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Tight Linkage between Translation and MHC Class I Peptide Ligand Generation Implies Specialized Antigen Processing for Defective Ribosomal Products

Shu-Bing Qian,* Eric Reits,† Jacques Neefjes,‡ Jeanne M. Deslich,* Jack R. Bennink,* and Jonathan W. Yewdell‡

There is mounting evidence that MHC class I peptide ligands are predominantly generated from defective ribosomal products and other classes of polypeptides degraded rapidly (t_{1/2} < 10 min) following their synthesis. The most direct evidence supporting this conclusion is the rapid inhibition of peptide ligand generation following cycloheximide-mediated inhibition of protein synthesis. In this study, we show that this linkage is due to depleting the pool of rapidly degraded proteins, and not to interference with other protein synthesis-dependent processes. Our findings indicate that in the model systems used in this study, MHC class I peptides are preferentially generated from rapidly degraded polypeptides relative to slowly degraded proteins. This conclusion is supported by the properties of peptide presentation from slowly degraded (t_{1/2} = 4 h) defective ribosomal products generated artificially by incorporation of the amino acid analog canavanine into a model viral Ag. We propose that specialized machinery exists to link protein synthesis with class I peptide ligand generation to enable the rapid detection of viral gene expression. The Journal of Immunology, 2006, 177: 227–233.

1 Abbreviations used in this paper: ER, endoplasmic reticulum; CHX, cycloheximide; TAP, transporter associated with antigen processing; DRiP, defective ribosomal product; FRAP, fluorescence recovery after photobleaching; NP, nucleoprotein; RDP, rapidly degraded polypeptide; SDP, slowly degraded polypeptide; VV, vaccinia virus.

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Materials and Methods

Cells, viruses, and reagents

L-Kb and MelJuSo cells were maintained in DMEM with 10% FBS. Recombinant vaccinia virus (rVV) expressing SIINFEKL-containing protein (nucleoprotein (NP)-GFP) has been described (6). Cells were infected at a...
multiplicity of infection of 10 or 20 PFU at 37°C in BSS/BSA, and then incubated at 37°C in complete growth medium for the remainder of the assay. The reversible proteasome inhibitor MG132 (chx-Leu-Leu-Leucin) and CHX were purchased from Sigma-Aldrich. Polyclonal Ab against GFP was purchased from BD Clontech. Purified 25-D1.16 mAb conjugated to AlexaFluor 647 has been described previously (6).

Metabolic radiolabeling

L-Kb cells were radiolabeled in suspension at 37°C with [35S]Met (500 μCi/ml; Amersham Biosciences) for 5 min in Met-deficient DMEM without prior Met starvation. Labeling was terminated by adding ice-cold PBS containing excess (1 mg/ml) Met. After washing twice, cells were chased for various times at 37°C in DMEM/10% FBS supplemented with 20 mM HEPES and 1 mg/ml Met. Whole cell extracts were precipitated with TCA or resolved in 10% SDS-PAGE gels, as described previously (14). Gels were fixed in 10% acetic acid, dried, and scanned by a Typhoon 8600 (Amersham Biosciences). Images were generated and data analyzed using ImageQuant software (Amersham Biosciences).

Cytofluorography

Kb-SINFEKL complex levels were measured by incubating cells for 30 min on ice with 25-D1.16 Ab conjugated to Alexafluor 647. Cellular GFP and Alexafluor 647 levels were determined on a FACScalibur cytofluorograph (BD Biosciences).

Immunoblotting

Cell lysates were prepared by heating for 10 min in SDS-PAGE sample buffer (50 mM Tris Cl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromphenol blue, and 10% glycerol). Proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Invitrogen Life Technologies). Membranes were blocked for 1 h in TBS (50 mM Tris Cl (pH 7.4), 150 mM NaCl) containing 5% BSA, followed by incubation with Abs, as indicated. After incubation with peroxidase-conjugated secondary Abs (Roche), immunoblots were developed using ECL (Pierce).

Acid strip assay

L-Kb (~5 × 10^6) cells were pelleted and resuspended in 0.5 ml of 0.2 M citric acid/NaHPO4 buffer (pH 3.0). After incubation on ice for 2 min, the sample was neutralized by adding excess of cold 0.1% BSA in PBS (pH 7.2) and cells were immediately collected by brief centrifugation. Cell surface Kb molecules were measured by flow cytometry after staining with HB176 and FITC-labeled anti-mouse IgG.

TAP-fluorescence recovery after photobleaching (FRAP)

Confocal microscopy of living cells was performed using a Leica TCS NT (Leica Microsystems) confocal system, equipped with an Ar/Kr laser (8). MelJuSo cells stably expressing TAP1-GFP were used to visualize lateral mobility of TAP complexes using FRAP. GFP-positive clones were selected for low to moderate fluorescence to ensure optimal assembly of TAP-fluorescence recovery after photobleaching (FRAP) structures (6). We examined this possibility by pulse radiolabeling L-Kb cells (L929 cells transfected with a cDNA encoding H-2Kb) with [35S]Met and chased for up to 2 h in the presence or absence of CHX. Protein degradation was measured by loss of TCA-precipitable radioactivity in total cell lysates. Control experiments established that CHX blocks protein synthesis within seconds of its addition to cells under these conditions (data not shown). As previously described (5, 10), ~25% of proteins (RDPs) are degraded within 30 min of their synthesis (t1/2 of ~10 min (Fig. 1A)). Remaining stable proteins (SDPs) are degraded with a t1/2 of >1000 min. Addition of CHX to the chase medium had no significant effect on the degradation of RDPs or SDPs.

We extended these findings to VV-infected cells by examining the effect of CHX on the degradation of chimeric protein consisting of GFP, the chicken OVA-derived Kb-binding peptide (SINFEKL), and influenza A virus NP containing a 30-residue KEKE-rich sequence inserted at NP333 (16). This protein misfolds and is degraded by proteasomes with a t1/2 of ~70 min (16). Cells were radiolabeled for 5 min with [35S]Met and chased in the presence or absence of CHX. Total cell lysates were directly analyzed by SDS-PAGE, and the NP(KEKE)-S-GFP band was quantitated by phosphor imager analysis. This revealed that CHX had no significant effect on proteasome-mediated degradation of NP(KEKE)-S-GFP (Fig. 1B).

These findings are consistent with numerous prior studies using a variety of cell types showing that CHX does not interfere with the degradation of SDPs unless cells are in a catabolic state induced by nutrient deprivation (reviewed in Ref. 17).

Results

CHX does not inhibit protein degradation

One possible confounding effect of CHX on Ag presentation is that it might inhibit protein degradation. Indeed, this assumption was built into recent mathematical modeling (15) of the effect of CHX on generation of Kb-SIINFEKL from NP-SIINFEKL-GFP constructs (6). We examined this possibility by pulse radiolabeling L-Kb cells (L929 cells transfected with a cDNA encoding H-2Kb) for 5 min with [35S]Met and chasing for up to 2 h in the presence or absence of CHX. Protein degradation was measured by loss of TCA-precipitable radioactivity in total cell lysates. Control experiments established that CHX blocks protein synthesis within seconds of its addition to cells under these conditions (data not shown). As previously described (5, 10), ~25% of proteins (RDPs) are degraded within 30 min of their synthesis (t1/2 of ~10 min (Fig. 1A)). Remaining stable proteins (SDPs) are degraded with a t1/2 of >1000 min. Addition of CHX to the chase medium had no significant effect on the degradation of RDPs or SDPs.

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These findings are consistent with numerous prior studies using a variety of cell types showing that CHX does not interfere with the degradation of SDPs unless cells are in a catabolic state induced by nutrient deprivation (reviewed in Ref. 17).
Generation of K$^b$-SIINFEKL complexes from NP-S-GFP DRiPs

We next studied the effect of CHX on Ag presentation from a defined Ag. We used a rVV that expresses wild-type NP genetically fused with the chicken OVA-derived K$^b$-binding peptide (SIINFEKL) and GFP at the COOH terminus. Like NP and GFP themselves, the fusion protein (NP-S-GFP) is highly stable, demonstrating negligible degradation within 4 h of its synthesis in L-K$^b$ cells, as determined by pulse-chase labeling with [35S]Met and monitoring degradation by SDS-PAGE of total cell lysates (see Fig. 4A). Consistent with this finding, levels of GFP fluorescence were constant for 4 h following the addition of CHX to VV-NP-S-GFP-infected cells (6) (see Figs. 2C and 4C).

We measured the generation of K$^b$-SIINFEKL complexes from NP-S-GFP using the 25-D1.16 mAb, which is specific for these complexes (18). Upon infection of L-K$^b$ cells with rVV-NP-S-GFP, the generation of K$^b$-SIINFEKL complexes reaches V$_{\text{max}}$ by 140 min postinfection (Fig. 2C). Complex generation is blocked within 60 min following addition of MG132 to cells and 100 min following CHX addition (Fig. 2C). The difference of 40 min between the effects of CHX and MG132 on complex delivery to the cell surface should approximate the time it takes for DRiPs to be completely degraded by the proteasome. Assuming DRiPs are degraded with first order kinetics, this implies a $t_{1/2}$ on the order of 10 min, because four $t_{1/2}$ are required to achieve >90% depletion of the peptide-generating pool ($((1/2)^4 \times 6.25\%$ remaining substrate).

Now for the crux of this experiment: if CHX blocks presentation solely by preventing the generation of NP-S-GFP DRiPs, then its action should be overcome if a pool of DRiPs has been created by transiently treating cells with MG132. We established that MG132 treatment does not significantly interfere with NP-S-GFP expression as determined by immunoblotting (Fig. 2B) or GFP fluorescence (Fig. 2C, left panel). To create a pool of NP-S-GFP DRiPs, we treated cells with MG132 between 180 and 300 min postinfection. We then removed MG132 and simultaneously added CHX (the drug treatment scheme is outlined in Fig. 2A). As seen in Fig. 2C (right panel), after a 60-min lag period, the generation of K$^b$-SIINFEKL complexes resumed. The 60-min delay cannot be attributed to delayed recovery of proteasome activity following MG132 removal, which commences within a few minutes, at most, after removing the drug (S. Qian, J. Bennink, and J. Yewdell, manuscript in preparation). Rather, the 60-min lag represents the time required for DRIP degradation, peptide loading, and transport of K$^b$-SIINFEKL complexes to the cell surface. Complex delivery continued for 100 min and reached ~50% the level achieved by cells that had not been exposed to MG132. The partial recovery of K$^b$-SIINFEKL expression after MG132 reversal relative to cells treated only with CHX is probably related, at least in part, to the observation that after removal of MG132, cells degrade only a fraction of the RDPs rescued from degradation by MG132 treatment (Qian et al., manuscript in preparation).

This experiment demonstrates that cells are capable of generating SIINFEKL from NP-S-GFP in the presence of CHX as long as the pool of DRiPs is expanded by temporarily blocking proteasome activity. Thus, in this system, the rapid inhibitory effect of CHX on the generation of K$^b$-SIINFEKL from NP-S-GFP indicates that peptides are generated from the relatively small pool of rapidly degraded DRiPs and not from the turnover of the much larger pool of native NP-S-GFP.

**FIGURE 2.** K$^b$-SIINFEKL complexes derive from rapidly degraded NP-S-GFP DRiPs. A. Experimental scheme. B. L-K$^b$ cells were infected with rVV-expressing NP-S-GFP and treated with MG132 and CHX, as diagrammed in A. Ten hours postinfection, levels of NP-S-GFP and β-actin (included as a control for cell number) were determined by immunoblotting. C. For the same cells used in B, both GFP fluorescence and K$^b$-SIINFEKL complex formation were determined by flow cytometry. Similar results were obtained in three additional experiments.

Generation of K$^b$ peptide ligands from RDPs

To generalize these findings to overall peptide ligand generation, we repeated this experiment using the HB-176 mAb, which recognizes conformed H-2K$^b$ molecules independently of the nature of their peptide ligand. Cells were pretreated with MG132 or DMSO for 2 h and then acid stripped to reduce the levels of K$^b$ staining and thereby increase the sensitivity of detecting newly arriving K$^b$ molecules. Note that after acid stripping the residual level of K$^b$ molecules was slightly decreased in cells treated with MG132 relative to control cells (Fig. 3A). Addition of CHX to cells resulted in steadily decreasing levels of K$^b$, which is most likely due to a combination of shedding, unfolding, and internalization of cell surface K$^b$. Including MG132 with CHX had little effect on the rate of cell surface K$^b$ loss. By contrast, transiently treating cells with MG132 for 2 h before adding CHX decreased the rate of K$^b$ loss. The net effect of MG132-induced accumulation of RDPs on K$^b$ delivery to the cell surface can be more clearly seen by transforming the data to account for the Kb cell surface loss contributed to delayed recovery of proteasome activity following MG132 removal, which commences within a few minutes, at most, after removing the drug (S. Qian, J. Bennink, and J. Yewdell, manuscript in preparation). Rather, the 60-min lag represents the time required for DRIP degradation, peptide loading, and transport of K$^b$-SIINFEKL complexes to the cell surface. Complex delivery continued for 100 min and reached ~50% the level achieved by cells that had not been exposed to MG132. The partial recovery of K$^b$-SIINFEKL expression after MG132 reversal relative to cells treated only with CHX is probably related, at least in part, to the observation that after removal of MG132, cells degrade only a fraction of the RDPs rescued from degradation by MG132 treatment (Qian et al., manuscript in preparation).

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**Generation of cellular K$^b$ peptide ligands from RDPs**

To more directly measure the generation of cytosolic peptides that are relevant to Ag presentation, TAP activity was determined by transforming the data to account for the Kb cell surface loss contributed to delayed recovery of proteasome activity following MG132 removal, which commences within a few minutes, at most, after removing the drug (S. Qian, J. Bennink, and J. Yewdell, manuscript in preparation). Rather, the 60-min lag represents the time required for DRIP degradation, peptide loading, and transport of K$^b$-SIINFEKL complexes to the cell surface. Complex delivery continued for 100 min and reached ~50% the level achieved by cells that had not been exposed to MG132. The partial recovery of K$^b$-SIINFEKL expression after MG132 reversal relative to cells treated only with CHX is probably related, at least in part, to the observation that after removal of MG132, cells degrade only a fraction of the RDPs rescued from degradation by MG132 treatment (Qian et al., manuscript in preparation).

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**FIGURE 2.** K$^b$-SIINFEKL complexes derive from rapidly degraded NP-S-GFP DRiPs. A. Experimental scheme. B. L-K$^b$ cells were infected with rVV-expressing NP-S-GFP and treated with MG132 and CHX, as diagrammed in A. Ten hours postinfection, levels of NP-S-GFP and β-actin (included as a control for cell number) were determined by immunoblotting. C. For the same cells used in B, both GFP fluorescence and K$^b$-SIINFEKL complex formation were determined by flow cytometry. Similar results were obtained in three additional experiments.
FRAP analysis using MelJuSo cells expressing TAP1-GFP (see Materials and Methods). As previously reported (8), cells treated with MG132 (Fig. 3C, top dashed line) demonstrated increased TAP diffusion (D) in the ER membrane relative to untreated cells (“control,” bottom dashed line) due to the decrease in cytosolic peptide supply. An effect of the same magnitude is achieved by incubating cells with a mixture of CHX and MG132 (data not shown). Removal of MG132 or MG132/CHX after 2-h pretreatment of cells results in full restoration of TAP D within 5–10 min (data not shown). Remarkably, pretreating cells for 2 h with MG132 and then removing MG132 simultaneously with adding CHX alone results in a burst of TAP activity (Fig. 3C, squares). This persists for between 10 and 15 min before returning to baseline levels, consistent with a 1/2 of ~7 min or less (three 1/2 in 20 min) for degradation of the RDP pool created by the MG132 blockade. By contrast, no such burst of peptide generation is seen if cells are incubated with both MG132 and CHX, cells pretreated with MG132 and CHX demonstrate a 15-min period of activity at levels associated with normally active cells (as indicated). Lines at the bottom and top of the figure depict the average TAP mobility, respectively, in untreated cells or cells incubated with MG132.

**FIGURE 3.** TAP and class I binding peptides selectively derive from RDPs and not SDPs. A, L-Kb cells pretreated with MG132 (or left untreated) were briefly incubated at pH 3 to decrease levels of Kb molecules. After neutralization, cells were cultured in the presence of CHX or MG132 as in Fig. 2A. Cell surface H-2Kb molecules were measured by flow cytometry. Similar results were obtained in a repeat experiment. B, Data from A are normalized for the level of Kb after acid stripping and the loss of cell surface Kb associated with CHX treatment. C, The diffusion coefficient D of TAP1-GFP is depicted for MelJuSo cells pretreated with MG132 or a combination of MG132 and CHX for 2 h. At time point t = 0, MG-132 or MG132/CHX is washed away and cells are chased in the presence of CHX. In contrast with cells pretreated with both MG132 and CHX, cells pretreated with MG132 demonstrate a 15-min period of activity at levels associated with normally active cells (as indicated). Lines at the bottom and top of the figure depict the average TAP mobility, respectively, in untreated cells or cells incubated with MG132.

Selected peptide generation from RDPs following deliberate DRiP generation

We next examined the capacity of cells to generate peptides from SDPs created from NP-S-GFP by incubating rVV-infected L-Kb cells with 15 mM canavanine. Canavanine is an Arg analog in which the δ-methylene group is replaced by oxygen. Canavanine replaces Arg on tRNAArg and is incorporated into proteins, where it induces misfolding and degradation (16, 19). Incorporation of canavanine into NP-S-GFP-destabilizes the protein, which now is degraded with a 1/2 of 4 h as determined by pulse-chase analysis using whole cell lysates resolved by SDS-PAGE to measure levels of NP-S-GFP (Fig. 4A). Degradation is mediated by proteasomes, as inferred by MG132 stabilization of NP-S-GFP. Canavanine does not affect the degradation of (R)NP-S-GFP (6), the N-end rule targeted form of NP-S-GFP (Fig. 4A), but does have a generalized destabilizing effect on viral and cellular proteins (Fig. 4A, right panel).

Addition of canavanine to cells 115 min postinfection results in decreased rate of synthesis of folded (i.e., fluorescent) NP-S-GFP, and a corresponding increase in Kβ-SIINFEKL complex formation (Fig. 4B). The latter cannot be attributed to canavanine-induced alterations in the efficiency of SIINFEKL liberation from NP or on general cell metabolism, because canavanine has little effect on Kβ-SIINFEKL generation from (R)NP-S-GFP (Fig. 4B).
We next examined the effect of CHX on Kb-SIINFEKL generation by cells incubated with canavanine from the start of infection with VV-NP-S-GFP. CHX caused an immediate cessation of GFP accumulation in both canavanine-treated and untreated cells. Interestingly, canavanine treatment did not result in destabilization of fluorescent NP-S-GFP, whose levels remained constant over the 3-h CHX incubation (Fig. 4C). This indicates that the destabilized canavanine-NP-S-GFP detected biochemically represents misfolded molecules that are nonfluorescent, reminiscent of a similar discrepancy we previously reported in the biochemical vs fluorescent stability of NP(KEKE)-S-GFP (6). Fluorescent NP-S-GFP synthesized in the presence of canavanine presumably lacks canavanine residues in the positions critical to induce misfolding sufficiently severe to result in degradation.

Most importantly, the effect of CHX on the generation of Kb-SIINFEKL complex formation is remarkably similar in canavanine-treated and untreated cells, with both attaining plateau values 120 min post-CHX addition. This can be most clearly seen by normalizing the data to account for the difference in efficiency of generating Kb-SIINFEKL complexes in the presence and absence of canavanine (Fig. 4D). Note that at the 120-min post-CHX addition time point there should still be a healthy pool of canavanine-misfolded, nonfluorescent NP-S-GFP as well as unloaded Kb in the ER to maintain complex generation (the latter as inferred from the data in Fig. 2C, right panel, which demonstrates that complexes can be delivered to the cell surface for at least 180 min following CHX). Thus, the highly similar kinetics observed following CHX treatment of canavanine-treated and untreated L-Kb cells is consistent with the idea...
that the nonfluorescent pool of misfolded canavanine-NP-S-GFP that is degraded with a $t_{1/2}$ of 4 h is an inefficient source of SIINFEKL.

This leads us to conclude that not all DRiPs are equal as sources of antigenic peptides. For NP-S-GFP, the canavanine-induced increase in $K^\alpha$-SIINFEKL generation appears to be due not to the slowly degraded DRiP pool evident in Fig. 4A, but to an increase in the pool of rapidly degraded NP-S-GFP DRiPs. This population is not apparent in the pulse-chase experiment, due either to: 1) low abundance (it would be difficult to detect a RDP population amounting to a few percent of the total); 2) migration at a different (and possibly heterogeneous) molecular weight due to downstream initiation or premature termination; or 3) a combination of 1 and 2.

**Discussion**

These findings imply that the vast majority of peptide ligands in L-Kb and MelJuSo cells are generated from RDPs. Although a number of studies have linked protein synthesis with peptide ligand generation, interpretation of the most direct experiments is based on the assumption that the effect of CHX on peptide ligand generation is strictly due to its inhibition of protein synthesis. Our findings demonstrate that CHX-mediated inhibition of protein synthesis for up to 2 h does not grossly interfere with protein degradation, peptide generation in the cytosol, or assembly and intracellular trafficking of MHC class I complexes to the cell surface, and therefore bolsters conclusions drawn from the use of CHX to discern between SDPs and DRiPs as sources of antigenic peptides from defined gene products.

The data in Fig. 3 provide clear evidence that overall peptide ligand generation from SDPs is limited in L-Kb and MelJuSo cells. This cannot be attributed to the number of SDP substrates degraded per unit time, because this represents $\sim$50% of the number of RDPs degraded per unit time in L-Kb cells (6). The logical implication is that peptide ligands are generated at much higher efficiency from RDPs than SDPs in MelJuSo cells.

There is published evidence for differences in efficiency of peptide ligand generation from different proteasome substrates. We previously reported that the efficiency of generating $K^\alpha$-SIINFEKL from two highly similar forms of NP-S-GFP differed by $>2$-fold (6). There is a 100-fold difference in the published efficiencies of peptide generation from biosynthesized viral proteins (1 complex generated for $\sim$2000 proteins degraded) and bacterial proteins secreted into the cytosol (1 complex generated for $\sim$20 proteins degraded) (6, 20). It is difficult to understand how cross-presentation of viral and cellular Ags could function at the inefficiency associated with endogenous Ag processing given the limited amounts of Ag delivered to the cytosol from endosomes/phagosomes (21).

We propose that the processing of SDPs into peptide ligands is highly inefficient to enable class I molecules to sample what is being translated rather than on what has been translated. This mechanism would enable $T_{CTM}$ to monitor the acute expression of viral genes with minimal competition from the turnover of SDPs. In rapidly dividing cultured cells, SDPs constitute $\sim$30% of proteasome substrates (6), and possibly more if the fraction of RDPs is lower than previously estimated, as has been recently suggested (22). In nondividing cells, however, long-lived proteins probably comprise more than half the proteasome substrates, because the rate of protein synthesis will be decreased by $\sim$40%, which represents the fraction of proteins synthesized by cultured cells to enable division every 24 h (6). By largely ignoring the turnover of SDPs, the immune system could potentially double its sensitivity in detecting a virus infection. This may seem like a marginal advantage, but given that some viruses can begin to release progeny within 4 h of infection, a doubling in the speed of detection may be critically important to control rapidly replicating viruses.

There is evidence that SDPs are not always a poor source of peptide ligands. We previously reported that NP-S-GFP itself and NP(KEKE)-S-GFP provide peptides from both rapidly and slowly degraded pools in L-Kb cells (6). For unknown reasons, using L-Kb cells derived from the same original clone, we now do not typically observe the continued generation of $K^\alpha$-SIINFEKL complexes at a reduced rate at the tail end of CHX treatment that was apparent in Fig. 3C in reference (6). Mature dendritic cells infected with influenza A virus have been reported to delay peptide generation from NP (23), presumably due to the storage of DRiPs in dendritic cell aggresome-like-induced structures (24). These findings suggest that cells possess the capacity to modulate peptide generation from RDP and SDP pools. In the case of dendritic cells, this appears to be accomplished by storing RDPs in dendritic cell aggresome-like-induced structure. The purpose of this, however, may not be to skew presentation toward SDPs, but rather to allow the dendritic cell Ag processing machinery to focus on exogenous Ags delivered to the cytosol for cross-presentation.

It would be a mistake to consider RDP and SDP pools as homogeneous, or to consign all DRiPs to the RDP pool. With a $t_{1/2}$ of 4 h, the major population of canavanyl-NP DRiPs falls into the SDP pool. The SDP pool of NP-S-GFP giving rise to the delayed Ag presentation in reference (6) is still probably DRiPs, because the $t_{1/2}$ of full-length NP is on the order of days, not hours. Regarding RDP heterogeneity, we recently reported that $\sim$20% of RDPs are degraded by proteasomes independently of ubiquitin or 19S regulators (7). Importantly, it appeared that such RDPs are a preferred source of antigenic peptides from NP-S-GFP. Alternative targeting signals might be used to deliver such RDPs to proteasomes particularly adept at generating antigenic peptides. One possibility is FAT10, a MHC-encoded ubiquitin-like protein (25) recently shown to function as a proteasome-targeting signal (26).

Alternatively, the intimate relationship between translation and Ag presentation might represent a physical linkage among ribosomes, proteasomes, and TAP to enable direct channelling of proteasome products to class I molecules in the loading complex (27). This would protect these peptides from highly active cytosolic endopeptidases and aminopeptidases (28) and would also limit competition from other peptides for binding to class I molecules. A direct linkage between ribosomes and class I molecules would provide some potential advantages. First, it could maximize the rapidity of detecting virus-infected cells. Second, it would be more difficult for viruses to block Ag presentation by overexpressing nonimmunogenic peptides with high affinity for class I molecules. Third, cells would broaden their repertoire of self peptide ligands by minimizing the monopolizing effects of cellular peptides of unusual abundance or class I binding affinity. This would be useful for thymic selection and for recognition of tumor cells.

What might such immunoribosomes look like? In response to multiple stress signals, including viral infections, cells block cap-dependent translation by phosphorylating translation eukaryotic initiation factor 2a. Cap-independent translation continues, however, and intriguingly, Shastri and colleagues (29) have found that translation of a CUG-initiated antigenic peptide might even be enhanced under these conditions. Nicchitta and colleagues (30, 31) recently reported that under conditions of eukaryotic initiation factor 2a phosphorylation induced by ER stress, translation of ER-bound ribosomes continued, while free ribosome translation was inhibited. Intriguingly, a subset of ER-bound ribosomes was found to be translating mRNA of cytosolic/nuclear proteins.

These findings demonstrate that translation is more heterogeneous than is generally appreciated. Another fact consistent with
this conclusion is the presence of ~500 tRNA genes in mammals. To some extent, this large number probably reflects the requirement to generate sufficient amounts of tRNAs needed to support translation (~10^5 copies per cell). There is an amazingly high degree of heterogeneity, however, among the tRNA genes, suggesting that tRNAs may be used on a selective basis for translation of certain mRNAs in specific locales under specific conditions. Remarkably, approximately one-third of all tRNAs reside in the MHC (32, 33), raising the possibility that these tRNAs are selectively used for some immune function, perhaps including the generation of RDPs dedicated for Ag processing.

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Disclosures

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