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Characterizing the Specificity and Cooperation of Aminopeptidases in the Cytosol and Endoplasmic Reticulum during MHC Class I Antigen Presentation

Arron Hearn,* Ian A. York,† Courtney Bishop,*† and Kenneth L. Rock*

Many MHC class I-binding peptides are generated as N-extended precursors during protein degradation by the proteasome. These peptides can subsequently be trimmed by aminopeptidases in the cytosol and/or the endoplasmic reticulum (ER) to produce mature epitope. However, the contribution and specificity of each of these subcellular compartments in removing N-terminal amino acids for Ag presentation is not well defined. In this study, we investigated this issue for antigenic precursors that are expressed in the cytosol. By systematically varying the N-terminal flanking sequences of peptides, we show that the amino acids upstream of an epitope precursor are a major determinant of the amount of Ag presentation. In many cases, MHC class I-binding peptides are produced through sequential trimming in the cytosol and ER. Trimming of flanking residues in the cytosol contributes most to sequences that are poorly trimmed in the ER. Because N-terminal trimming has different specificity in the cytosol and ER, the cleavage of peptides in both of these compartments serves to broaden the repertoire of sequences that are presented. The Journal of Immunology, 2010, 184: 4725–4732.
of nonproteosomal proteases in vivo in the generation of CTL epitopes should enhance our understanding of epitope generation and modeling of this process. This led us to initiate the current study to systematically examine the specificity of trimming N-terminal sequences from antigenic precursors in vivo.

Materials and Methods

Plasmids

To express N-terminal SIINFEKL (OVA254–264) (S-L) precursors in the cytoplasm, synthetic complementary oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) were annealed and inserted into the plasmid pUG-1 (11). This generates a plasmid consisting of Ub with (X)9-S-L (where n is a variable number of residues) fused to the C terminus. The oligonucleotides for cytoplasmic expression were, for Ub-XXS-L, 5′-XXX XXX TCC ATC ATC AAC TTC GAG AAG CTG TAA TAGCTG-CA and for Ub-4N-X-S-L (Ub-LeQXLXS-L), 5′-CTG GAG CAG CTG XXX TCC ATC AAC TTC GAG CAG CTG TAA TAGCTG-CAGGATCC-3′. A PstI site (CTGCAG) was used for selection. XXX is a codon that encodes for an amino acid. The most common codons for each amino acid, based on the frequency of usage of each codon (per thousand) in human coding regions, were used: ala (A), GCT; arg (R), CGC; asn (N), AAC; asp (D), GAC; cys (C), TGT; gln (Q), CAG; glu (E), GAG; gly (G), GGC; his (H), CAC; ile (I), ATC; leu (L), CTG; lys (K), AAG; met (M), ATG; phe (F), TTC; ser (S), TCC; and thr (T), ACC. All plasmids were sequenced to confirm correct sequences and reading frames. Generation of signal sequence (ss) XXXS-L plasmids were described previously (32).

Cells

HeLa-Kb and HeLa-Kb-ICP47 (HeLa-Kb-A3–47 cells) [HeLa cells stably transfected with H-2Kb, HLA-A3 and ICP47] cells were derived previously (19, 33). Mouse embryonic fibroblasts (MEFs) were prepared from wild-type C57BL/6 (two independent lines) and ERAP1 knockout mice for use in experiments. This analysis revealed that N-terminal trimming amino acids had a marked influence on the levels of presented peptide detected on the cell surface (Fig. 1A). S-L from all constructs was presented, but at different levels, such that there was a highly reproducible hierarchy of presentation that depended on the specific N-terminal residues. Amino acids, such as alanine, cysteine, tyrosine, and methionine, seem to be removed efficiently from precursors to generate the mature S-L epitope. In fact, 16 of the 19 aa (including alanine) were associated with presentation that was ≥40% of that observed with Ub AAS-L (the construct leading to the greatest presentation), demonstrating that many amino acids are removed rather efficiently. Only glutamate, aspartate, and glycine were associated with lower presentation: <40% of that observed with Ub AAS-L. However, although the acidic amino acids glutamate and aspartate seem to be associated with low presentation, the hierarchy of presentation did not otherwise correlate with the chemical nature of the side chain. Thus, among the various classes of amino acids (e.g., basic, hydrophobic, etc.), there was a broad range of efficiencies of presentation within each group (Fig. 1B).

If the Ub C-terminal hydrolases were cleaved the various Ub XXS-L constructs imprecisely or at different rates, this could contribute to the differential presentation of the various Ub XXS-L Ags. Although these proteases are thought to cleave precisely and independently of the P residue (35), we sought to evaluate this issue. In an attempt to exclude the possibility that differences in presentation between the various constructs was due to the specificity of the Ub hydrolases, we sought to generate XXS-L in another way. For these experiments, we generated a subset of minigenes that consisted of a Kozak consensus sequence followed by an initiating methionine, and the sequence XXS-L (we excluded X = M from this analysis because of the potential for multiple translation initiation sites) were X-encoded amino acids that were processed efficiently (L and N) and inefficiently in the cytoplasm (E, G, and I), as well as amino acids that lie between these two extremes (F, K, Q, and V). When these constructs were transfected into HeLa-Kb cells, they were trimmed and presented on H-2Kb (Fig. 1C). Again, there was a hierarchy of presentation of the various constructs; importantly, this hierarchy was essentially the same as that observed with Ub XXS-L (Fig. 1C). The concordance of the data from the Ub XXS-L and MAXXS-L constructs argues strongly that the hierarchy in presentation is not due to differences in the generation of precursors but rather is due to processing of the precursors to the mature peptide.

Given that the identity of the amino acid repeated twice N-terminally of an epitope markedly influences its presentation when
expressed in the cytosol, we next examined whether a single amino acid showed the same effect as the corresponding doublet. To this end, we generated a subset of Ub XS-L constructs that encoded amino acids that were processed efficiently (L, M, and Y) and inefficiently in the cytosol (D, E, and G), as well as amino acids that lie in between these two extremes (K and R). The data obtained with these constructs (Fig. 1D) are largely consistent with those obtained with the corresponding doublet, although the absolute differences in the level of presentation were smaller, presumably due to the reduction in the number of residues that needed to be removed to generate mature epitope (Fig. 1A).

Ub XXS-L trimming by ERAP1

Antigenic precursors produced in the cytosol could potentially be trimmed in the cytosol and/or in the ER after transport by TAP. The aminopeptidase ERAP1 seems to be the only important peptide-trimming enzymatic activity in the ER of HeLa cells, as demonstrated using S-L precursors with the natural flanking sequence (LEQLE) (19, 22). Therefore, we sought to determine how much ERAP1 contributes to the presentation of the various Ub XXS-L constructs. These precursors were transfected into HeLa-Kb cells treated with ERAP1-specific siRNA under conditions in which ERAP1 protein expression is reduced by \(90\%\) (19), and presentation was compared with cells treated with a control irrelevant siRNA. Ub C-terminal hydrolase cleavage of the control Ub S-L in the cytosol generates mature S-L directly. Because this mature epitope requires no ER trimming for presentation, its presentation should not be dependent on ERAP1. Treatment with ERAP1 siRNA did not affect presentation from this precursor (Fig. 2A), demonstrating that the siRNA does not cause off-target effects on Ag

**FIGURE 1.** Ag presentation in HeLa-Kb cells transfected with cytoplasmic targeted N-terminal S-L precursors. Cells were transfected with Ub XS-L, Ub XXS-L, or MAXXS-L (A, inset), where X represents one of the 20 aa. H-2K\(^{b}\)-S-L presentation was determined by staining cells with 25.D1.16. Data are arranged in order of efficiency of presentation, from highest to lowest, left to right (A) or are grouped according to the chemical nature of the amino acid side chain (B). Bar graphs represent the average mean fluorescence intensity (MFI) of two independent transfections. Error bars represent the difference within each group. Correlation of S-L presentation from Ub XXS-L and MAXXS-L (C) or Ub XS-L (D).
presentation. In contrast, the presentation of S-L from most of the Ub XXS-L constructs was reduced in ERAP1-silenced cells, suggesting a role for this enzyme in epitope generation from cytosolic precursors. Interestingly, there was a range of ERAP1-dependent presentation from these various N-terminally extended precursors. For example, presentation from Ub GGS-L was severely reduced in ERAP1-silenced cells, whereas presentation from Ub WWS-L was unaffected (again, a finding suggesting no off-target effects of the siRNA-treatment) (Fig. 2A). Dependence on ERAP1 was approximately similar for the other 17 constructs and fell between these two extremes (Fig. 2B). Overall, the magnitude of the ERAP1 dependence ranged from 0–80%.

The presentation that occurs in ERAP1-silenced cells presumably reflects trimming occurring in the cytosol and possibly some contribution from residual ERAP1. The large difference in ERAP1 dependence of the various XXS-L constructs suggests that there is sequence specificity for trimming by other peptidases responsible for the generation of S-L. Although ERAP1 siRNA reduces ERAP1 expression by \( \approx 90\% \) (19), a small amount of residual ERAP1 could still process precursors under these conditions. To test the potential contribution of residual ERAP1, we measured the presentation of a subset of peptides in MEFs prepared from wild-type (ERAP1\(^{+/+}\)) or ERAP1 knockout mice, in which ERAP1 is completely absent. Again, the dependence on ERAP1 for presentation was different for different constructs (Fig. 2C), and the pattern of dependence on ERAP1 correlated strongly between MEFs and HeLa cells (\( R^2 = 0.82 \) (Fig. 2D)). The magnitude of ERAP1 dependence in MEFs was greater than in HeLa cells, ranging from \( \approx 30\% \) to nearly 100%, suggesting that the small amounts of ERAP1 in siRNA-treated HeLa cells may contribute slightly to peptide processing. Alternatively, MEFs may have a slightly different spectrum of peptidases, such that ERAP1 is more important than in HeLa cells. In any case, the strong correlation between HeLa cells and MEFs suggests that peptide processing is remarkably similar between different cell types, as well as between different species.

Interestingly, even in MEFs, the absence of ERAP1 only modestly reduced the presentation of the Ub WWS-L construct. Therefore, a cytosolic peptidase can play a dominant role in the removal of tryptophan residues upstream of S-L prior to transport into the ER.

**Presentation and ERAP1-dependence of precursors expressed in the cytosol versus the ER**

It was of interest to compare the presentation of XXS-L constructs in the cytosol versus ER. To do this, we compared the presentation of the Ub XXS-L peptides with ER-targeted peptides that we had previously generated and characterized in HeLa-Kb cells stably transfected with ICP47 (32). ICP47 blocks the TAP transporter and prevents cytosolic peptides from gaining access into the ER (36),
allowing specific analysis of ER-specific trimming. These ER-targeted constructs consisted of the XXS-L sequences that were fused to an N-terminal signal sequence (ss XXS-L). Upon synthesis, these constructs are transported via the sec61 translocon into the ER, where the leader peptide is removed by the signal sequence peptidase; our previous studies found that differences in the presentation of these constructs were not due to differences in the efficiency of cleavage of the various constructs by the signal sequence peptidase (32).

We previously examined the presentation of the ER-targeted constructs in HeLa-Kb cells in which TAP was inhibited (32). In HeLa-Kb cells with a functional TAP transporter, the difference between highest and lowest presentation of Ub XXS8L and ss XXS8L constructs was much greater for the ER-targeted precursors than for the cytosolic precursors, and presentation from many of the epitope precursors was greater in relative terms if expressed in the cytosol than if targeted to the ER (Fig. 3A). As a result, when the averages of the results obtained in Fig. 3A were plotted, there was a poor correlation between presentation of the same constructs targeted into ER compared with the cytosol (Fig. 3B). Presentation from cytosol precursors is probably the result of a combination of editing in the cytosol and the ER (Fig. 2), whereas processing of ER precursors is only dependent on the specificities of the aminopeptidase(s) in this compartment (32). ERAP1 seems to play a major role in ER precursor editing in HeLa cells stably transfected with ICP47 (32), as well as in many cases of cytosolic precursors in HeLa cells with an active TAP transporter (Fig. 2). We next compared the role of ERAP1 in the processing of cytosolic precursors (Fig. 2) with that of ER precursors in HeLa cells with an active TAP transporter. Fig. 3C shows that all of the ER-targeted precursors (including WWS-L) rely much more heavily on the activity of ERAP1 than do the majority of the cytosolic precursors. Thus, a large percentage of presentation from cytosolic precursors is due to an ERAP1-independent component, and the differences in presentation efficiency must reflect trimming within the cytosol.

**Presentation from precursors with and without TAP activity**

To further assess the compartment in which the various cytosolic or ER precursors are trimmed, we compared the presentation of S-L in HeLa-Kb or HeLa-Kb-ICP47 (HeLa cells stably transfected with ICP47) cells (33). No presentation was observed when SIINFEKL, the mature epitope, was expressed within the cytoplasm (Ub S-L), indicating efficient TAP inhibition, whereas ER-targeted S-L (ss S-L), which bypasses the need for TAP transport, was presented efficiently (Fig. 4A). As expected, the presentation of all of the cytosolic precursors (Ub XXS-L constructs) was essentially

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Comparison of precursors expressed in the cytosol and the ER. A, HeLa-Kb cells were transfected with ER- (ss; black bars) or cytosol- (Ub; white bars) targeted XXS-L and stained with 25.D1.16. Data are arranged in order of efficiency of presentation from ER-targeted precursors from highest to lowest, left to right. B, Correlation of S-L presentation from Ub XXS-L and ss XXS-L (from the mean data in A). Following treatment with control or ERAP1 siRNA, HeLa-Kb cells were transfected with ss XXS-L or Ub XXS-L. C, The percentage of S-L presentation that is ERAP1 dependent was calculated [100 - (MFI with ERAP1 siRNA/MFI with control siRNA) × 100]. The Ub XXS8L data in C are from Fig. 2B and are regraphed for comparison with the new ss XXS8L data. Data represent the average percentage of two independent experiments.

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Effect of TAP inhibition on S-L presentation from cytosol and ER precursors. A, HeLa-Kb-ICP47 cells were transfected with Ub XXS-L for 24 h and stained for surface H-2Kb-S-L. ER-targeted S-L (ss S-L) and cytoplasmic S-L (Ub S-L) were used as a positive control and negative control, respectively. B, Comparison of S-L presentation from ER-targeted precursors following transfection of HeLa-Kb cells (black bars) and HeLa-Kb-ICP47 cells (white bars). Bar graphs represent the average percentage of two independent experiments using the MFI of cells transfected with ss MMS-L as 100% presentation. Error bars represent the difference within each group. C, Using the data in B, the percentage TAP dependence of each ss XXS-L construct was calculated.
completely inhibited in HeLa-Kb-ICP47 cells, because these cytosolic peptides require TAP for transport into the ER. In contrast, the presentation of S-L from most ER-targeted precursors was efficient (Fig. 4B). Unexpectedly, however, the presentation of S-L from many ER-targeted constructs (e.g., those with D, W, and E) was partially to almost completely inhibited by ICP47 (Fig. 4B, 4C), implying that the presentation of these ER-targeted precursors remained at least partially dependent on TAP.

**XXXS-L cytosol dependence**

Several studies have raised the possibility of retrotranslocation of epitope precursors from the ER into the cytoplasm for trimming, followed by reimport of peptides into the ER for presentation (37–39). In this study, some of the ER-targeted precursors (ss XXS-L) that show a strong reliance on the cytosol for presentation (i.e., are inhibited by ICP47; Fig. 4B), also require ERAP1 for processing (Fig. 3C). This suggests that epitope production may be dependent on trimming in the cytosol and ER. Assuming that the ER-targeted construct is not mistargeted into the cytosol, the epitope precursor must be retrotranslocated into the cytoplasm for processing. Thus far, our findings suggest that for ER-targeted precursors that require ERAP1 and TAP, these XXS-Ls are poor substrates for ERAP1 and must be converted into XS-L in the cytosol (XXXS-L → XS-L) for ERAP1-dependent removal of X (XXS-L → S-L) or that following the ERAP1-dependent generation of XS-L in the ER (XXXS-L → XS-L), S-L must be generated in the cytosol (XS-L → S-L), because these XS-Ls are poor substrates for ERAP1. To distinguish between these possibilities, we compared the presentation from ss D/W/ES-L and ss DD/WW/EES-L, the latter of which is dependent on ERAP1 and the cytosol for presentation (Figs. 3C, 4B, 4C) in HeLa-Kb cells with and without ICP47. ss MS-L and ss MMS-L, the latter of which is ERAP1 dependent and cytosol independent (Figs. 3C, 4B, 4C), were used as controls. Presentation from ss MS-L and ss MMS-L was not TAP dependent, implying that M and MM can be removed efficiently in the ER for S-L production (Fig. 5A). Interestingly, although presentation from ss DD/WW/EES-L was inhibited when TAP was inactivated, as shown previously, the presentation from ss W/ES-L and, to a lesser extent, ss D S-L was not affected (Fig. 5A). This suggests that the W/E residue can be efficiently removed from W/E S-L in the ER, without the need for any other component. To test this further, precursors were transfected into HeLa-Kb cells that were treated with control or ERAP1-specific siRNA (Fig. 5B). S-L presentation was reduced in ERAP1 knockdown cells transfected with ss D/W/E/M S-L as well as ss DD/DD/EE/MM/MMS-L, demonstrating that ERAP1 was required for removal of the N-terminal amino acids in all of these precursors. Taken together, this is suggestive of a mechanism that involves XXS-L retrotranslocation into the cytoplasm for XS-L generation, followed by transport back into the ER for ERAP1-specific generation of S-L.

**Discussion**

Proteasomes generate many N-terminally extended precursors. For these precursors to be presented on an MHC class I molecule, they must be trimmed by peptidases to mature epitopes. Previous studies showed that the removal of amino terminal flanking residues occurs in living cells. This N-terminal trimming was “downstream” (independent) of proteasomes and mediated by aminopeptidases. It was clear that this peptide editing could, in principle, occur in the cytosol, where peptides were first generated by proteasomes, and/or after transport into the ER by TAP. However, the relative contribution of these two compartments to trimming and their specificity had been incompletely understood.

![FIGURE 5.](https://doi.org/10.1088/1473-4409/aa6622) ER-targeted precursors require cytosol trimming. A, HeLa-Kb cells (black bars) and HeLa-Kb-ICP47 cells (white bars) were transfected with ss XS-L or ss XXS-L. Bar graphs represent the average percentage presentation of two independent wells using the MFI of cells transfected with ss MMS-L as 100% presentation. Error bars represent the difference within each group. B, HeLa-Kb cells were treated with siRNA targeting ERAP1 (white bars) or ctrl siRNA (black bars) and transfected with ss X/S-L or ss XXS-L. Bar graphs represent the average percentage presentation of two independent experiments using the MFI of cells treated with control siRNA and transfected with Ub MMS-L as 100% presentation. Error bars represent the difference within each group.

The specificity of trimming in these compartments in vitro has been studied using fluorescent substrates and purified cytosol or ER (31). However, in vivo, the effect that sequences N-terminal to an epitope have on processing and presentation on MHC class I molecules has only been systematically studied in the ER (32). This is important to define for cytosolic trimming, because extracts may not faithfully reproduce the conditions in living cells (e.g., concentrations and ionic conditions are changed, enzymes may be activated or inactivated, and metabolic pathways are inhibited); presumably, because of this, the specificity of trimming that we and Reits et al. (9) observed in vivo is not identical to that reported by Schatz et al. (31). Defining what is occurring in vivo is important biologically, because the specificity of trimming can clearly influence the magnitude of responses and overall immunodominance hierarchies. In this study, we analyzed the trimming of precursors in the cytoplasm of living cells and compared it with the trimming of the same precursors in the ER.

Our experimental approach was to express, in living cells, N-extended precursors in which we systematically varied the amino acids at the P2 and/or P1 position N-terminal to the SL8 epitope. Our findings clearly demonstrate that trimming of these precursors can occur in the cytosol and in the ER; the efficiency of cytosolic trimming process, like that of ER trimming (32), is affected by the N-terminal residues, i.e., it has specificity; the specificity of cytosolic trimming is distinct from that in the ER; recycling of peptides from the ER to the cytosol may occur, potentially allowing sequential trimming of peptides in both compartments in either order; and the
net effect of cytosolic trimming is to broaden the repertoire of peptides that can be presented on MHC class I molecules.

Our experimental approach makes certain assumptions that are worth discussing. We expressed a series of peptides from minigenes that were transfected into APCs. Our interpretation of the results assumes that the transcription, translation, and, for Ub-X constructs, the posttranslational Ub cleavage, are similar for all constructs. Because the Ub construct bicistronically expresses GFP, we are able to gate on cells expressing similar levels of GFP that should also be expressing similar levels of the Ub fusion proteins. To further test this assumption, we compared presentation from minigene constructs that are processed very differently and obtained very similar results using MAXXSL, Ub-XXS-L, and Ub-XS-L constructs. This rules out the possibility that differences in presentation arise from differential Ub-X cleavage and makes it highly unlikely that one (X) or two (XX) residues placed at different locations (2 or 76 residues) from the translational start site would affect translation (or transcription) and do so in the exact same way. Therefore, differences in translation, transcription, and or Ub cleavage are unlikely to account for the differences in presentation that were seen with different specific sequences.

Although it is possible that these upstream residues may also affect TAP transport of cytosolic precursors, the residues that are consistently associated with high-level presentation of our model epitope are not particularly preferred for TAP translocation (40, 41). Moreover, we saw very similar results in mouse and human cells, although mouse and human TAP have somewhat different preferences for translocation (42–45). Therefore, we interpret that presentation observed with the various epitope precursors reflects aminopeptidase specificity within the cell. However, it should be pointed out that, even if TAP selectivity contributes to some of the observed differences, our results still define the overall specificity of Ag presentation within the cell (i.e., cytosolic trimming, TAP transport, and ER trimming). In addition, for the ER-targeted constructs we assume that XX residues do not influence cleavage by the signal peptidase, which liberates the epitope precursor from the signal peptide. This is supported by two pieces of data that show that the same results are obtained for sequences that are adjacent (ss X-S-L) or six residues away (ss LEQLXS-L) from the signal sequence cleavage site and that presentation from minigenes and trimming of the same sequences by purified ERAP1 correlate well with one another (32).

Our data show that much of the trimming of precursor peptides occurs in the ER, even when the peptides are originally generated in the cytosol, but the relative importance of ER versus cytosolic trimming depends on the specific N-terminal residues. In one case, extensive trimming of a cytosolic precursor can occur in the cytosol (WWS-L). This N-terminal flanking sequence must be removed very efficiently in the cytosol, because when this same construct was targeted into the ER directly, it was efficiently trimmed by ERAP1. In general, cytosolic trimming is more important for those sequences that are more poorly trimmed in the ER, broadening the sequences that can be efficiently processed and presented. As a result, the difference between the best and the worst presented constructs was smaller when N-extended peptides were expressed in the cytosol compared with the ER. Only N-terminal glycine was associated with relatively poor presentation in cytosolic- and ER-targeted constructs. Nevertheless, the trimming that occurs in the cytosol does have specificity. There is a reproducible hierarchy, observed in human and mouse cells, in the trimming and presentation of the various constructs based on the identity of their N-terminal residues.

Many of the precursors that were targeted by a signal sequence through SEC61 into the ER that were efficiently trimmed were presented equally well in control cells and cells in which TAP was inhibited with ICP47, as expected. However, a remarkable finding was that the presentation of some ER-targeted precursors was strongly inhibited by ICP47. It is possible that these were ones that failed to translocate through SEC61. However, this explanation seems unlikely, because SEC61 can clearly transport the particular residues when they are in other locations (e.g., E in S-L) or even when the residue is present as a singlet (ss XS-L), and SEC61 obviously transports proteins that have all 20 aa. Another possibility is that the signal peptidase failed to cleave the signal peptide from certain precursors, and these had to be retrotranslocated to the cytosol for the signal peptide to be removed. However, whether cleavage occurs depends mainly on features of the signal peptide that remain unchanged in all of the constructs used throughout this study, particularly the amino acids at positions −3 and −1 N-terminal of the cleavage site (reviewed in Ref. 46). It seems more plausible that these sequences need export and trimming in the cytosol because they are very poorly trimmed in the ER. Indeed, the residues that are ICP47 inhibitable are the ones that are poorly removed by ERAP1 (32) and are particularly poorly removed when they are present in tandem.

Several previous studies raised the possibility of export followed by reimport of peptides into the ER for presentation. For example, in vivo, epitopes are generated in a TAP-dependent mechanism from the signal sequence of LCMV gp33 protein (37), as are HLA-E–binding epitopes generated from the signal sequences of MHC class I molecules (38). Such TAP-dependent presentation could be due to failed translocation of the signal sequence into the ER or retrotranslocation of peptide precursors from the ER into the cytosol. An elegant study by Altrich-VanLith et al. (39) showed this to be the case for an epitope derived from tyrosinase. In this system, TAP inhibition significantly diminished presentation from ER-targeted precursors with two-residue extensions containing histidine. His is one of the amino acids poorly removed in the ER, and it required cytosolic trimming in our experimental system (ss HHS-L). In addition, earlier studies of isolated microsomes also demonstrated that ER luminal peptides could be retrotranslocated out of the ER and recycle back in a TAP-dependent fashion (47). Thus, there is growing evidence that ER-to-cytosol peptide translocation can occur, although the biological importance of this phenomenon was unknown. In these earlier studies, it was unclear why retrotranslocation might be important for presentation. Our findings suggest that ER-to-cytosol recycling may be important in situations in which ERAP1 does not trim particular sequences down to mature epitopes. Our data further suggest that recycling of peptides between the ER and cytosol is likely to be a general phenomenon that occurs physiologically and contributes to MHC class I Ag presentation.

In conclusion, our findings reveal that the amount of MHCrepeptide complex presented to the immune system is determined by the identity of amino acids upstream of the epitope precursor and the ability of aminopeptidases to remove them. In many cases, mature epitope generation is a combination of processing that has occurred in the cytosol and the ER. Taken together, this demonstrates an important role for aminopeptidases in influencing the specificity of CD8$^+$ T cell responses.

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Disclosures
The authors have no financial conflicts of interest.
References


Supplementary Figure 1

**Representative gating of Ub XX SIINFEKL transfected HeLa-Kb cells.** Following transfection of HeLa-Kb cells with Ub HH SIINFEKL, cells were stained with 25.D1.16 and analyzed by flow cytometry. Live cells were first gated on (A) followed by gating on GFP-expressing cells to limit the analysis to transfected cells (B). Cells expressing large amounts of GFP were gated out due to saturation of the H-2K\(^b\)-S-L signal.