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Substrate-Induced Protein Stabilization Reveals a Predominant Contribution from Mature Proteins to Peptides Presented on MHC Class I

Jeff D. Colbert, Diego J. Farfán-Arribas, and Kenneth L. Rock

The origin of the MHC class I–presented peptides are thought to be primarily from newly synthesized but defective proteins, termed defective ribosomal products. Most of the data supporting this concept come from studies in which inhibitors of protein synthesis were found to rapidly block Ag presentation even when cells contained a pool of mature proteins. However, these data only indirectly address the origin of presented peptides, and in most studies, the contribution of mature functional proteins to the class I peptide pool has not been directly quantified. In this report, we address the efficiency and contribution of mature proteins using a tetracycline-inducible system to express Ags that are conditionally stabilized upon ligand binding. This system circumvents the use of general inhibitors of protein synthesis to control Ag expression. Moreover, by controlling Ag stabilization, we could investigate whether the degradation of mature Ags contributed to Ag presentation at early and/or late time points. We show that mature proteins are the major contributor of peptides presented on class I for two distinct antigenic constructs. Furthermore, our data show that the protein synthesis inhibitors used previously to test the contribution of defective proteins actually block Ag presentation in ways that are independent from blocking Ag synthesis. These data suggest that for the constructs we have analyzed, mature functional proteins, rather than defective ribosomal products, are the predominant source of MHC class I–presented peptides. The Journal of Immunology, 2013, 191: 5410–5419.

Major histocompatibility complex class I molecules display peptides predominantly derived from a cell’s endogenously synthesized proteins. This feature allows CD8+ T cells to monitor the genes expressed in cells and detect ones that are mutated or of viral origin. A majority of these MHC class I–presented peptides are generated from the hydrolysis of endogenously expressed proteins by proteasomes (1). Because the proteasome is responsible for degrading the majority of endogenously synthesized proteins (2), it was originally thought that class I–presented peptides were generated as the proteome was catalyzed by the ubiquitin-proteasome pathway (3).

Within the proteome, different proteins are turned over at different rates, resulting in half-lives that vary from minutes to days. It was suggested that if turnover was the source of presented peptides, then the immune system would be slow to detect and respond to stable proteins with long lifetimes. However, it was noted that soon after infection, the immune system recognizes and responds very rapidly to many stable viral proteins (4). This led to the idea that there might be a mechanism for rapidly generating and presenting peptides from otherwise stable proteins (5).

It was hypothesized that rapid presentation of peptides from all Ags could occur if mistakes took place during protein synthesis that led to the rapid degradation of a fraction of all newly synthesized proteins. Indeed, it was known that the proteasome rapidly hydrolyzes abnormal proteins, including ones that have abnormal sequence (e.g., from puromycin incorporation or from nonnatural amino acids like canavanine), or those that are misfolded, mislocalized, or prematurely terminated (6). It was initially proposed that such mistakes would come from inherent defects in translation. Such products were called defective ribosomal products (DRiPs) (5). Key to the DRiP hypothesis is the idea that these immature and defective proteins are rapidly degraded by the proteasome within minutes of their synthesis (7). Although presentation from defective nascent proteins has been documented, the frequency of these errors under natural conditions and their contribution to peptides presented on class I remain controversial (8, 9).

Much of the strongest evidence that DRiPs contribute to Ag presentation came from experiments evaluating the effect of terminating protein synthesis on the generation of presented peptides. Upon cessation of Ag synthesis, it was observed that Ag presentation very rapidly stopped even if cells contained a large pool of previously synthesized mature Ag (7, 10–14). This seemed to indicate that presented peptides were coming from newly synthesized Ags rather than older, mature proteins. It was estimated that the half-life of the newly synthesized Ags contributing to Ag presentation was <5 min and that this was independent of the stability of the mature protein. These results were interpreted to indicate that newly synthesized Ags, rather than the proteome, were the dominant source of presented peptides (7, 10, 11, 15).

Other findings, such as the observation that Ag presentation coincides with the appearance of viral proteins in cells (16), have
be interpreted to support the DRIP model. However, most of the data in support of DRIPs being the dominant source of processed peptides are indirect. Likewise, few data directly quantify the efficiency of presentation from newly synthesized versus old Ags or from DRIPs versus functional proteins. To address these issues, we have used an Ag expression system that allows for the control of Ag expression and turnover without affecting other cellular components. In addition, we address the effects of inhibitors of protein synthesis on presentation of peptides from mature proteins. Our data demonstrate that mature proteins are the primary and highly efficient source of peptides presented on class I molecules for the two constructs we have analyzed. Furthermore, we show that inhibitors of protein synthesis that were previously used to conclude that DRIPs contribute to class I presentation block MHC class I presentation by interfering with steps beyond Ag synthesis.

Materials and Methods

Cell lines and constructs

The hamster cell line E36 and human cell line HeLa expressing the murine class I MHC H-2 Kβ were described previously (hereafter, E36 Kβ and HeLa Kβ) (17, 18). Also used in the current study is the murine lymphoma EL4, which endogenously expresses H-2 Kβ. For the copepod GFP (copGFP) (19) and expressed in the vector pPItuner (Clontech). The immunodominant class I epitope from ovate, SIINFEKL (S8L), and terminal HA-tag were incorporated into primers at the C-terminus end of the fusion. The final product was then cloned into the lentiviral vector pTRIPz (Open Biosystems) following digestions with AgeI and MluI. FKBP was removed from the above vector to form ΔcopGFP by overlapping PCR. For the EGFP construct, fluorescent protein was amplified from the pEGFP N1 vector (Clontech), and the S8L epitope was incorporated into overlapping primers used to fuse FKBP to EGFP. A single HA-tag was included upstream of EGFP and downstream of pTRIPz. A diagram illustrating these two Ag constructs is shown in Fig. 1A and Fig. 2A. Cells expressing Ag constructs cloned into pTRIPz were selected with puromycin following lentiviral transduction. E36 Kβ cells transfected with plasmids (pDNA 3.1) carrying FKBP-S8L-EGFP without the tetracycline (Tet)–inducible promoter were selected with hygromycin. Clones were generated in E36 Kβ and HeLa Kβ cells by limiting dilution or colony lift and screened for GFP expression and 25-D1.16 staining (described below) following culture in 1 μg/ml doxycycline (Dox) (Sigma-Aldrich) and 5 μM Shield (CheminPharma). Transduced EL4 cells were sorted by flow cytometry for both EGFP expression and for presentation of S8L in the context of Kβ using the 25-D1.16 Ab.

MHC class I Ag presentation

Ag presentation was monitored on a single-cell level using the mAb specific for Kβ-S8L complexes (25-D1.16) (20). Total GFP expression was monitored by Abs targeting the C-terminally fused HA epitope (Cell Signaling) by Western blot and by flow cytometry for folded GFP expression. Flow cytometry data were collected on an LSRII instrument using FACS DIVA software (Becton Dickinson) or on a FACS Calibur using CellQuest software (Becton Dickinson). Data were analyzed from live cell gates using FlowJo software (TreeStar). Geometric mean fluorescence intensities (MFIs) of Ags were then subjected to acid stripping (0.132 M citric acid, 0.06 M ethanol and 0.1% DMSO). Cells were analyzed for GFP expression and 25-D1.16 MFI as described above and plotted in Prism (GraphPad). Percent inhibition of Ag presentation was determined by comparing 25-D1.16 MFI expressed in cells treated with Dox alone with 25-D1.16 MFI expressed in those treated with 8–16 μM Shield, as indicated.

For class I presentation of mature protein in the absence of protein synthesis, GFP expression in E36 Kβ cells was induced with 0.1 μg/ml Dox and 5 μM Shield in RPMI for 24 h. Cells were then washed with cold RPMI and exposed to 5 μM Shield alone (in the absence of Dox to stop newly synthesized Ags) for another 24 h. Cells were then subject to acid strip to remove preformed surface Kβ-S8L complexes, as described above. Cells were further cultured in RPMI containing either 5 μM Shield alone or 10 μM MG132 (Enzo) alone, or containing the carrier controls 0.02% ethanol and 0.1% DMSO. Cells were analyzed for GFP expression and presentation of S8L (25-D1.16), as described above.

Efficiency of class I presentation

To determine the efficiency of Ag presentation from old proteins compared with newly synthesized proteins, E36 Kβ cells expressing copGFP were cultured in the presence of 0.1 μg/ml Dox, 5 μM Shield, and 10 μM MG132 over time. The time required to generate equivalent copGFP proteins (in MFI) during synthesis (in the presence of Dox), compared with an old cohort of proteins that had been stabilized with Shield in the absence of synthesis (in the absence of Dox), was noted. In parallel, we followed the generation of Kβ-S8L complexes from newly synthesized proteins by culturing cells in 0.1 μg/ml Dox alone over time. Next, we quantified Kβ-S8L complexes generated during Ag synthesis at the times at which equivalent copGFP was expressed, as described above. We added the time required for newly formed Kβ-S8L complexes to transit from the endoplasmic reticulum (ER) to the cell surface (30 min) (Supplemental Fig. 2B). Ag presentation in the absence of synthesis (from old proteins) was compared with Ag presentation from newly synthesized (25-D1.16 MFI) and calculated as percent efficiency of class I presentation from mature proteins.

An alternative way of measuring the presentation efficiency from newly synthesized proteins compared with the efficiency of the turnover of mature proteins in the absence of synthesis was as follows. E36 Kβ cells expressing copGFP were induced with Dox (50, 100, or 150 ng/ml) in the presence or absence of 1 μM Shield. Induction was started at 20-min intervals of mixing. For each set of cultures, cells were then harvested at indicated time points containing the drugs mentioned above. Cells were harvested after induction times of 180, 200, 220, 240, 260, 280, and 300 min. Thus the time range covered was 120 min. This short induction experiment allowed us to calculate the efficiency of presentation from newly synthesized protein over a 2-h period. The samples with Shield were used to calculate the amount of protein synthesized and degraded (GFP MFI), whereas the samples without Shield provided the corresponding S8L presentation levels (25-D1.16 MFI). The efficiency of presentation was assessed by plotting S8L presentation versus GFP fluorescence for all time points, and data points were fit by linear regression analysis using Prism (GraphPad). For mature proteins in the absence of synthesis, Ags were induced and stabilized for 24 h and then cultured in Shield alone for an additional 24 h, as mentioned above. After this, cells were acid stripped and kept on ice. Every 20 min, cells were transferred to media containing a low concentration of Shield (0.1 μM) to allow for the linear degradation of the protein pool previously built up. The degradation times were 60, 80, 100, 120, 140, 160, and 180 min, covering a range of 2 h, as in the short induction experiment described above. All cells were stained with 25-D1.16 to assess presentation of S8L. Cells were fixed in 4% PFA for 5 min, washed with PBS, and then analyzed in a FACSCalibur flow cytometer (BD Biosciences). With FlowJo software, GFP+ cells were gated and their geometrical MFIs for GFP and 25-D1.16 were obtained. Linear regression lines were fitted using Prism. The plots comparing the presentation efficiency from these experiments are shown in Supplemental Fig. 2C–F.

Loading of exogenous Ags

E36 Kβ and EL4 cells were pretreated for 1 h with 20 μM (11.1 μg/ml) emetine (Calbiochem), 40 μM (11.4 μg/ml) cycloheximide (CHX) (Sigma-Aldrich), and 100 μM (11.4 μg/ml) DMSO. OVA257–264 was introduced into E36 Kβ and EL4 cells by hypertonic loading followed by osmotic lysis in hypotonic media (22). Alternatively, in this same experimental design, 2 μg OVA was loaded into cells using the PULSin protein delivery reagent (Polyplus Transfection) in accordance with the manufacturer’s instructions, in place of hypertonic loading. Following Ag loading, cells were then incubated for 90 min in the presence or absence of the inhibitors described above. Alternatively, cells were then subject to acid strip to remove preformed surface Kβ-S8L complexes, as described above. Cell viability was assessed by trypan blue exclusion and was found not to be affected by the treatment conditions noted above. Cells were then washed extensively to remove inhibitors and treated with 5 μg/ml BFA to prevent...
the egress of new peptide-loaded class I molecules out of the ER. Protein synthesis inhibitor treatment never exceeded 2.5 h in the experiments listed above.

Ag presentation of exogenous peptides was performed as described previously (21). Briefly, E36 Kb and EL4 cells were pretreated for 1 h using the same inhibitor treatment listed above. Performed class I was then removed by acid stripping or left untreated. Next, cells were treated with inhibitors for an additional 90 min, followed by washing in cold PBS and fixation in 4% PFA. Fixed cells were left alone or exposed to increasing concentrations of S8L peptide, as indicated, for 30 min at room temperature, followed by extensive washing to remove unbound peptide.

Ag presentation was detected by coculturing APCs overnight with the T cell hybridoma RF33-Luc, as described. Luciferase activity was determined using the luciferase assay kit (Promega) as described and read on a FLUOstar OPTIMA plate reader (BMG Labtech). The efficiency of Ag loading was assessed by immunoblotting E36 Kb cell lysates for intracellular OVA. For hypertonic loading, mock loaded cells were subjected to the same conditions in the absence of Ags. As a control, cells were also cultured with OVA in normal media. For PULSin-treated cells, control samples were treated in the presence of OVA without the PULSin reagent. None of the control conditions resulted in luciferase activity above background.

**Real-time PCR**

E36 Kb cells expressing copGFP were cultured for 24 h in the presence or absence of 1 μg/ml Dox and 5 μM Shield. Cells were then washed in PBS and cultured in the absence of Dox over time. RNA was harvested using the Qiagen RNaseasy kit (Qiagen) and DNase I (Qiagen). cDNA was then synthesized using the iScript cDNA synthesis kit (Bio-Rad). Real-Time PCR was performed with iQ SYBR Green Supermix (Bio-Rad) containing 25 ng cDNA from each sample using the iCycleer from Bio-Rad. Specific primers used include cop-GFP (5′-TATGCGCTACCCTTCTAC-3′) and 5′-CCTGCTGTCGTTGAAG-3′; hypoxanthine phosphoribosyltransferase (HPRT) (5′-ACCAGTCAACAGGGGACATAAA-3′), hypoxanthine phosphoribosyltransferase (HPRT) (5′-ACCAGTCAACAGGGGACATAAA-3′), hypoxanthine phosphoribosyltransferase (HPRT) (5′-ACCAGTCAACAGGGGACATAAA-3′), and 5′-GTCTGCAATGTCTTTGCCAGTGC-3′, and 18S RNA (5′-CAGCCAGCCGAGATTTGACCA-3′ and 5′-TAGTGACCCGACCGGCTTG-3′). Samples were normalized to either 18S RNA or HPRT expression at each time point. Fold expression was determined by comparing Dox-induced samples with samples cultured without Dox.

**Western blot**

Samples were washed in PBS and lysed in cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% [v/v] Triton X-100, 1% [w/v] sodium deoxycholate, 0.1 [w/v] % SDS, pH 7.4) with Complete Protease Inhibitor mixture (Roche). Quantitation of total protein was performed by bicinchoninic acid protein assay (Pierce). Proteins were separated on 10 or 12% NuPAGE gels before transfer. Membranes were probed for expression of copGFP (anti-HA, Cell Signaling Technology), or OVA (MP Biomedicals). GAPDH Abs (Millipore) were probed on the same membranes as the Ags to confirm equivalent total protein loaded.

**Results**

**Inhibition of Ag presentation by ligand-induced protein stabilization**

To address the contribution of mature proteins to class I presentation, we developed an experimental system that allowed us to 1) selectively and reversibly control Ag synthesis without affecting synthesis of other cellular proteins, 2) measure the fraction of mature protein and its turnover, 3) quantitatively distinguish presentation from defective protein versus mature functional protein, and 4) determine the efficiency of presentation from Ags at various times after synthesis. Our approach took advantage of a mutant form of FKBP that was rapidly degraded by the proteasome even when expressed as a fusion protein. Importantly, rapid degradation of the mature FKBP fusion could be blocked by the addition of a fully reversible cell-permeable ligand, called Shield (19). To bind Shield, these constructs must be properly folded and therefore by definition are not DRiPs (11, 19). Shield was shown to selectively stabilize FKBP fusions without affecting other cellular processes (19, 23, 24) and has been used by others to study Ag presentation (11, 12, 25). Therefore, we fused FKBP to copGFP, allowing for us to follow mature proteins by monitoring GFP fluorescence, a process requiring precise folding for the formation of the fluorophore (26). To monitor class I Ag presentation, we introduced the T cell epitope S8L from OVA at the C terminus of the fusion protein followed by a terminal HA-tag that was used to monitor Ag expression regardless of protein conformation (Fig. 1A). Finally, the presence of an inducible transcriptional activator (Tet) allowed for specific and reversible activation of Ag transcription in response to Dox (Supplemental Fig. 1A).

We transduced E36 cells, expressing the murine class I molecule H-2 Kb, with lentivirus carrying the copGFP fusion construct (fusion protein referred to as copGFP hereafter) and isolated clones with Dox-inducible GFP expression. To assess the effects of protein stabilization on Ag presentation, we induced the expression of the copGFP fusion with Dox and titrated Shield for 1 h, a time when DRiPs might be expected to be a more dominant source of presented peptides. As shown in Fig. 1B (left panel), increasing the concentration of Shield resulted in a concomitant increase in protein stabilization, as shown by the increase in copGFP fluorescence (Fig. 1B, black) and immunoreactive protein levels (Supplemental Fig. 1B). These data fit well with previous work demonstrating protein stabilization of FKBP fusions with Shield (19). Importantly, protein stabilization was accompanied by a dose-dependent inhibition of class I presentation, as assessed by staining cells with an Ab (25-D1.16) recognizing S8L bound to the murine class I molecule H-2 Kb (Fig. 1B, white). We examined whether inhibition continued over 3 h (Fig. 1B, middle panel) and 6 h (Fig. 1B, right panel) following induction of protein synthesis. Multiple experiments revealed that the inhibition of class I presentation at 1 h was 90.3% (±5.2% SEM) and 93.0% (±3.3% SEM) at 3 h, with slightly lower inhibition occurring at the later time points (Fig. 1C).

The striking inhibition of class I presentation in the presence of Shield, we sought to confirm that Shield was specifically affecting our FKBP–copGFP fusion and not other processes required for Ag presentation. To do this, we made an identical construct shown in Fig. 1A, except lacking the FKBP domain (ΔcopGFP) and induced expression of this variant construct in the presence of increasing concentrations of Shield. As expected, addition of Shield had no effect on GFP fluorescence from this construct lacking the Shield binding domain (Fig. 1D, black). Similarly, Ag presentation was also unaffected by the addition of Shield (Fig. 1D, white), indicating that the effects observed in Fig. 1B were due only to the stabilization of FKBP with Shield and not a pleotropic effect of this agent. Consistent with this conclusion, we also found that Dox and Shield did not induce cellular stress, as assayed by the induced splicing of the transcription factor XBP-1 (27) (Supplemental Fig. 1C); affect cell viability/metabolic activity, as measured by MTS assay (Supplemental Fig. 1F); or inhibit expression of class I molecules (Supplemental Fig. 1E). Our data confirmed what other groups have already shown concerning the target specificity and lack of cellular toxicity from these agents (19, 23, 24, 28). Together, these data show that when mature proteins are selectively stabilized by Shield, Ag presentation is markedly inhibited, demonstrating a predominant contribution of this pool of functional (ligand-binding) proteins to class I presentation.

**Source of protein and epitope location do not affect presentation from mature proteins**

The observation that mature proteins are the primary source of MHC class I–presented peptides is at variance with an earlier study that used a similar experimental approach. In this report, Dolan et al. (11) showed that stabilizing mature proteins in EL4 cells reduced Ag presentation by a much smaller degree (±36%). One difference in this earlier study was the use of EGFP rather than copGFP as the source of Ag. These are very different proteins,
with the copepod (*Pontellina plumata*) sequence having only 19% amino acid identity with the jellyfish (*Aequorea victoria*) GFP (29). Other differences include the incorporation of the peptide S8L at the opposite end of the fluorescent protein and very different sequence flanking the epitope (Fig. 2A). These differences raised the possibility that the contribution of DRiPs versus functional mature proteins to class I presentation could vary based on the protein and/or epitope location. To test this possibility, we generated a new construct that was essentially identical to the one used by Dolan (Fig. 2A, EGFP) (11). E36 Kb clones were isolated as described for the copGFP-expressing cells. Surprisingly, we observed a similar effect of Shield on protein stabilization and Ag presentation that we observed for copGFP. As shown in Fig. 2B, E36 Kb cells expressing EGFP displayed a pronounced increase in protein stabilization (black) and a corresponding inhibition of Ag presentation (white) with increasing concentrations of Shield (83.6 ± 4.2% SEM). These data further demonstrate that the source of Ags and location of epitopes do not influence the contribution of mature proteins to class I presentation, at least for these two constructs.

Next, we assessed whether cell type or species influenced the contribution of mature proteins to Ag presentation in this experimental system. We transduced the murine lymphoma cell EL4 with an inducible construct expressing the EGFP fusion described previously [same cells and Ags as used by Dolan et al. (11)] and determined whether increasing concentrations of Shield inhibited

![Figure 1](image1.png)

**FIGURE 1.** Loss of MHC class I Ag presentation upon Ag stabilization. (A) Illustration of the construct used to follow protein maturation and Ag presentation. (B) Kinetics of copGFP expression (black, GFP MFI) and S8L presentation (white, Kb:S8L MFI) in E36 Kb cells at 1 h (left panel), 3 h (middle panel), and 6 h (right panel) following the induction of protein synthesis (Dox) and protein stabilization in the absence (0 μM) or presence of increasing concentrations of Shield. Graphs shown are representative of at least three independent experiments. (C) Quantitation of the percent Ag presentation inhibited after Dox induction in the presence of increasing concentrations of Shield at 1, 3, and 6 h after induction. Results shown are the mean ± SEM comparing untreated cells (Dox alone with 0.05% ethanol) with cells treated with 8 μM Shield (n = 3). (D) Shield titration in E36 Kb cells expressing copGFP without FKBP (ΔcopGFP). GFP fluorescence (black) and Kb:S8L expression (white) are shown from a representative experiment (n > 3).

![Figure 2](image2.png)

**FIGURE 2.** Effects of Shield are independent of the expressed protein, location of the epitope, or cell type expressing the Ag. (A) Illustration of constructs used to test whether the origin of proteins or location of epitopes alters class I presentation from mature proteins. (B) E36 Kb cells expressing EGFP were induced with 0.1 μg/ml Dox and stabilized with increasing concentrations of Shield for 3 h, as described in Fig 1B. (C) EL4 cells expressing EGFP were induced with 0.25 μg/ml Dox for 3 h or HeLa Kb cells expressing copGFP (D) were induced with 0.1 μg/ml Dox for 3 h with an increasing concentration of Shield. (E) EL4 cells expressing EGFP were induced with 0.5 μg/ml Dox alone over time. Preformed MHC class I molecules were removed by acid stripping, followed by culture with Dox and increasing concentrations of Shield for the final 3 h. Shield titrations in cells pretreated for 72 h with Dox (left panel) or without pretreatment (right panel) are shown. (F) Statistics from three independent experiments performed at 3, 24, 48, and 72 h, as described in (E), are shown (mean ± SEM). GFP fluorescence (black) and S8L presentation (white) are shown in (B)–(E) from representative experiments (n = 3).
Ag presentation. Surprisingly, and in contrast to the previous report, Ag presentation of S8L in EL4 cells was strongly inhibited (92.2 ± 3.9% SEM) (Fig. 2C). Similar results were observed in human cells (HeLa, 80.5 ± 3.2%) expressing murine class I MHC K\(^b\) and the Ag construct copGFP (Fig. 2D). Therefore, for the constructs we have analyzed, mature proteins make a major contribution to Ag presentation in both human and rodent cells, regardless of the protein source or location of the epitope.

Another difference between our experiments and those of Dolan et al. is that we added Shield at the same time that Ag synthesis was initiated, whereas Dolan et al. (11) added Shield just after acid stripping cells that had been continuously synthesizing Ags. To investigate whether continuous Ag expression for one to several days might account for the differences shown in this article and those reported previously, we induced Ag synthesis for up to 72 h, then acid stripped the cells and added both Dox and Shield. Under these conditions, we observed less Shield-induced inhibition of Ag presentation (Fig. 2E), similar to the findings of Dolan et al. (11). These data suggest that over time, some Ag accumulates that cannot be stabilized by Shield. However, because this Shield-resistant component is not seen upon the initiation of synthesis but takes >6 h to accumulate, it is by definition almost certainly not arising from DRiPs (Fig. 2F).

**Do old, fully mature proteins contribute to MHC class I presentation in the absence of new Ag synthesis?**

Thus far, our data have shown that stabilization of a mature protein during protein synthesis (with Dox) results in the inhibition of class I Ag presentation. However, as stated above, previous experiments from several groups have shown that blocking Ag synthesis rapidly inhibits MHC class I Ag presentation despite the presence of a pool of previously synthesized Ags (7, 10–14). These data have been offered as support for the importance of DRiPs to Ag presentation and, in any case, have suggested that MHC class I–presented peptides come predominantly from newly synthesized Ags rather than older proteins. Our experimental system allowed us to examine this proposition in the absence of protein synthesis inhibitors because we could stabilize a cohort of proteins in the presence of Shield and then examine whether it could contribute to Ag presentation long after it was synthesized. To do this, we induced and stabilized copGFP by culturing cells in the presence of Dox and Shield for 24 h to build up a pool of fully mature proteins. Next, we removed Dox to stop further synthesis of the Ags and continued to culture cells in the presence or absence of Shield. Fig. 3A shows the dramatic loss of copGFP fluorescence following the removal of Dox and Shield (white), but not in the continued presence of Shield alone (black), indicating that Shield stabilized the mature proteins for an extended period after synthesis was terminated. Although nearly all of the proteins were lost within 6 h (with a half-life of 1.2 h) following the removal of Dox and Shield, remaining mRNA transcripts may further contribute to protein expression (30). Therefore, we measured the lifetime of copGFP mRNA following the removal of Dox by quantitative RT-PCR. Fig. 3B shows that mRNA was degraded to background levels by 18 h after Dox had been withdrawn, indicating that proteins must be stabilized for at least 18 h to have a cohort of fully mature proteins without any newly synthesized Ag molecules.

With this information, we designed experiments aimed at determining the ability of an old cohort of mature proteins to contribute to MHC class I presentation (Fig. 3C). E36 K\(^b\) cells were induced to express copGFP and to stabilize its expression with Shield for 24 h. Next, Dox was removed to selectively stop transcription of the Ag construct and the culture was continued in the presence of Shield to allow the Dox-induced mRNA to degrade, whereas the old, mature proteins were stabilized for an additional 24 h. To exclusively address the role of mature proteins in Ag presentation, we first removed preformed surface class I–peptide complexes that were generated during the initial buildup and stabilization of Ags. This treatment had no effect on stabilized GFP expression (Fig. 3D, right panel) but was efficient at removing the preformed complexes (Fig. 3D, left panel). Next, we either continued to stabilize proteins by culturing cells in Shield alone (Shield), withdrew Shield and allowed the Ags to degrade (DMSO), or cultured cells in the presence of proteasomal inhibitor (MG132) to inhibit Ag degradation by the proteasome for an additional 6 h. As shown in Fig. 3E, treatment with Shield alone continued to stabilize mature proteins (black, right panel). Under these conditions, the stabilized mature proteins did not contribute to class I presentation (compare Fig. 3D with Fig. 3E, gray), as expected. In contrast, when Shield was removed and replaced with control media (DMSO), the Ags were degraded and K\(^b\):S8L complexes were rapidly generated (Fig. 3E, left panel, and Supplemental Fig. 2A). As predicted, the dramatic loss of copGFP fluorescence occurred due to proteasomal degradation, because in the presence of MG132, protein turnover and presentation of the Ags were completely inhibited (Fig. 3E, MG132). Overall, these data show that long after new Ag synthesis (24 h following Dox removal), mature proteins can contribute peptides to class I presentation.

**Presentation of mature proteins is a highly efficient source of antigenic peptides**

We next sought to determine whether any difference could be noted in the efficiency of generating presented peptides from newly synthesized proteins versus older mature proteins in the absence of protein synthesis, as shown in Fig. 3E. To accomplish this, we first followed new copGFP expression over time by measuring fluorescence in the presence of Dox, Shield, and MG132 (to prevent degradation) (Fig. 3F, left panel). In parallel to the kinetics of protein expression, we measured the amount of K\(^b\):S8L complexes generated from the newly synthesized proteins when they were allowed to degrade in the absence of Shield (Fig. 3F, right panel). As described in detail in Materials and Methods, the time required to achieve equivalent proteins from the newly synthesized proteins compared with the old cohort of Ags was used to determine the efficiency of class I presentation from these pools of proteins. Our data demonstrate that class I presentation from Ags processed and presented during synthesis (Fig. 3F, 758 K\(^b\):S8L MFI) was as efficient as presentation from old, mature Ags in the absence of synthesis (Fig. 3E, 749 K\(^b\):S8L MFI). The data presented in Supplemental Table I details the efficiency of Ag presentation from old, mature proteins compared with newly synthesized proteins (97.5 ± 2.9% SEM, n = 3).

We also assessed the efficiency of Ag presentation from old and newly synthesized proteins by comparing the rate at which K\(^b\):S8L complexes appeared on the cell surface as the copGFP protein was degraded over a 2-h period (see Materials and Methods for full details). The results clearly show the presentation efficiencies are essentially no different, indicating that mature proteins are as efficient a substrate for class I presentation as rapidly degraded, newly synthesized proteins for the Ags tested in this study (Supplemental Fig. 2E, 2F).

**Ag-independent effects of protein synthesis inhibitors on Ag presentation**

Our data are at variance with previous reports suggesting that newly synthesized defective proteins are the main contributor to class I presentation (7, 10, 11, 15) and that the contribution of mature proteins to class I presentation is inefficient (11, 13). Many reports supporting the role of DRiPs as a primary source of class I–presented
FIGURE 3. Ag presentation from old proteins are as efficient as newly synthesized proteins. (A) E36 K\textsuperscript{b} cells expressing copGFP were cultured for 24 h with 0.1 \mu g/ml Dox and 5 \mu M Shield. Dox and Shield were removed by cold PBS wash, and then the culture continued in the presence of 5 \mu M Shield alone (black) or in control media (0.02\% ethanol) (white) for 6 h. Cells were harvested at the given times to assess GFP fluorescence by flow cytometry. (B) E36 K\textsuperscript{b} cells expressing copGFP were cultured as described in (A). Cells were harvested at the given times, and copGFP mRNA expression was assessed by quantitative RT-PCR, as described in Materials and Methods. Samples were normalized to 18S RNA or HPRT expression and graphed as fold over the untreated control (−). Values shown are means (± SEM) for three independent experiments. (C) Experimental scheme used to analyze the role of mature proteins contributing to class I presentation in the absence of Ag synthesis. (D) Buildup of total fluorescence (GFP MFI, black) and Ag presentation (K\textsuperscript{b}: S8L MFI, gray) over 48 h before (−) or after (+) the removal of preformed class I peptide complexes by acid stripping. (E) Class I presentation in the absence of synthesis. Cells were cultured for an additional 6 h following acid strip in the presence of 5 \mu M Shield alone (Shield), 10 \mu M MG132 alone (MG132), or 0.1\% DMSO and 0.1\% ethanol control (Control). Changes in GFP fluorescence (GFP MFI, black) and class I presentation (K\textsuperscript{b}:S8L MFI, gray) from mature proteins are shown as the mean of triplicates (± SEM) from one of three independent experiments. (F) Efficiency of Ag presentation from mature proteins. The kinetics of new protein expression was determined by culturing E36 K\textsuperscript{b} cells in the presence of 0.1 \mu g/ml Dox, 5 \mu M Shield, and 10 \mu M MG132 (F, left). The kinetics of presentation was determined by culturing cells in 0.1 \mu g/ml Dox alone (F, right) over 6 h. Proteins stabilized in the absence of protein synthesis (E, 1) were compared with new proteins generated during protein synthesis (F, 2). The time required to generate equivalent levels of proteins (F, 3) plus 30 min for transport of complexes from the ER (F, 4) was used to determine the number of K\textsuperscript{b}:S8L complexes formed in this time (F, 5). Efficiency of presentation from mature proteins was calculated by dividing K\textsuperscript{b}:S8L complexes (MFI) formed from old proteins (E, 6) in the absence of synthesis by those formed during synthesis (F, 5). Data shown are one representative experiment from three. Refer to Supplemental Table I for the remaining experimental data.

peptides have relied on protein synthesis inhibitors to form these conclusions (7, 10–14). In these reports, it was found that inhibitors of protein synthesis quickly blocked Ag presentation despite the presence of a pool of mature Ags in the cell, implying that presented peptides came only from newly synthesized proteins rather than old, mature proteins. However, this conclusion assumed that blocking protein synthesis did not inhibit other cellular processes necessary for Ag presentation apart from Ag synthesis. Therefore, we examined whether inhibiting protein synthesis had any effect on Ag presentation in situations in which the Ags were pre-existing (mature) and did not require continued synthesis. We reasoned that if these inhibitors are affecting only Ag synthesis, as previously believed, then they should not block class I presentation from fully mature proteins, as shown in Fig. 3E.

To test this, we subjected E36 K\textsuperscript{b} cells to increasing amounts of the irreversible inhibitor of protein synthesis emetine, or the reversible inhibitor CHX, to determine the dose necessary for complete inhibition of Ag synthesis. As expected, emetine concentrations as low as 20 \mu M (11.1 \mu g/ml) and CHX concentrations as low as 40 \mu M (11.4 \mu g/ml) were sufficient to completely block copGFP expression (Supplemental Fig. 3A) without inducing general cellular toxicity (Supplemental Fig. 1C, right panel, and 1D, right panel). These conditions and results are similar to those published previously (31, 32). Next, we expressed a cohort of Shield-stabilized mature proteins in the absence of new Ag synthesis, as described in Fig. 3C (Fig. 4A). Cells with stabilized Ag were then treated with inhibitors of protein synthesis or a carrier control (0.04\% DMSO) for 1 h in the continued presence of Shield. Following removal of class I:peptide conjugates by acid stripping, the cells were further cultured in control medium (DMSO), or in the presence of protein synthesis inhibitors, and Shield was withdrawn to allow the cohort of old, mature proteins to degrade. For reasons that are currently unclear, we consistently observed an increase in protein levels with cells cultured in the presence of emetine (black) compared with control-treated cells (white) (Fig. 4B, left panel). However, importantly, protein synthesis inhibitors blocked class I presentation by 60–80\% (Fig. 4B, middle panel), despite the fact pre-existing mature proteins were the only source of Ags.

We extended these studies to another situation in which the presented Ags were not synthesized by the APCs, but in this case introduced by loading exogenous Ags into the cytosol (22). E36 K\textsuperscript{b} cells not expressing the Ags were first pretreated in the presence of emetine or a carrier control (0.04\% water) for 1 h, followed by incubation with a hypertonic solution containing mature OVA. Loaded Ags were then released into the cytosol by culturing cells in hypotonic media (22). Ag-loaded cells were cultured for an additional 90 min in the presence of emetine to allow for processing of mature proteins. Presentation of new peptides was stopped by the addition of BFA, and cells were cultured for 18 h with a T cell hybridoma specific for K\textsuperscript{b}:S8L (RF33-Luc) (21). As shown in Fig. 4C, E36 K\textsuperscript{b} cells treated with OVA in the presence of emetine (black square) showed a nearly complete inhibition of
was then released into the cytosol by osmotic lysis of endosomes, as described in Materials and Methods. Co-cultured with the T cell hybridoma RF33-Luc for 18 h, and NFAT–luciferase activity was assessed by luminescence (RLU) (n = 5). The presentation of mature proteins in the absence of Ag synthesis. (B) E36 Kb cells were pretreated for 2 h with control media (DMSO, white), 40 μM CHX (gray), or 20 μM emetine (black), as indicated. Cells were acid stripped and then cultured again in control media (0.04% DMSO), 40 μM CHX, or 20 μM emetine for 6 h. GFP expression (left panel), Kb:SSL expression (center panel), and total class I levels (right panel) were assessed by flow cytometry from samples taken every 60 min. Data shown are representative of one of three independent experiments. (C) E36 Kb cells pretreated with 20 μM emetine (squares) or 0.02% water control (circles) were subjected to hypertonic loading in the presence of mature OVA (black) or control (white). Full-length OVA was then released into the cytosol by osmotic lysis of endosomes, as described in Materials and Methods. Ag-loaded E36 Kb cells were diluted 2-fold and cocultured with the T cell hybridoma RF33-Luc for 18 h, and NFAT–luciferase activity was assessed by luminescence (RLU) (n = 5). (D) E36 Kb cells were pretreated as described above and then loaded with OVA using the PULSin protein delivery reagent, as described. Symbols are the same as described in (C) (n = 3). (E) EL4 cells were treated with 40 μM CHX (squares) or 0.1% DMSO (circles) for 1 h prior to hypertonic loading of OVA (black), as described for E36 Kb cells in (C) (n = 3). (F) E36 Kb cells (left) and EL4 cells (right) were pretreated for 60 min with 0.1% DMSO (white), 40 μM CHX (gray), or 20 μM emetine (black). Cells were subjected to acid stripping to remove preformed MHC class I and followed by an additional 90 min treatment in the presence of the inhibitors listed above. Cells were then washed and fixed with 4% PFA, pulsed with SSL peptide titrated by 3-fold serial dilutions, as shown. Unbound peptide was removed by extensive washing, and then cells were cocultured with RF33-Luc overnight (n = 3).

Ag presentation compared with those exposed to Ags in the absence of emetine (black circle). To rule out the possibility that emetine treatment impaired osmotic loading of the Ags into these cells, we lysed cells following Ag loading and performed Western blot analysis. Supplemental Fig. 3B shows that loading was not affected by inhibitor treatment but that protein levels were somehow stabilized in the presence of emetine. These data are reminiscent of the increase in protein levels observed previously when emetine was present (Fig. 4B, left panel). We used another method to introduce full-length protein into E36 Kb cells (PULSin protein delivery reagent), but at a lower protein concentration, and observed the same inhibition in the presence of emetine as observed before (Fig. 4D). The effects of protein synthesis inhibitors on class I presentation were not specific to emetine or E36 cells, as we observed the same results when EL4 cells were treated with CHX (Fig. 4E, Supplemental Fig. 3C).

Protein synthesis is necessary for all components of the Ag processing and presentation machinery, and inhibitors of synthesis will have greater effect on those proteins that are limiting or that have shorter half-lives. Therefore, we addressed whether the protein synthesis inhibitors could inhibit levels of MHC I on the cell surface or the ability of the cells to present exogenous peptides. We found that class I levels modestly decrease over time (Supplemental Fig. 3D), whereas the ability to present exogenous peptides (Supplemental Fig. 3E) was largely unchanged after inhibition of protein synthesis. However, if preformed class I was removed by acid stripping prior to treatment with synthesis inhibitors, recovery of surface MHC class I levels was markedly reduced (Fig. 4B, right panel), as was the ability to present exogenous peptides (Fig. 4F).

Together these data demonstrate that inhibitors of protein synthesis affect Ag presentation by a mechanism or mechanisms distinct from just inhibition of Ag synthesis, and further highlight the difficulty in interpreting the contribution of DRiPs to class I presentation when inhibitors of protein synthesis are used.

**Discussion**

The data presented in this report address three major questions. First, is the primary source of peptides presented on class I MHC molecules derived predominantly from newly synthesized defective proteins (DRiPs) or functionally mature proteins? Second, are newly synthesized Ags a more efficient source of presented peptides than old, mature proteins? Finally, we address whether inhibitors of protein synthesis, a widely used tool in experiments that support the DRiP hypothesis, also affect class I presentation of mature proteins.

To address these questions, we used a Tet-induced Ag expression system encoding a reversible destabilizing domain. Owing to the specificity of Shield for folded FKBP fusions, our system allowed mature proteins to be stabilized without altering the formation and proteolysis of DRiPs. Our data demonstrated that Shield-stabilized proteins were the primary source of presented peptides from newly synthesized Ag. Is it possible that these Shield-stabilized constructs are actually DRiPs rather than mature proteins? By definition, DRiPs are not functional but instead represent defective proteins that never achieve a mature conformation (33). In contrast, the Shield-stabilized Ags bind ligands and are fluorescent, and therefore are not defective (11). Moreover, DRiPs are rapidly poly-ubiquitinated, a feature that was first used to identify and quantify these abnormal proteins (7). In contrast, we and others detect no substantial ubiquitin laddering of the Shield stabilized constructs (34). In fact, proponents of the DRiP hypothesis agree that such Shield-stabilized proteins are not DRiPs (11, 25).

Although our data clearly demonstrate that mature proteins are the more abundant contributors to the class I peptide pool for the two Ags we have analyzed, these results contrast with other recently published reports that used similar Ag constructs. Dolan et al. (11) showed that most (60–70%) of the peptides feeding into the class I presentation pathway originated from an FKBP–Ag fusion that could not bind Shield and be stabilized. Given this observation and the fact that presentation from these constructs...
was rapidly blocked when protein synthesis was inhibited by CHX, it was suggested that DRiPs were the major source of the presented peptides. However, our data demonstrate that the concentration of Shield used in the Dolan study (11) (2–5 μM) does not fully stabilize the Ags. In fact, our data show that as much as 16 μM of Shield was necessary to fully stabilize the FKBP–EGFP fusion proteins, as indicated by the continual increase in GFP fluorescence and decrease in Ag presentation as the concentration of Shield increased. In addition, Dolan et al. (11) used cells that had been continuously synthesizing Ag before exposure to acid stripping and Shield. Our data suggest that with extended Ag synthesis, cells may accumulate a Shield-resistant form of Ag; this form of Ag is not seen for >6 h after Ag synthesis is initiated. Moreover, we generalized our findings to two different FKBP Ags (with >80% difference in amino acid sequence) and in different APCs, including ones from rodents and humans.

In addition to addressing the dominant source of presented peptides from our newly synthesized Ag constructs, we also investigated whether, and to what extent, old, mature proteins (“retirees”) were a source of peptides when they were degraded. We found that the presented peptides from our constructs were as efficiently generated from old, mature proteins as newly synthesized ones. As we show in this article, these results are likely due to the fact that the majority of peptides being presented during synthesis were generated from mature proteins. These findings are different from what was originally described by others. Dolan and colleagues (11) initially reported that the contribution of peptides from mature proteins was small compared with neosynthesized Ags. This observation suggested that peptides generated from newly synthesized DRiPs might have preferential access to the MHC class I pathway, and led to the idea that the processing and presentation of DRiPs were somehow compartmentalized. However, a subsequent paper from the same group, and using the same expression system, reported that endogenously expressed mature proteins were actually more efficient at contributing peptides to class I synthesis than newly synthesized proteins (12). The basis for these discrepant results was not addressed, although the latter findings are more similar to the ones we report in this article. We calculated the efficiency of Ag presentation without using protein synthesis inhibitors, whereas other studies have used these agents; it is likely that artifacts derived from the use of these inhibitors (see below) may have contributed to some of the discrepancies mentioned above.

Although our findings demonstrate a major contribution from mature proteins, these results may be limited to our Ag constructs, and it is possible that DRiPs and/or newly synthesized Ags are a more important source of presented peptides from other proteins or from other systems of expression (e.g., from viruses). However, our data are consistent with another recently published paper from our laboratory where mature Ag was a dominant source of presented peptides for another two Ag constructs (17) and with older data showing that mature proteins introduced directly into the cytosol were efficiently presented on class I molecules (3, 22, 36). Also consistent with our findings, an earlier study using SILAC to follow the kinetics of class I–presented peptides in a human cancer cell line showed that the majority of the class I peptides analyzed continued to be generated and presented for six or more hours posttransfection, a time period wherein DRiPs should have been long gone (37).

The majority of data supporting DRiPs come from experiments wherein inhibitors of protein synthesis were shown to rapidly block presentation despite the presence of substantial pools of mature proteins. However, as shown in this article, these are complex experiments, as protein synthesis is required for class I presentation in ways other than just Ag synthesis. This is perhaps not surprising because all components of the class I pathway need to be synthesized by the cell and there may also be other, yet unidentified short-lived proteins that are needed for the pathway; some of these components may become limiting if they are not replenished by synthesis. Moreover, blocking protein synthesis has long been known to have indirect effects on other cellular processes (38), and it is possible that these indirect effects also alter class I Ag presentation. For example, several genetic studies in Saccharomyces cerevisiae showed that CHX affected ubiquitin-modifying proteins and components of the proteasome after short incubation times (39–41). These results may partially explain why we observed stabilization of Ag levels following treatment with emetine. Moreover, the necessity of protein synthesis for class I presentation may explain why some viruses (e.g., vaccinia virus, EBV) target protein synthesis as a mechanism of evasion.

In at least one previous study, control experiments were performed to assess the Ag-independent effects of protein synthesis inhibitors on class I presentation. Qian et al. (13) treated cells with the reversible proteasome inhibitor MG132 to build up DRiPs (and other forms of Ags) and then found that when they removed MG132 to allow Ag degradation, CHX did not completely block Ag presentation. This finding was interpreted to indicate that CHX had minimal effects on peptide turnover and class I presentation. However, their data showed that when MG132 was removed CHX inhibited ~50% of class I presentation. These data, like ours and those from earlier studies (42–45), point to the problems associated with using protein synthesis inhibitors to study the contribution of newly synthesized proteins to Ag presentation.

In addition to the inhibitor experiments described above, a few experiments have used Tet-regulated systems to study the contribution of Ag neosynthesis to Ag presentation. This approach circumvents the problems with pleotropic effects associated with general inhibitors of protein synthesis. For lymphocytic choriomeningitis virus (nucleoprotein) and EBV (EBNA1) viral Ags, it was reported that when Tet was removed and Ag synthesis was terminated, the presentation of some peptides decreased at times when there was still a substantial pool of mature proteins (46, 47). Similarly, while this article was in preparation, it was reported for a Tet-inducible FKBP–EBV Ag system that mature Ags did not contribute to Ag presentation (25). It is possible that these Ags are ones for which the contribution from DRiPs is more substantial. However, an important limitation of these experiments is that the mechanism by which the pools of long-lived mature proteins were degraded was not reported. In fact, in other studies, at least two long-lived EBV-derived proteins, including EBNA1, were found to be degraded by autophagy (48, 49), whereas other studies have shown that several other EBV Ags are degraded by an alternative ubiquitin-proteasome–independent pathway (50, 51). This observation is not surprising because autophagy is a pathway that degrades a number of stable cytosolic proteins. Importantly, autophagy degrades proteins in vacuoles that are likely inaccessible to the proteasome and MHC class I Ag presentation pathway. Therefore, to interpret these experiments it is essential to determine how the mature Ags are degraded, and this issue was not addressed in any of these Tet-regulated experiments.

Overall, the data presented in this article do not argue against the concept that defects in protein synthesis, protein folding or targeting occur, or that the degradation of these mistakes does not somehow contribute to Ag presentation. However, our findings argue that the magnitude of this contribution is small in the experimental systems we have examined. Whether this is true more generally will need to be determined. In our system, Shield inhibits...
the presentation of newly synthesized Ags by 80–95%. It is possible that the portion of this response that is not inhibited by Shield comes from DRiPs that could not be stabilized by Shield. However, it is also possible that the noncovalent nature by which Shield binds to FKBP allows for transient destabilization, rendering a fraction of the fusion proteins susceptible to proteasomal processing and finally presentation. The DRiP model was proposed to explain how stable proteins could get rapidly presented. It was suggested that Ag presentation from proteins with long half-lives would be significantly delayed and that the few peptides that might be generated would be outcompeted by all of the peptides generated from endogenous Ags. However, it has long been appreciated that the kinetics of degradation for mature Ags with either short or long half-lives begins immediately after synthesis, following first-order rate kinetics (52–54). In other words, no delay occurs in this process. With the efficient presentation of peptides from a few mature proteins as shown in this article and elsewhere (12, 55) and the sensitivity of CD8+ T cells to recognize a small fraction of MHC:peptide complexes (<10 complexes per cell) (56), peptides from proteins expressed at low copy should be sufficiently presented for recognition by CD8+ T cells. Moreover, there is no evidence that competition exists among antigenic peptides under physiological conditions. Therefore, rapid presentation of viral Ags may not come just from the degradation of DRiPs.

Disclosures

The authors have no financial conflicts of interest.

References

Corrections


The authors wish to add an equal contribution footnote to their article. The footnote and the corrected author line are shown below.

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¹J.D.C. and D.J.F.-A. contributed equally to this work.
Supplemental Figure 3

A

GFP MFI

10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} [Inhibitor] μM

B

Emetine

- - + +

Ovalbumin

- + - +

C

CHX

- - + +

Ovalbumin

- + - +

D

Kb MFI (Y3)

0 1 2 3 4 5 6 Time (hrs)

E

NFAT-Luc (RLU x 10^4)

E36 Kb cells

0 10^{-1} 10^{0} 10^{1} 10^{2} [SIINFEKL] (ng/ml)

NFAT-Luc (RLU x 10^4)

EL4 cells

0 10^{-1} 10^{0} 10^{1} 10^{2} [SIINFEKL] (ng/ml)
### Supplemental Table I. Quantitation of the Efficiency of Antigen Presentation from Mature Protein

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Rate of synthesis&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Rate of Ag pres.&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Mature protein&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Mature Ag pres.&lt;sup&gt;4&lt;/sup&gt;</th>
<th>Calculated Ag pres.</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>y=3437x-4423</td>
<td>y=198x-201</td>
<td>7010 MFI</td>
<td>554 MFI</td>
<td>557 MFI</td>
<td>99.5%</td>
</tr>
<tr>
<td>2</td>
<td>y=4913x-6178</td>
<td>y=268x-325</td>
<td>11218 MFI</td>
<td>749 MFI</td>
<td>758 MFI</td>
<td>98.8%</td>
</tr>
<tr>
<td>3</td>
<td>y=2453x-4036</td>
<td>y=219x-238</td>
<td>8866 MFI</td>
<td>964 MFI</td>
<td>1023 MFI</td>
<td>94.2%</td>
</tr>
</tbody>
</table>

Abbreviations: Exp., experiment number; pres., presentation; MFI, mean fluorescent intensity

<sup>1</sup>Data from experiments shown in Fig 4A. <sup>2</sup>Data from experiments shown in Fig 4B. <sup>3</sup>Data from experiments shown in Fig 3D. <sup>4</sup>Data from experiments shown in Fig 3E.
Supplemental Figures

Supplemental Figure 1- **Dox and Shield efficiently induce expression of antigenic proteins without nonspecific global cellular stress.**

A, E36 K^b^ cells stably expressing copGFP were exposed to 5μM Shield and an increasing concentration of Dox for 15hrs. GFP fluorescence was assessed by flow cytometry (n=3). B, E36 K^b^ cells were treated as in Fig 1B, followed by cell lysis and separation on SDS-PAGE. Representative western blot probed with anti-HA antibody to evaluate total antigen expression. GAPDH serves as a loading control for equivalent total protein levels (n=2). C, In order to assess the effects of different treatments on cellular stress E36 K^b^ cells were assayed for the presence of the stress-induced spliced form of XBP1 mRNA by RT-PCR after the following treatments. E36 K^b^ cells were left untreated (None) or treated with 0.1μg/ml Dox alone, 16μM Shield alone, or both Dox and Shield for 6hrs (left panel). E36 K^b^ cells were treated with 0.1μg/ml Dox, 5μM Shield or both treatments for 24hrs (center panel). E36 K^b^ cells were treated with 0.1% DMSO, 40μM CHX or 20μM emetine for 6hrs (right panel). D, EL4 cells were either left untreated (None), or treated with 0.5μg/ml Dox alone, 16μM Shield or both for 6hrs (left panel). EL4 cells were treated 0.1% DMSO, 40μM CHX or 20μM emetine for 3hrs (right panel). For a positive control of cellular stress in C and D, cells were treated with (5μg/ml) tunicamycin for 6hrs. Following treatment in C and D, RT-PCR of XBP-1 was performed from equivalent amounts of mRNA. Cellular stress induced XBP-1 mRNA splicing is indicated with an arrowhead.
GAPDH PCR serves as a loading control (n=3). E, To determine the effects of Dox and Shield on class I levels, cells were acid stripped and then either left untreated (None), treated with Dox alone (0), or with Dox and increasing concentrations of Shield for 6hrs, as shown in Fig 1 and 2. Surface MHC class I levels were assessed by FACs analysis (anti-H-2 K\textsuperscript{b} (Y-3) (n=3). F, To determine the effects of Dox and Shield on cell viability, cells were treated as in E, followed by MTS assay (Promega) in accordance to manufacturer protocol. Values shown are the difference in absorbance after background subtraction (510nm-655nm) (n=3).

Supplemental Figure 2- **Efficiency of class I presentation of mature protein.**

A, MHC class I complexes are rapidly generated from mature protein. Antigen (copGFP) expressed in E36 K\textsuperscript{b} cells was stabilized for 24hrs in the absence of protein synthesis as described in Fig 3C. Preformed surface class I molecules were then removed by acid strip followed by culture in RPMI for 6hrs, during which time the kinetics of antigen degradation (black) and class I presentation (white) were measured. Data are representative of 3 independent experiments. B, E36 K\textsuperscript{b} cells were treated with 0.1 \mu g/ml Dox and 5\mu M Shield for 24hrs. Preformed K\textsuperscript{b}:S8L complexes were removed by acid strip. Cells were then cultured in RPMI alone (black) or 5\mu g/ml BFA (white) to block transport of new K\textsuperscript{b}:S8L complexes out of the ER and the appearance of K\textsuperscript{b}:SL8 complexes were measured. C, Experimental design used to determine the efficiency of antigen presentation from old mature protein (gray) versus newly synthesized protein.
(black). ‘Low dose Shield’ (0.1μM) was used during the degradation phase to keep the rate of degradation linear. Dashed lines indicate 20 min time intervals where class I presentation (Kb:S8L) and antigen expression (GFP) were assessed, as described in the materials and methods. D, The kinetics of GFP synthesis from new antigen (solid black) or degradation of old antigen (solid gray) was assessed over time (2hrs total). The kinetics of antigen presentation from newly synthesized antigen (open black) or old mature antigen (open gray) was assessed by 25-D1.16 staining over time (2hrs total). E, Scatter plot shows the amount of antigen (GFP MFI) relative to antigen presentation (Kb:S8L) from old mature antigen (gray) and newly synthesized (black) antigen over the time period shown in D. F, Bar graph represents the absolute slope of newly synthesized protein (black) and old protein (gray). Data shown in D and E are representative of at least 3 experiments. Data shown in F are the means (±SEM) of at least 3 independent experiments.

Supplemental Figure 3- Effect of protein synthesis inhibitors on presentation of exogenously loaded protein and peptide.
A, E36 K$b$ cells stably expressing copGFP were treated with 0.1 μg/ml Dox, 5μM Shield and either CHX (gray) or emetine (black) for 6hrs and then GFP expression was assessed by flow cytometry. Data shown represent a series of 3-fold serial dilutions of the inhibitors and are representative of 3 independent experiments. Western blot analysis of ovalbumin expression in E36 K$b$ cells (B) and EL4 cells (C) following hypertonic loading as described in Fig 5C. Cell
lysates (5 μg protein) were separated by SDS-PAGE, transferred to membranes and then probed for ovalbumin (Ova) and GAPDH. D, E36 K\(^b\) cells were treated with control media (DMSO, white), 40μM CHX (gray) or 20μM emetine (black) over the indicated time and then K\(^b\) expression was assessed by flow cytometry (Y-3 antibody). Data shown are representative (n=3). E, E36 K\(^b\) cells (left) or EL4 cells (right) were treated with 0.1% DMSO (white), 40 μM CHX (gray) or 20 μM emetine (black) for 2.5hrs. Cells were then washed and fixed in 4% PFA followed by exposure to increasing concentrations of S8L peptide as indicated. Unbound peptide was removed by washing, followed by overnight culture with the T cell hybridoma RF33-Luc and then NFAT luciferase activity was measured, as described (n=3).