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The Impact of Misfolding versus Targeted Degradation on the Efficiency of the MHC Class I-Restricted Antigen Processing

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Evidence suggests that most epitopes presented by MHC class I molecules are derived from those newly synthesized proteins that are defective due to errors during manufacture. We examined epitope production from model cytosolic and exocytic proteins modified in various ways. Substrates containing a degradation targeting sequence demonstrated very rapid turnover and enhanced epitope production, as was the case for substrate retargeted from endoplasmic reticulum to cytosol. For less radical alterations, including point mutation and deletion and elimination of glycosylation sites, despite detectable changes in folding, half-life was only moderately decreased and there were no significant increases in epitope production. Puromycin, which causes premature termination of protein synthesis, also had no impact upon epitope production. It appears that most defective proteins are not rapidly dispens with and the targeting of most nascent proteins for Ag processing is not tied to quality control. The Journal of Immunology, 2005, 174: 2763–2769.

Materials and Methods

Chemicals

General chemical supplies were obtained from Sigma-Aldrich. Molecular biology reagents were obtained from New England Biolabs. Isotopes were purchased from ICN Biomedicals and lactacystin was purchased from Boston Biochemicals. Monoclonal anti-nucleoprotein (NP) Abs (clones H16 and H24) were provided by Dr. W. Gerhard (Wistar Institute, Philadelphia, PA), anti-Tac hybridoma (clone 7G7B6) were obtained from American Type Culture Collection; monoclonal anti-hemagglutinin (HA) tag Abs (clone 12CA5) were purchased from Roche Diagnostic Systems. PCR primers were synthesized at the Kimmel Cancer Center Nucleic Acid Facility (Philadelphia, PA).

Cell lines and mice

L-K8 (L.929 transfected with H-2K\(^{b}\)) and L-K6 (L.929 transfected with H-2K\(^{d}\)) were maintained in DMEM with 5% FBS. Six- to 8-wk-old female inbred BALB/c (H-2\(^{b}\)) and C57BL/6 (H-2\(^{b}\)) mice were obtained from Taconic Farms or The Jackson Laboratory and maintained in the Thomas Jefferson University Laboratory Animals Facility (Philadelphia, PA).

Viruses

Generation of the triple tandem HA-tag and addition of OVA257–264 epitope with five flanking amino acids on both sides to NP\(^{13–498}\) based Ags was done by the two-step PCR using consequent primers: CCTGACTATGGGGGTCATACCCATACGATGTTCCAGATTACGCTGGATCTCTCTGCCTGACTATGCGGGGTCA.charges. This notion has been tested in many different systems that compare the presentation of wild-type Ag with variants designed to enhance the rate of degradation. Strategies include mutation to induce misfolding (9, 10), relocation to a different subcellular compartment (11, 12), and attachment of a degradation signal (degron) (4, 11–16). However, the second two strategies, while almost universally demonstrated to enhance peptide generation, may not accurately predict the fates of DRiPs because degron-mediated degradation is a highly precise and rapid process. At the same time, errors in subcellular mistargeting appear to be rare (17, 18). To determine the relative influence of these and other modifications upon epitope supply, we assessed the production of two distinct epitopes incorporated into two model Ags targeted to the cytosol and to the endoplasmic reticulum (ER). 3

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3 Abbreviations used in this paper: ER, endoplasmic reticulum; NP, nucleoprotein; HA, hemagglutinin; Ubi, ubiquitin; vac, vaccinia virus.
TCGACCCACATGATCCGATGTCGCTCCCGACTACCGAGGGTC GTATCTCATGATGACGTCGACTATGACGCGGACTAT for Tac targeted to the cytosol.

Addition of the ubiquitin (Ubi) moiety and changing the N-terminal amino acid to Arg was done using the primer CTAAGCTTACCGTCCGACGT CTTCCACTTAAAGACGGTGACGGGG adding Arg to the C-ter-

nus of Ubi followed by the Nhel site. All HA tags were constructed such that they contained an Nhel site immediately after the ATG initiation codon thus allowing for substitution with Ubi-Arg using SolI/Nhel. The genes were cloned into the modified pSCEII plasmid, containing β-glyco-
sidase gene. Sequencing, using β-cyanoethyl phosphorylaminates chemistry (Applied Biosystems) and conducted by the Kimmel Cancer Institute Nu-
cleic Acid Facility, confirmed the integrity of each construct. Recombina-
tion into vaccinia virus (vac) and titration of vac (in duplicate) was con-
ducted as described (20).

Flow cytometric analysis

L-Kb cells were infected for 1 h at 37°C with vaccinia recombinants at 10 PFU/cell at a concentration of 10^6 cells/ml in balanced salt solution contai-
ning 0.1% BSA. After 1 h, RPMI 1640 plus 10% FBS media was added and the cells were incubated for additional time. Aliquots were removed every hour and stained for K^b/OVA257–264 complexes on the surface with 25-D1.16 mAbs specific for K^b/OVA257–264 complex (obtained from A. Porgador, University of Ben-Gurion, Beer-Shiva, Israel) and FITC-labeled goat anti-mouse Abs (Vector Laboratories), and then were fixated in 2% solution of paraformaldehyde (Electron Microscopy Sciences) in PBS.

In the experiments with puromycin, 200 μM puromycin was added after 1 h of infection and removed after 20 min of incubation; the cells were then

washed twice with RPMI and resuspended in RPMI. The infection then proceeded, and the aliquots were removed and stained as earlier described.

When needed, permeabilization of the cells was performed as previously described (21): the cells were first fixed in 4% solution of formaldehyde in PBS for 10 min, washed three times with PBS, then permeabilized in 2 mg/ml n-octyl-β-D-glucopyranoside (Calbiochem) for 10 min, and washed three times before staining.

CTL assay

Epitope-specific T_{CDS}^{H-2} were derived from C57BL/6 or BALB/c mice, re-
spectively, as described elsewhere (22). Briefly, mice were immunized by i.p. injection of 5 × 10^7 PFU of a vac expressing the isolated OVA257–264 epitope in the case of C57BL6, or a vac expressing the isolated NP134–155 epitope in the case of BALB/c mice. After at least 2 wk, spleens from

appropriate mice were harvested, and one-third of the cells were infected with A/PR/8/34 influenza virus or WSN/33-Ova influenza virus with

OVA257–264 and H-2Kb-restricted NP 147–155 (Fig. 1). These

two epitopes were chosen because, for all earlier constructs as well as those used here, they demonstrate strikingly different sensitivity to proteasomal inhibitors; while OVA257–264 presentation is profoundly inhibited by proteasome inhibitors, NP 147–155 presentation is either unchanged or increased depending upon context (16, 19, 22). The basis for this latter presentation phenotype is the presence of both chymotryptic-like and tryptic-like proteasomal cleavage.

Western blotting

Laemmli SDS-PAGE and transfer to nitrocellulose membrane were per-
formed as previously described (23). The protein bands were visualized by

consequent incubating with anti-HA Abs (1 μg/ml) (Roche), HRP conju-
gated goat anti-mouse Abs dilution 1/10,000 (Vector Laboratories) and

LumiGLO Chemiluminescent Substrate System (Kirkegaard & Perry Lab-
oratories) with subsequent autoradiography.

Proteasease K treatment

The cells were metabolically labeled as described elsewhere (20), lysed in the lysis buffer (0.01M Tris-HCl, pH 7.5, 0.14 M NaCl, 0.5% Nonidet

P-40), and the constructs were immunoprecipitated with anti-HA Abs immo-

zized on rProtein A-agarose beads (RepliGen). The gel slurry were washed once with the lysis buffer and three times with PBS. The samples were placed on ice, and 1 μg/ml protease K was added to the gel slurry with immobiliized Ags. Aliquots were removed at indicated times and 5 mM PMSF was added to stop the reactions. The slurry were washed then once with PBS and boiled 5 min in the Laemmli buffer. Proteolytic frag-
ments were separated by SDS-PAGE in 12% gels and visualized by

autoradiography.

Results

Generation and biochemical characterization of the cytosolic and exocyotic Ags and their variants—folding and half-lives

NP from influenza virus A/PR/8/34 depleted of the twelve N-ter-

minal amino acids that target it to the nucleus (NP_{13–499}) was used as a model cytosolic Ag. The loss of amino acids 1–12 does not impact folding as demonstrated by continued reactivity with a large panel of anti-NP mAbs nor is there an effect upon presenta-
tion efficiency (data not shown). The α-chain of human IL-2R (Tac

Ag) was used as a model glycoprotein that would be subject to

quality control within the ER. Both groups of model Ags were

appended with an HA-tag in tripleicate which does not disturb fold-
ing of the core proteins (data not shown) and allows for the re-

trieval of Ag independent of its folding state. Each Ag was further

engineered to contain the same two epitopes: H-2Kb-restricted

OVA257–264 and H-2Kb-restricted NP_{47–155} (Fig. 1). These

epitopes were chosen because, for all earlier constructs as well as those used here, they demonstrate strikingly different sensitivity to proteasomal inhibitors; while OVA257–264 presentation is profoundly inhibited by proteasome inhibitors, NP_{47–155} presentation is either unchanged or increased depending upon context (16, 19, 22). The basis for this latter presentation phenotype is the presence of both chymotryptic-like and tryptic-like proteasomal cleavage.

Metabolic labeling and immunoprecipitation

Metabolic labeling and immunoprecipitation were performed as previously described (20). Gels were dried and exposed to LE Storage Phosphor Screen (Molecular Dynamics), and the luminescence was measured using Typhoon 8600 Variable Mode Imager and analyzed using Image Quant software.

Metabolic labeling and immunoprecipitation were performed as previously described (20). Gels were dried and exposed to LE Storage Phosphor Screen (Molecular Dynamics), and the luminescence was measured using Typhoon 8600 Variable Mode Imager and analyzed using Image Quant software.

FIGURE 1. Model Ags based on NP_{13–499} (targeted to the cytosol) and on Tac (targeted to the ER).
sites within NP147–155 sequence (TYQRTRALV) (19). This suggests the possibility, supported by preliminary data (E. J. Wherry, T. N. Golovina, S. E. Morrison, G. Sinnataumby, M. S. McElhaugh, L. C. Eisenlohr, manuscript in preparation) that nonproteasomal proteases (24–26) are involved in the generation of NP147–155.

Cytosolically targeted influenza virus NP (NP13–498) was altered to induce misfolding in two ways: 1) substitution of Val280 and Tyr281, located in the center of a hydrophobic domain spanning residues 250–301, with arginine residues (NP13–498/RR), a manipulation based upon previous reports that similar changes decrease stability (27, 28); and 2) deletion of the entire NP250–301 hydrophobic domain (NP13–498/Δ). The constructs were incorporated into vac, which allow for expression in a wide range of cell lines.

Compared with NP13–498, NP13–498/RR, and NP13–498/Δ are more rapidly digested by proteinase K, and the digestion products are different for all three (Fig. 2A), indicating altered folding states. This is also apparent when comparing immunoprecipitation of the constructs with the conformation-independent anti-HA mAb vs the conformation-sensitive anti-NP mAb H19-S24-4; precipitation of NP13–498/RR with H19-S24-4 is clearly impaired compared with

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NP_{13-498} while NP_{13-498Δ} does not interact with this Ab at all (Fig. 2A). The protein was also targeted for programmed degradation by replacing the initiating methionine with arginine and preceding the construct with a Ub moiety that is posttranslationally removed by Ub C-terminal hydrolase (29). According to the N-end rule (30), an arginine residue at the N terminus constitutes a degron that mediates rapid, Ub-dependent degradation. As anticipated by published results (11), Ubi-Arg-NP_{13-498} was completely degraded within several min (Fig. 2E, discussed below). This precluded analysis of its folding using proteinase K sensitivity assay. However, immunoprecipitation analysis, demonstrating relatively weak interaction with the anti-NP Ab H19-S24-4, indicates that this construct is also misfolded (Fig. 2A).

The Tac-based Ag was initially modified in three ways. First, we mutated both sites of asparagine-linked glycosylation because sugar moieties play an important role in the quality control of glycoproteins (31). Although Tac_{cHu}, can be immunoprecipitated by a conformation-dependent anti-Tac Ab, the quantity precipitated diminishes with longer chase periods following metabolic labeling. This is in contrast to precipitation with the conformation-sensitive anti-HA-tag Ab that declines at a slower rate, indicating a deterioration of the structure over time (Fig. 2C). Second, the signal peptide was removed, forcing delivery to the cytosol (cytoTac), a location lacking the environment and machinery necessary for the proper folding of this protein. A similar modification to influenza HA (11) results in profound misfolding and instability. As expected, cytoTac is rapidly digested by proteinase K (results not shown) and is precipitable with the anti-HA-tag Ab but not the conformation-sensitive anti-Tac 7G7B6 Ab (Fig. 2D). Third, we used a modification described by Bonifacino et al. (32), in which L_10 within the transmembrane domain was substituted with arginine (Tac_{R10}). Although charged residues in transmembrane domains often serve to drive oligomerization, in the case of a monomeric protein such as Tac, such residues usually constitute a degradation signal (32, 33). The folding of Tac_{R10} appears to be similar to that of wild-type Tac because they are essentially identical in terms of proteinase K sensitivity and digestion patterns (Fig. 2B).

The misfolded NP-based constructs (NP_{13-498RR} and NP_{13-498Δ}) had only moderately decreased half-lives (1.8 and 2 h, respectively) compared with 2.8 h for NP_{13-498} while the half-life of Ubi-Arg-NP_{13-498} is dramatically lower (10 min) (Fig. 2E, top panel). Indeed, it has been reported that N-end rule substrates can be targeted for degradation even before translation has been completed (34). Among the Tac-based constructs, the half-life of Tac_{cHu} was identical to that of wild-type Tac (2.3 h). Despite its unstable folding noted above, this construct passes quality control within the ER and is transported to the cell surface (Fig. 2F). Tac_{R10} has a moderately decreased half-life of 30 min which is sufficient for the proper folding of its extracellular domain (Fig. 2E, bottom panel) but, as anticipated (32), this construct is not transported to the cell surface (Fig. 2F), and is likely targeted to the cytosol for degradation (18, 35–38). CytoTac was most rapidly degraded, with a half-life of ~15 min (Fig. 2E, bottom panel). Thus, among the described model Ags, only the variant with profound misfolding due to relocation within the cell (cytoTac), and those containing degrons (Ubi-Arg-NP_{13-498} and Tac_{R10}) were rapidly degraded. The other variants, more representative of the errors that would arise during transcription, splicing, or translation are degraded at a rate not much faster than that of wild-type, a remarkable fact taking into consideration that these changes do significantly impact the protein structure.

Finally, it was important to determine the degree to which defective products are made during the expression of the wild-type proteins. If rapidly degraded, the products of such errors might be undetectable by the analyses shown thus far. Most importantly, if such products are abundant, then the imposed changes might affect a relatively minor increase in the total amount of defective proteins available for class I processing. To this end, we used an approach similar to that taken by others (3). Cells were infected with the various recombinant viruses in the presence or absence of the proteasome inhibitor lactacystin. Following lysis, relative steady state levels were determined by PAGE followed by Western blotting and staining with anti-HA Abs (Fig. 3). Addition of lactacystin revealed that some truncated polypeptides were produced during biosynthesis of the wild-type NP_{13-498} but not a wild-type Tac. These truncated products would appear to have little significance in terms of epitope production as addition of a degradation signal to NP_{13-498} does not result in production of more truncated products, yet, as shown below, this modification greatly enhances epitope supply. The same is true for the Tac Ags; with the difference that in contrast to the stable Tac, destabilized Tac derivatives showed a ladder pattern consistent with multibiquitylation (Fig. 3).

**Impact on epitope presentation—the cytosolic NP-based constructs**

OVA_{257-264} epitope presentation was assessed in two ways: surface staining with Abs specific for OVA_{257-264}/H-2^Kb complexes (clone 25-D1.16) and 51Cr release cytotoxic assay in which surface complexes were limited through the use of brefeldin A treatment (39, 40). Staining with OVA_{257-264}/Kb-specific Abs revealed that the first OVA_{257-264}/H-2^Kb complexes from either NP_{13-498}, NP_{13-498RR}, or NP_{13-498Δ} were detectable after 3 h of infection while the OVA_{257-264}/H-2^Kb complexes from Ubi-Arg-NP_{13-498} are readily apparent within the first 2 h of infection (Fig. 4A). 51Cr release assay results closely correlated with the Ab staining results (Fig. 4B).

NP_{147-155} epitope presentation was evaluated by the 51Cr release assay only as NP_{147-155}/H-2^Kb-specific Ab is not available. Despite the fact that this epitope differs radically from OVA_{257-264} in being indifferent to or enhanced by proteasome inhibitor (16, 19, 22), its presentation pattern was essentially identical (Fig. 4C); the rapidly degraded, degron-containing Ubi-Arg-NP_{13-498} generated the highest levels of epitope while the misfolded versions of NP_{13-498} were indistinguishable in this regard.

**Impact on epitope presentation—the exocytic Tac-based constructs**

Within the Tac group of model Ags, the trend was similar in that only those versions that are profoundly unstable due to circumstances beyond simple misfolding allow for greater epitope production. However, after this first level of assessment, results were

**FIGURE 3.** Effect of lactacystin on the expression and degradation of the model Ags. The cells were infected with the indicated vac for 4 h with and without 50 μM lactacystin and then lysed. The lysates were separated by SDS-PAGE, Western blotted, and stained with anti-HA-tag Abs.
less straightforward. Elimination of N-linked glycosylation had no impact on presentation of either epitope (Fig. 4, D and F), in line with the lack of effect upon degradation rate. The appearance of OVA257–264/H2-Kb complexes from cytoTac and TacR10 was more rapid compared with the wild-type Tac construct, although after 4 h of infection the number of surface complexes was appreciably lower in the case of cytoTac compared with TacR10 (Fig. 4D). Given the extreme misfolded state of cytoTac, one explanation for its lower contribution of epitope could be that it is subject to degradation by other cellular proteases that might diminish the amount of substrate for “productive” proteasomal degradation. However, addition of various individual protease inhibitors of different specificities or protease inhibitor mixtures does not enhance OVA257–264 presentation from cytoTac at later time points (data not shown). Therefore, we considered the possibility that the cellular machinery associated with degron-mediated destruction produces epitope more efficiently than the machinery associated with the destruction of misfolded proteins, however profound the degree of misfolding. If true, then addition of a degron to cytoTac should enhance epitope production. To test this, we converted cytoTac to an N-end rule substrate by substituting its N-terminal Met with Arg. Ubi-Arg-cytoTac became the most unstable construct of all those tested (data not shown), but the modification increased OVA257–264 production only slightly (Fig. 4D). Thus, the efficient generation of epitope from TacR10 cannot be explained by degradation rate or qualitative aspects of degron-mediated destruction.

For OVA257–264 presentation, results were similar in 51Cr-release assay although the differences were not so prominent (Fig. 4E). Interestingly, the pattern of presentation for the NP147–155 epitope was distinct, with appreciably more efficient generation of the epitope from all three modified Tac variants (Fig. 4F) despite considerable differences in degradation rates.

Impact of premature translation termination on epitope production

It has been shown by others that brief termination of protein synthesis with puromycin increases the presentation of influenza matrix 58–66 epitope in low molecular protein-deficient cells, suggested by the authors to be due to an increase in the amount of defective products available for processing (9). Following very similar experimental conditions, we investigated the impact of puromycin on presentation of our epitopes from various constructs. Consistent with results shown above, though puromycin pretreatment induced a number of shorter products (Fig. 5A), it did not increase the level of OVA257–264 or NP147–155 presentation in either context (Figs. 5, B and C); rather, it slightly decreased the level of presentation from the more rapidly degraded constructs. Under similar experimental conditions Gileadi et al. did observe an increase in the presentation of epitope 58–66 from influenza virus Matrix protein (9), but it was in a different system, namely, in low molecular protein-deficient cells, and matrix 58–66 epitope was not presented in these cells if expressed in the context of wild-type matrix protein.

Discussion

The initial goal of this project was to test the prediction that misfolding of our constructs would differentially affect the presentation of NP147–155 and OVA257–264 based upon the opposite impact that proteasome inhibitors have on their production and the prevailing models. Thus, if misfolding leads to enhanced proteasome-dependent degradation, we anticipated increased presentation of OVA257–264 but not NP147–155. However, in almost all cases, misfolding, either through radical amino acid substitutions, substantial deletion, or puromycin treatment had no impact on generation of either epitope. Only through addition of a degron or retargeting...
from the exocytic pathway to the cytosolic pathway was epitope production consistently enhanced. This agrees with the notion proposed by others that defective proteins are not turned over immediately. Rather, the folding machinery of the cell makes several attempts at creating a functional protein, allowing for structural solutions that can compensate for some mutations (41) and possibly providing a holding area for defective proteins when higher priority substrates, such as those containing degrons, tax the degradation machinery. Although cytoTac does not contain a degron, an exocytic protein delivered to the cytosol, with limited possibilities of disulfide bonding and glycosylation, may be quickly recognized by the quality control machinery as irreparable and, with the lack of even subdomain folding, immediately targeted for disposal. This notion is supported by the work of Wong et al. who evidently observed that only extensive rearrangement of HIV gag or employment of the N-end rule resulted in enhanced degradation and increased epitope production. Less radical changes were not destabilizing, and epitope production was not increased (10). Thus, it appears that if a protein is substantially defective it can be targeted for rapid degradation without a degron. How such proteins are targeted and the frequency with which they arise can only be answered with further study. Obviously our sample size, although representing two distinct types of proteins, is relatively limited.

Assuming, for the sake of this discussion, that the types of processing substrates represented by cytoTac are relatively rare, how can our findings be reconciled with the observation that nascent polypeptides are the major source of class I-restricted peptide (3)? It has been demonstrated that both the degradation and folding machineries of the cell can access nascent polypeptides before termination of translation (34, 42). Thus there is a competition for any nascent prefolded protein between the folding and degradation machineries of the cell. We propose that epitopes are derived from the cases, likely rare, where the degradation machinery prevails. The ultimate success of a protein in terms of folding would have no bearing on this competition, explaining why most of our point mutations and deletions have no impact on epitope supply. In contrast, the degron-containing polypeptides are almost uniformly targeted for destruction either during or after translation independent of folding state.

Based upon much of the work reported here, appending a degron to an Ag may seem a reasonable strategy for enhancing T<sub>CD8</sub> activation, but the task is clearly more complex. The most rapidly degraded protein (Ubi-CytoTac) of the group we tested does not produce the highest level of epitope, indicating qualitative aspects of Ag processing that remain poorly understood. In addition, as has been shown recently, (10, 43), the relative epitope production from two versions of an Ag does not predict the relative level of T cell response, due to nebulus in vivo phenomena such as cross-presentation. Indeed, preliminary in vivo experiments with the constructs described here indicate that efficiency of epitope production is not predictive of immunogenicity (T. N. Golovina, unpublished observations). This contrasts with earlier studies where we modulated expression levels of a single model Ag, and under such circumstances epitope production is essentially predictive of the magnitude of the T<sub>CD8</sub> response (44). The collective data indicate that protein context is an important determinant of both epitope production and immunogenicity, and that these two properties are differentially impacted. A deeper understanding of this disconnect should contribute significantly to the rational engineering of Ags to optimize T<sub>CD8</sub> responses.

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2769


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