytic assays that were used and the relatively low levels of epitope that are needed at the cell surface for maximal TCD8+ stimulation in such assays. In the cases of the glycoproteins, it is also difficult to determine whether cytosolic delivery enhances presentation due to relocation or due to a significantly decreased half-life, because degradation rate has been reported by many (19, 20, 22–24), though not all (25), groups to cause enhanced MHC class I-restricted presentation.

A requirement for cytosolic processing was established at a time when it was generally held that protein trafficking across the ER membrane is unidirectional (cytosol to ER) and that a separate, ER-resident degradation system exists to eliminate exocytic proteins that fail quality control (26). In this light, several models were proposed to explain the processing of exocytic proteins, including 1) the misdirection of a minor fraction of nascent protein to the cytosol perhaps through translation on free ribosomes, 2) saturation of the translocation machinery (17, 27), and 3) failure of the ER targeting signal sequence to be synthesized (21, 28). In any of these models, presentation would be relatively inefficient, as only a small fraction of total Ag is accessed by the processing machinery. It is now clear from a number of cases that degradation of exocytic proteins involves retrograde transport through the translocon (termed “retrotranslocation” here and elsewhere (29)).
and delivery to the proteasome (29, 30). In some cases it appears that the proteasome can play an active role in retrotranslocation (31, 32). This also appears to be the primary route for the generation of most glycoprotein-derived epitopes. Central to this conclusion has been the study of epitopes that contain N-linked glycosylation sequences and whose presentation can be monitored by T cell responses that are prevented by glycosylation. In almost all cases studied, T cell activation occurs when Ags containing these epitopes are targeted to the cytosol, but is significantly reduced or lost with targeting of the Ag to the ER, the site of glycosylation (21, 33–35).

Despite the elucidation of this pathway, it still seems reasonable to assume that presentation of exocytic Ags will be less efficient than presentation of their cytosolic counterparts due to the additional handling that is required. It might also be expected that, with the exception of the translocation step, processing of exocytic and cytosolic Ags is qualitatively similar. Considering the wide variety of models that have been proposed for class I-restricted Ag processing and the significance of the targeting issue for vaccine design, we felt it important to test these expectations under conditions that allow for a fair comparison of relative presentation levels. Thus, we have assessed the production of three influenza virus A/PR/8/34 NP-derived epitopes when NP is targeted to the cytosol or to the ER. Using a system for limiting, in controlled fashion, levels of Ag expression, we find that presentation of two proteasome inhibitor-sensitive epitopes (NP50–57 and NP366–374) is not compromised by ER targeting while that of a proteasome inhibitor-insensitive epitope (NP147–155) is significantly reduced. Furthermore, addition of proteasome inhibitor enhances presentation of NP147–155 when NP is targeted to the ER but has no effect when NP is targeted to the cytosol. Thus, ER localization is not necessarily a detriment to efficient presentation, and processing is qualitatively different for Ags targeted to the two compartments.

Materials and Methods

Chemicals

General chemical supplies were obtained from Sigma-Aldrich (St. Louis, MO). Molecular biology reagents were obtained from New England Bio-labs (Beverly, MA). Isotopes were purchased from Amersham (Arlington Heights, IL) and lactacyclin was purchased from the laboratory of Dr. J. E. Corey (Harvard University, Boston, MA). Carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone (Z-LVS) (36) was a generous gift of Dr. H. Ploegh (Harvard University). Monoclonal anti-NP Abs were provided by Dr. W. Gerber (Wistar Institute, Philadelphia, PA). PCR primers were synthesized at the Kimmel Cancer Institute Nucleic Acid Facility (Philadelphia, PA).

Cell lines

MC57G (H-2D b ), L929 (H-2K k ), L-K d (L929 transfected with H-2K d ), and 3T3 cells were maintained in RPMI 1640 with 10% FBS. L-D b (L929 transfected with H-2D b ), and 3T3 cells were maintained in RPMI 1640 with 10% FBS. L-D b (L929 transfected with H-2D b ), and 3T3 cells were maintained in RPMI 1640 with 10% FBS.

Animals

Six- to 8-wk-old female inbred C3H (H-2k), BALB/c (H-2d), and C57BL/6 (H-2b) mice were obtained from Taconic Farms (Albany, NY) or The Jackson Laboratory (Bar Harbor, ME) and maintained in the Thomas Jefferson University Laboratory Animal Facilities (Philadelphia, PA).

Viruses

The recombinant vaccinia viruses (rcvacs) encoding NP and minigenes NP50–57, NP147–155, and NP366–374 have been previously described (28).

Generation of the NP13–49 and ER-NP13–496 genes was by one-step PCR, essentially as described elsewhere (40). Primers for NP13–496 were GTC GAC TCT TAC GAA CAG ATG GAG ACT and ATT AGC TTG CCG CCA GAT CT, allowing substitution of a Sali/Ill fragement. ER-NP13–496 was prepared by attaching the signal sequence from the influenza virus strain A/WSN/33 hemagglutinin to the NP gene in front of Met1. Primers for this one-step reaction were ACG CTG CTG CCA CCA CCA TGA AGG CAAC AGC TAC TGG TCC TCG TAT AGC CAT TGG TTC CCG GCC AGC AGG CTA GCA TGG AGA CTG ATG GAC AAC AGG and CCT CTT TTG ATT GTT GTC TTT, allowing substitution of a Sali/I عمير. The Q to N substitutions in the NP147–155 epitopes of NP13–496 and ER-NP13–496 were conducted by one-step PCR using the downstream primer GAA CAA GGG CCC TTG TCG TAT TAT AAG TTG and upstream primer GGT AAG GAA GTA GGA TCA T. allowing the substitution of Sali/I عمير. The clones were cloned into modified versions of the pSc11 plasmid, containing heteroduplexes (hairpins) (HP) of different lengths (16, 18, and 20 bp) in the 5′ untranslated region of the recombinant gene (28, 41, 42). Sequencing, using β-erythroxyethyl phosphorimidates chemistry (Applied Biosystems, Foster City, CA) and conducted by the Kimmel Cancer Institute Nucleic Acid Facility, confirmed the integrity of each construct. Reombination into vaccinia virus (vac) and titration of vac (in duplicate) was conducted as described (28, 40).

CTL assay

Epitope-specific T cells were derived from C57BL6, C3H, or BALB/c mice, respectively, as described elsewhere (28, 40). Briefly, mice were immunized by i.p. injection of 106 PFU of a vac expressing the isolated NP50–374 epitope in the case of C57BL6, a vac expressing the isolated NP147–155 epitope in the case of BALB/c, and a vac expressing the isolated NP147–155 epitope in the case of C3HeB. After at least 2 wk, spleens from appropriate mice were harvested and one-third of cells were infected with A/PR/8/34 influenza virus for restimulation. Secondary cultures were incubated at 37°C/6% CO2 for 6–7 days before harvesting for effector population.

Two MHC class I typing assays were performed, as previously described (28, 40, 42). L929, L-K d, or MC57G cells were used as APCs for H-2K k, H-2D b, and H-2D d–restricted responses, respectively. APC were infected for 1 h at 37°C with vaccinia recombinants at 10 PFU/cell at a concentration of 107 cells/ml in balanced salt solution containing 0.1% BSA. For evaluation of TAP dependence, T2-K d or T2-K k cells were infected with 10 PFU/cell vac and 10 PFU/cell of a control vac or TAP2I2-expressing vac. After 1 h, 2 μl of 2% Triton X 100 plus 10% FBS were added and the cells were incubated for another 3 h with rotation. Cells were pelleted and resuspended with 50 μl/106 cells of RPMI 1640 with 10% FCS containing 100 μl of Na3Citrate (Amersham) and incubated for 1 h at 37°C. APC were then washed three times with PBS and resuspended in RPMI 1640 plus 10% FBS and combined with CTL populations in round-bottom plates at 104 cells/well. APCs and CTL were cocultured for 4 h at 37°C before 100 μl of supernatants were collected and counted in a gamma detector (Pharmacia Biotech, Uppl-sala, Sweden). The data are presented as percentage of specific 51Cr release, defined as 100 × ((experimental cpm – spontaneous cpm)/total cpm – spontaneous cpm)). When needed, lactacystin was added to a final concentration of 50 μM during the first 5 h of the assay.

Metabolic labeling, immunoprecipitation, and Endo H treatment

Metabolic labeling and immunoprecipitation were performed essentially as described elsewhere (28), except that a mixture of two anti-NP mAbs was used (H16-L10-IR5 and H19-S24-4). [35S]Methionine/cysteine at 50–100 μCi/108 cells was used in all cases. Endoglycosidase H (Endo H; Boehringer Mannheim, Mannheim, Germany) experiments involved a 20-min pulse at 4 h postinfection, and Endo H treatment following immunoprecipitation was performed as described elsewhere (43). Briefly, 20 μl of the reaction mixture (0.1 M sodium acetate (pH 6), 5 μM EDTA, 2 μM PMSF, 1 μM Endo H) were added to 10 μl of the protein in Laemml sample buffer (44); the reaction mixture without Endo H was added to the control samples. The reaction was allowed to proceed for 12 h at 31°C. Proteins were separated by SDS-PAGE in 10% gels and visualized by autoradiography.

Subcellular fractionation

Subcellular fractionation was performed as described elsewhere (45). Briefly, cells were resuspended in homogenization buffer (0.25 mM sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA (pH 7.4)) and disrupted in a Dounce homogenizer (50 strokes, pestle type A). After removal of nuclei, the homogenate was first centrifuged at 10,000 × g for 30 min and the supernatant was recentrifuged at 100,000 × g for 60 min. The 10,000 × g pellet from subcellular fractionation was resuspended in homogenization buffer (pH 7.4), 0.25 mM sucrose in the presence or absence of 0.2 mg/ml protease K. A total of 0.5% NP40 was added to a control sample. The samples were then incubated for 1 h at 4°C and the reaction was stopped by addition of PMSF to a final concentration of 2.
mM. Immunoprecipitation, SDS-PAGE, and autoradiography were performed.

**Immunofluorescence**

A total of 5 \times 10^4 3T3 cells were seeded onto coverslips in 24-well plates and incubated overnight. Cells were infected with 5 PFU/cell indicated vac and incubated for 4 h at 37°C/9% CO_2. The monolayers were washed three times with ice-cold PBS and fixed-permeabilized in methanol/acetone (1/1) for 1 min. Between each of the remaining steps the coverslips were washed three times with PBS. Anti-NP Abs H16-L10-4R5 and H19-S24-4 were used as primary Abs and were incubated with the cells overnight at 4°C. Fluorescein-labeled horse anti-mouse Ig (IgG) (Vector Laboratories, Burlingame, CA) was used as secondary Abs with incubation for 30 min at 37°C. For negative control wells, the secondary (FITC-labeled) reagent alone was used. All images were viewed at the Kimmel Cancer Institute Confocal Facility.

**Peptide elution**

Peptide elution was performed as described elsewhere (46). Briefly, 2 \times 10^4 MC57G cells were infected with the corresponding vacs at 10 PFU/cell for 5 h. The cell pellet was then sedimented at 1,000 \times g for 10 min. The cells were resuspended in 0.5% trifluoracetic acid in deionized water in the presence of 1 mM of PMSF and 10 \mu M leupeptin and were disrupted in a Dounce homogenizer (50 strokes, pestle type B). The cell debris was sedimented by centrifugation at 15,000 \times g for 20 min, and the resulting lysate was filtered through Centriplus (Amicon, Beverly, MA) units with >6,000-kDa exclusion. The low molecular filtrate was separated using reversed phase HPLC using a 218TP column (C18, 5-\mu M particle size; Vydac, Hesperia, CA). Fractions (0.6 ml) were collected, dried, and resuspended in 150 \mu l of PBS. Fifty microliters of these fractions or their 2-fold serial dilutions were added to wells of 96-well plates. MC57G cells, which had been preincubated at 26°C for at least 24 h to maximize expression of the peptide-receptive MHC class I molecules on their surfaces, were added at a density of 5 \times 10^5/150 \mu l to each well containing HPLC fractions and incubated for 3 h at 26°C. NP 366–374-specific D^2-restricted T cell hybridoma DBFZ25 (47) (1 \times 10^4/well) was added then, and the plates were incubated for 24 h at 37°C. Activated T-hybridoma cells were assayed for \beta-galactosidase (lacz) production as described (47). The retention time of the NP 366–374 epitope was evaluated by comparison with the model peptide ASNENMETM corresponding to the influenza virus A/PR/8/34 NP 366–374 epitope.

**Results**

**Generation and characterization of NP variants directed to the cytosol and ER**

NP expressed either in isolation or during an influenza infection exists in both the cytosol and nucleus, and the Ag processing capabilities in these two compartments may differ significantly. For example, the 11S Reg (also termed PA28) caps of nuclear proteasomes contain \gamma subunits while those of the cytosol contain primarily \alpha and \beta, and 11S Reg composition has been shown to alter the cleavage specificity of the proteasome (48). To eliminate this complication, we abrogated trafficking of NP to the nucleus. This was accomplished by removing the first 36 bases that encode an unconventional nuclear localization signal (49, 50). Confocal immunofluorescence studies of cells infected with a vac expressing this variant, termed NP_{13–498}, demonstrate that the protein is located almost entirely in the cytosol, in stark contrast to cells infected with vac expressing wild-type NP (Fig. 1A). To direct NP_{13–498} to the exocytic compartment, we attached the leader sequence of influenza A/WSN/33 hemagglutinin to its amino terminus, generating ER-NP_{13–498}. Again, immunofluorescence microscopy confirmed the predicted location of this form (Fig. 1A). The constructs were then subjected to a series of biochemical studies. The initiating codons of NP_{13–498} and ER-NP_{13–498} are surrounded by different bases and the proteins might therefore be synthesized at different rates (51), a situation that would complicate interpretation of any observed differences in Ag presentation. To address this possibility, we conducted brief (5 min) metabolic labeling of cells infected with equal amounts of NP_{13–498} or ER-NP_{13–498} expressing vac followed by immunoprecipitation with a NP-specific mAb mixture, SDS-PAGE analysis, and autoradiography. NP_{13–498} contains one predicted site of N-linked glycosylation near the N terminus that can be used when NP_{13–498} is delivered to the ER (Fig. 1B). To resolve ER-NP_{13–498} to a single band for direct comparison with NP_{13–498}, the immunoprecipitate was treated with Endo H before SDS-PAGE. Densitometry analysis of the gel (data not shown) revealed the NP_{13–498} and Endo H-treated ER-NP_{13–498} bands to be of essentially identical intensity (Fig. 1B), indicating very similar rates of synthesis. Differences in stability between the two forms of NP could also complicate interpretation as decreased stability has been linked with enhanced Ag presentation by many (19, 20, 22–24, 52), though not all (25), groups. However, the immunofluorescence and biochemical studies shown indicate the high stability of both NP_{13–498} and ER-NP_{13–498}. Prolonged chase times reveal that a portion of labeled ER-NP_{13–498} remains unglycosylated (data not shown). This might be due to a fraction of ER-NP_{13–498} being mistargeted to the cytosol, though it seems much more likely to be due to failure of...
some ER-NP<sub>13–498</sub> molecules to be modified at Asn<sup>9</sup> (with reference to NP<sub>13–498</sub>) due to its proximity to the N terminus of the mature protein and, consequently, poor access to the glycosylation machinery. First, analysis of cell extracts expressing this protein following rVac infection indicate that virtually all detectable ER-NP<sub>13–498</sub> is microsome associated (Fig. 2A). Furthermore, all versions of ER-NP are resistant to proteinase K digestion unless the microsome fraction is first treated with detergent (Fig. 2B), indicating location of ER-NP<sub>13–498</sub> in the lumen of the microsomal fraction. This is in contrast to NP<sub>13–498</sub> which partitions almost entirely in the cytosolic fraction. The small amount of NP<sub>13–498</sub> that is microsome associated is accessed by proteinase K without prior detergent treatment (Fig. 2B), indicating location at the cytosolic face of the microsomal membrane. Second, efficient delivery of full-length NP to the ER following N-terminal attachment of a signal sequence has been previously demonstrated, using glycosylation-sensitive T<sub>CD8+</sub> activation as a readout. In this case, the H-2K<sup>d</sup>-restricted NP<sub>147–155</sub> epitope was modified to allow for N-linked glycosylation at position 149. Epitope-specific T<sub>CD8+</sub> kills targets expressing the mutated NP when it is expressed in the cytosol but not when it is directed to the ER. The basis for this finding is the N-linked glycosylation of the epitope in the ER, followed by peptide:N-glycanase-mediated deglycosylation in the cytosol (53). This process converts the glycosylated asparagine to aspartic acid, a change that significantly reduces recognition. Due to obvious differences between full-length NP and NP<sub>13–498</sub>, particularly in terms of trafficking, and our use of a different signal sequence to target NP<sub>13–498</sub> to the ER, we elected to confirm ER targeting of ER-NP<sub>13–498</sub> at the level of T<sub>CD8+</sub> activation. Thus, the same Q149 to N149 mutation was conducted for NP<sub>13–498</sub> (NP<sub>13–498<sup>N</sup></sub>) and ER-NP<sub>13–498</sub> (ER-NP<sub>13–498<sup>N</sup></sub>). Metabolic labeling experiments show the mutated version of ER-NP<sub>13–498</sub> (ER-NP<sub>13–498<sup>N</sup></sub>) to be fully glycosylated at either one or two sites (Fig. 3A), confirming our prediction that the unglycosylated ER-NP<sub>13–498<sup>N</sup></sub> cohort is exocytic. When we assayed for recognition of NP<sub>13–498<sup>N</sup></sub> and ER-NP<sub>13–498<sup>N</sup></sub> by T<sub>CD8+</sub> specific T<sub>CD8+</sub> was recognized but ER-NP<sub>13–498<sup>N</sup></sub> was not (Fig. 3B), as anticipated. Thus, essentially all of the ER-NP<sub>13–498</sub> that is processed for presentation is first delivered to the ER.

It was also important to confirm that presentation of ER-NP<sub>13–498</sub> requires processing in the cytosol, i.e., retrotranslocation. This was previously shown to be the case with full-length NP, using TAP-deficient cell lines as target cells (21). Using a similar approach, we observed TAP-dependence for presentation from ER-NP<sub>13–498</sub> of two of the three epitopes, NP<sub>50–57</sub> (SDYEGRLI, in single letter code) and NP<sub>147–155</sub> (TYQRTRALV), that were investigated (data not shown). Attempts to ascertain the role of TAP in the presentation of the third epitope, NP<sub>366–374</sub> (ASNENMETM), were consistently hampered by high background lysis of the target cells. However, obligate delivery of the Ag to the cytosol for generation of this epitope is strongly implied by the effects of proteasome inhibitors as described below.

**Relative production of three epitopes from NP<sub>13–498</sub> and ER-NP<sub>13–498</sub>**

Having established the biochemical properties of NP<sub>13–498</sub> and ER-NP<sub>13–498</sub> and having determined that presentation of epitopes from ER-NP<sub>13–498</sub> requires retrotranslocation, we were ready to carry out a careful comparison between the presentations of epitopes from these two forms of NP. Such an analysis is frequently impossible with standard assays, owing to the sensitivity

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**FIGURE 2.** Biochemical localization of NP<sub>13–498</sub> and ER-NP<sub>13–498</sub> inside the cell. A, Subcellular distribution of NP<sub>13–498</sub> and ER-NP<sub>13–498</sub>. L-K<sup>+</sup> cells were infected with the indicated rVac. After 4 h of infection the cells were homogenized and the nuclei were removed; the homogenate was centrifuged at 10,000 × g for 30 min (10,000g pellet), and the supernatant was then centrifuged at 100,000 × g for 60 min (100,000g pellet and 100,000g supernatant). NPs were then immunoprecipitated from these fractions and separated by SDS-PAGE. B, Sensitivity of microsome-associated NP<sub>13–498</sub> and ER-NP<sub>13–498</sub> to proteinase K treatment. Microsomes were treated with proteinase K either in the presence or absence of detergent (0.5% NP40). NP<sub>13–498</sub> and ER-NP<sub>13–498</sub> were immunoprecipitated, separated in a 10% polyacrylamide gel, and visualized by autoradiography.

**FIGURE 3.** A, Characterization of NP<sub>13–498</sub>, NP<sub>13–498<sup>N</sup></sub>, ER-NP<sub>13–498</sub>, and ER-NP<sub>13–498<sup>N</sup></sub>. MC57G cells were infected with indicated rVac and NPs were immunoprecipitated, treated with Endo H where indicated, separated by SDS-PAGE, and visualized by autoradiography. B, The effect of Q149 to N149 substitution upon presentation of NP<sub>147–155</sub>. The E:T ratios were 50:1, 17:1, and 5.6:1.
of T<sub>CD8+</sub> to very low levels of Ag (28, 54, 55). We therefore placed the genes encoding NP<sub>13–498</sub> and ER-NP<sub>13–498</sub> behind a series of sequences designed to create thermostable RNA duplexes or HP 16, 18, and 20 bp in length. These structures limit, in graded fashion, the biosynthesis of Ag and, consequently, the amount of epitope presented (28, 41, 42). The various HP constructs were recombined into the vac genome and the resulting viruses were tested for their ability to sensitize target cells for killing by T<sub>CD8+</sub>-specific for NP<sub>147–155</sub>, and two other well-defined epitopes within NP (56, 57). In agreement with previous findings (21), presentation of the H-2K<sup>d</sup>-restricted NP<sub>147–155</sub> epitope was diminished when the construct was delivered to the ER (Fig. 4B). This is observed even with no HP in place. In contrast, presentation of NP<sub>50–57</sub> (H-2K<sup>d</sup>-restricted, Fig. 4A) and NP<sub>366–374</sub> (H-2D<sup>b</sup>-restricted, Fig. 4C) is not compromised by delivery of NP to the ER, a finding clearly and consistently observed under conditions of limited Ag expression (HP20). Indeed, presentation of these epitopes often appears to be slightly better from ER-NP<sub>13–498</sub> as in the assay shown in Fig. 4. To confirm this last, unexpected observation, we assessed the relative amounts of NP<sub>366–374</sub> generated from the two forms of NP with a biochemical approach that has become standard protocol (46). This involved acid elution of peptides from whole cell extracts 5 h after infection, HPLC separation of low-molecular mass peptides (<10 kDa), and coincubation of fractions with target cells and the DBFZ.25 (47) T-hybridoma that produces β-galactosidase upon activation by the H-2D<sup>b</sup>/NP<sub>366–374</sub> complex. Peptide levels from both forms of NP were very similar in all of the active fractions (Fig. 5), a conclusion that we further strengthened by testing dilutions of the peak fractions (Fig. 5, inset).

Effects of proteasome inhibitors on presentation of NP<sub>13–498</sub> and ER-NP<sub>13–498</sub>

In addition to showing that the presentation of some epitopes is not compromised by delivery of Ag to the exocytic compartment, the data in Fig. 4 also suggest that the processing of NP<sub>13–498</sub> and ER-NP<sub>13–498</sub> is qualitatively different, because delivery to the cytosol favors production of NP<sub>147–155</sub> but not NP<sub>50–57</sub> or NP<sub>366–374</sub>. This possibility was reinforced by observations on the relative impact that proteasome inhibitors have upon the generation of epitopes from the two NP forms. We have observed that inhibitors of proteasome cleavage decrease presentation of NP<sub>50–57</sub> and NP<sub>366–374</sub> from wild-type NP (E. J. Wherry, T. N. Golovina, and L. C. Eisenlohr, manuscript in preparation), consistent with the original observation (58) and many subsequent publications suggesting a critical role for the proteasome in epitope generation. In contrast, presentation of NP<sub>147–155</sub> is unaffected and in some cases significantly enhanced by treatment of infected target cells with the same inhibitors (40, 59). Fig. 6 shows that for both cytosolic and exocytic versions of NP, presentation of NP<sub>50–57</sub> and NP<sub>366–374</sub> is significantly inhibited by lactacystin. Note that this is readily apparent only under conditions of limited Ag expression in the case of NP<sub>50–57</sub> (HP20). In contrast, lactacystin treatment has no effect upon presentation of NP<sub>147–155</sub> from NP<sub>13–498</sub> and significantly elevates presentation of this epitope from ER-NP<sub>13–498</sub>. Similar results were obtained for all three epitopes using the inhibitors N-acetyl-leucyl-leucyl-nor-leucynal and Z-L3VS (data not shown).

Discussion

Early models of T<sub>CD8+</sub> recognition focused upon membrane-bound glycoproteins as providing the primary targets for MHC class I-restricted responses. Subsequent discoveries, such as the peptide nature of T<sub>CD8+</sub> recognition and the critical roles for TAP and the proteasome in most cases of presentation, deci-
terms of presentation efficiency when our model Ag was targeted to the ER.

How can our results be reconciled with the earlier reports (19, 20, 60) showing significant increases in presentation following re-targeting of HA and HIV envelope? Townsend et al. (19) showed that presentation of an H-2k-restricted HA-derived epitope was lost when expression was driven by a late vaccinia promoter, and that presentation under these conditions could be regained by redirecting HA to the cytosol through removal of the signal sequence. At that time it was suggested that vaccinia late gene product(s) interferes with Ag processing, but recent evidence suggests that the explanation lies with the limited expression of host proteins involved in presentation that is possible during late phases of the vac replication cycle (61). A simple explanation is that the HA and envelope epitopes assayed for are NP147–155-like in this aspect of Ag processing, an idea that cannot be tested at present because we do not understand the basis for the NP147–155 presentation phenotype. However, if the two notable properties of NP147–155—higher presentation from cytosolic Ag and insensitivity to proteasome inhibitors, are linked, then this explanation would be less likely. Most epitopes (including the murine HIV envelope epitope (62)), are sensitive to the proteasome inhibitors. Of note, a human epitope from HIV envelope that is enhanced by retargeting to the cytosol is presented in a TAP-independent manner, making it difficult to compare with our epitopes (63, 64). Another factor to consider is the relative stabilities of the proteins in the two compartments. Many exocytic proteins, including HA and HIV envelope, are highly unstable when targeted to the cytosol (19, 20, 29, 60), while, as shown in this study, NP13–498 is relatively stable in both locations. A number of groups have reported a positive correlation between degradation rate (including NP) and Ag presentation efficiency (19, 20, 22–24, 52). Thus, the enhanced presentation of retargeted glycoproteins may be due to an increase in degradation rate rather than more direct access to Ag processing machinery. We have tested this possibility preliminarily by destabilizing NP13–498 with point mutations predicted to cause misfolding. This manipulation does lead to a significantly higher degradation rate but does not appreciably influence the presentation of the three epitopes described here (T. N. Golovina, unpublished observations). A third consideration is the egress of exocytic proteins from the ER to the plasma membrane, a step that likely jeopardizes the possibility of efficient class I-restricted Ag processing. ER-NP13–498 appears to fold properly, based upon binding to several monoclonal anti-NP Abs, and is secreted in detectable

![Figure 5](http://www.jimmunol.org/DownloadedFrom/p50465461475645.png)

**FIGURE 5.** Peptide elution profile. Vac recombinants expressing NP13–498, ER-NP13–498, or the control virus were used to infect MC57G cells. Peptides were eluted from the cells with 0.5% trifluoroacetic acid, and the low molecular portion of the lysate was fractionated using reverse phase HPLC on Vydac 218 TP column. MC57G cells were incubated with the fractions for 3 h at 26°C before adding DNP366–374-specific DBFZ.25 T-hybridoma cells. Inset, Titration curves of the fractions. This experiment was conducted twice with essentially identical results.

![Figure 6](http://www.jimmunol.org/DownloadedFrom/p50465461475645.png)

**FIGURE 6.** The effect of treatment with lactacystin on the presentation of NP50–57 (A), NP147–155 (B), and NP366–374 (C) epitopes. Vac recombinants expressing NP13–498 and ER-NP13–498, either with or without HP20 preceding the open reading frames, were used to infect target cells expressing the indicated MHC class I molecules. Target cells were then tested for the ability to be lysed by epitope-specific TCD8/H11001 in a standard 51Cr release assay. The assay was conducted in the presence (+) or absence (−) of 50 μM lactacystin. The E:T ratios for the data shown were 50:1, 17:1, and 5.6:1 for H-2Kd and H-2Db; and 21:1, 7:1, and 2.3:1 for H-2Kb.
amounts, though its release from the ER appears to be slow, based upon the rate at which Endo H resistance is acquired (T. N. Golovina, unpublished observations). This may be due to aberrant interactions between the naturally cytosolic/nuclear protein and the quality control system of the ER and might increase the opportunity for retrotranslocation. However, egress of HIV envelope is also relatively slow (65), and presentation of this protein is markedly enhanced by retargeting to the cytosol (20). Thus, it is presently difficult to gauge the impact that the duration of ER residence has upon presentation efficiency. Experiments are under way to resolve these various issues, but a key point that can be concluded from the data presented here is that the ER membrane provides little impediment to the Ag processing pathway, suggesting that retrotranslocation is a very efficient process. This makes some sense if, as it now appears, retrotranslocation provides the only means for turnover of most ER-resident proteins that fail quality control. A less-efficient process might allow for the accumulation of misfolded and other defective species in the ER.

While the identification of an efficient retrotranslocation system has obviated the need for other models to explain processing of glycoproteins, mistargeting to the cytosol does appear to be the explanation for the presentation of some epitopes. This is the case for an epitope within HIV envelope that cannot be recognized by a particular clone when modified by glycosylation. Despite 100% glycosylation at this site, the epitope is presented, implying direct delivery of an undetectable fraction of the synthesized protein to the cytosol (66). The prevalence and relative efficiency of this pathway remains to be determined, but it is clearly not operable for NP147–155 and several other epitopes that have been studied here (Fig. 3) and in experiments that also exploited glycosylation/de-glycosylation-mediated conversion of asparagine to aspartic acid within the epitope (21, 33–35).

One might have assumed that once a protein is retrotranslocated it undergoes qualitatively similar processing as that of default cytosolic proteins. Our results suggest that this is not the case. While NP306–374 are generated with comparable efficiency from both locations, NP147–155 is clearly generated with more efficiency from cytosolic NP. In addition, proteasome inhibitors enhance presentation of NP147–155 from ER-targeted NP but not from cytosolic NP. There are several possible explanations for this differential processing. One is that retrotranslocated proteins have little or no access to nonproteasomal proteases that have a positive impact upon the generation of NP147–155 due to direct delivery of most retrotranslocated material to the proteasome. Indeed, the proteasome appears to participate in the retrotranslocation of at least some proteins (31, 32). Current efforts are aimed at determining whether nonproteasomal proteases do play a role in the generation of NP147–155. The neutral or enhancing effects of proteasome inhibitor on presentation of this epitope and our identification of a major proteasome cleavage site within the epitope would appear to support a role for other proteases. However, the issue is complicated by the recent realization that current proteasome inhibitors do not inhibit all proteasomal activities and can, in fact, alter specificity (67). Thus, the inhibitors might block the activity that destroys NP147–155 while preserving or enhancing the activity that is necessary for its generation. If proteasomes are solely responsible for the generation of NP147–155 from either form of NP, another potential explanation for the differential production of NP147–155 from NP13–498 and ER-NP13–498 is the delivery of these two forms to different proteasome populations that generate NP147–155 with differing efficiency. IFN-γ stimulation alters the composition of the proteasome by replacing the constitutive catalytic β subunits of the 20S core (X, Y, and Z) with their induced counterparts (LMP2, LMP7, and MECL1) and the 19S cap (attached to one or both ends of the 20S core) with the 11S Reg (PA28) cap (68). Both modifications have been reported to alter proteolytic specificity (69–71). An analysis of unstimulated primary rat hepatocytes revealed significant underrepresentation of the constitutive Z subunit and overrepresentation of the IFN-γ-inducible LMP2 subunit (which can be detected in many unstimulated cell types) in the microsomal fraction (72). Thus, we have speculated that immunoproteasomes (68), predisposed to the ER membrane, are less efficient in generating NP147–155 compared with constitutive proteasomes that are more available in the cytosol. However, we find no difference in presentation of NP147–155 under limiting conditions of expression, in IFN-γ-treated and untreated cells (T. N. Golovina, unpublished observations). Another possibility is that proteasomes with the same subunit composition process both forms of NP but that association with the ER membrane in some way alters specificity, as has been shown in the case of proteasome 20S core association with 11S Reg (PA28) cap complex (71, 73).

A number of compelling questions about retrotranslocation as it pertains to Ag presentation remain. ER-NP13–498 is not tethered via a transmembrane domain to the ER membrane as are many other proteins used in the study of this process, making its efficient presentation all the more notable. It will be interesting to determine how tethering and other modifications such as glycosylation, shown to impact retrotranslocation efficiency (32), affect epitope production. Work on such questions may reveal new avenues in the design of vaccines intended to optimize TCD8+–mediated responses.

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