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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 online version of this article contains supplemental material.

Abbreviations used in this paper: ER, endoplasmic reticulum; ERAP1, endoplasmic reticulum aminopeptidase 1; MEF, mouse embryonic fibroblast; MFI, mean fluorescence intensity; S-L, chicken ovalbumin peptide SIINFEKL; siRNA, small interfering RNA; ss, signal sequence; TAP, transporter associated with Ag presentation; TOP, thimet oligopeptidase; Ub, ubiquitin.

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Major histocompatibility complex class I molecules present a large variety of peptides generated by intracellular protein degradation to the immune system. This allows the identification and elimination of cells synthesizing abnormal or “foreign” proteins, which may be a result of mutation or infection by virus (1–5).

Peptides that can stably bind MHC I molecules are generally 8–10 residues in length and are generated by a complex pathway involving many components in several cellular compartments. The first step in this pathway is degradation of proteins by the proteasome in the nucleus or cytosol. This multisubunit protease is capable of generating mature epitopes (~<5% of product) (6). However, the majority of epitope-containing peptides (15–25% of proteasome product) (6) are extended on the N terminus and are incapable of MHC binding unless processed further (7). A large number of aminopeptidases and endopeptidases within the cytosol have the potential to create or destroy MHC-binding peptides from the precursors generated by the proteasome (8–17). Within seconds, the majority of peptides in the cytosol are hydrolyzed into amino acids that can then be reused in the synthesis of new proteins (18). However, some escape destruction and are shuttled by the transporter associated with Ag presentation (TAP) into the endoplasmic reticulum (ER). In addition, some MHC class I-presented peptides may be generated through the trimming of long antigenic precursors in the cytosol (19). TAP translocates peptides longer than 16 residues inefficiently in vitro (20); therefore, trimming of long precursors by peptidases within the cytosol may also be critical for the generation of peptides suitable for TAP binding (21).

A substantial amount of the trimming of N-extended precursors is performed in the ER by the IFN-γ-inducible metallopeptidase ER aminopeptidase 1 (ERAP1) (ER aminopeptidase associated with Ag processing) (19, 22, 23). ERAP1 prefers substrates 9–16 residues long (19, 24), which corresponds to the length of peptides efficiently transported by TAP (20). ERAP1 seems to be particularly adapted to generate peptides for MHC class I presentation, because it rapidly trims precursors down to eight or nine residues before cleavage slows or stops completely. Thus, unlike other aminopeptidases that continually cleave peptides until only one free amino acid remains, ERAP1 acts with a molecular ruler to generate peptides of the optimal length for MHC class I binding (24). It was also suggested that ERAP1 cooperates with MHC class I molecules to generate mature epitopes, although the available data do not distinguish whether MHC class I molecules are actually involved in the trimming process or only bind and protect mature epitopes from destruction (25). In cells lacking ERAP1, many antigenic precursors are trimmed poorly, if at all, in the ER (19, 22, 26–28); mice lacking ERAP1 had markedly different presentation of a variety of epitopes (26–29), leading to differences in immunodominance hierarchies (26, 29, 30). However, precursor peptides generated in the cytosol are still presented to various extents in ERAP1-deficient cells, indicating that some trimming of presented peptides occurs in the cytosol (19).

Schatz et al. (31) recently investigated the role that the ER and cytosol may play in epitope generation in cell extracts, and we examined the role of ER trimming in intact cells (32). These studies suggest that trimming in the ER plays an important part in generating antigenic peptides, but it does not account for all peptide processing. Understanding the contribution and specificity...
of nonproteasomal proteases in vivo and 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)n-PL (where n is a variable number of residues) fused to the C terminus. The oligonucleotides for cytoplasmic expression were synthesized by the manufacturer’s protocols. For Ub-XS-L, 5′-XXX XXX TCC ATC ATC AAC TTC GAG AAG CTG TAA TAGCTG-3′ and for Ub-4N-X-S-L (Ub-LEQLXS-3′), 5′-CTG GAG CAG CTG XXX TCC ATC ATC AAC TTC GAG AAG CTG TAA TAGCTG-CAGGATCC-3′. A PstI site (CTGGAC) 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) XS-L plasmids was 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 described previously (19, 33). Mouse embryonic fibroblasts (MEFs) were prepared from wild-type C57BL/6 (two independent lines) and ERAP1 knockout mice (two independent lines), as previously described (26).

Small interfering RNA

Cells were transfected with small interfering RNA (siRNA) for ERAP1 or with the control siRNA murine thimet oligopeptidase (TOP) (directed against murine TOP in a region that differs from human TOP) using oligofectamine (Invitrogen, Carlsbad, CA) as previously described (19).

Abs and flow cytometry

HeLa cell lines were transiently transfected with plasmid (0.25–1 μg) using TransIT HeLa Monster (Mirus, Madison, WI), according to the manufacturer’s protocol. MEFs were transfected with FuGene6 (Roche, Indianapolis, IN), according to the manufacturer’s protocols. After transfection, cells were incubated for 24–48 h, and H-2Kb-S-L complexes on the cell surface were detected using mAb 25.D1.16 (34). The cells were analyzed by flow cytometry on a FACSCalibur apparatus (BD Biosciences, San Jose, CA) with CellQuest (BD Biosciences) or FlowJo (Tree Star, Ashland, OR) software, gating on GFP-expressing cells to limit the analysis to transfected cells (Supplemental Fig. 1). Unless otherwise stated, data are representative of three independent experiments.

Results

The effect of N-terminal flanking residues on the presentation of cytosolic precursors

Because antigenic epitope precursors are first generated in the cytosol, which contains resident aminopeptidases that can trim peptides, we sought to analyze the specificity of trimming of N-extended precursors in this compartment.

To systematically examine the specificity of trimming of N-extended precursors of MHC class I-presented peptides, we generated a series of minigenic constructs containing the model epitope SIINFEKL (S-L) (the immunodominant H-2Kb–restricted epitope from chicken OVA). Minigenes were constructed to encode 19 different amino acids (proline was excluded for reasons described below) on the N terminus of the mature epitope. To amplify any differences between constructs in the rate of removal of their flanking residues, they were expressed with two identical amino acids upstream of the epitope, generating XSS-L, where X represents any amino acid. These sequences were ligated in frame to the 3′ region of ubiquitin (Ub) so that they would be expressed as fusion proteins (Ub XSS-L) in the cytosol. Ub C-terminal hydrolases in cells efficiently and precisely cleave the peptide bond between the Ub moiety and any fused sequence [unless the residue flanking the scissile bond is a proline (35), which is why proline was omitted from our series]; thus, when expressed in cells, these constructs efficiently generate XSS-L in the cytoplasm and avoid the need for an initiating methionine on the N terminus of the antigenic peptide. The Ub XSS-L sequences were cloned into a plasmid that bicistrionically expressed GFP, allowing the direct correlation of GFP and Ub XSS-L expression. The Ub XSS-L constructs were transiently transfected into HeLa-Kb cells, and the amount of S-LK complexes on the cell surface of cells expressing similar levels of GFP was quantified by staining with the mAb 25.D1.16, which recognizes the combination of H-2Kb and S-L (34). When N-extended precursor peptides are expressed in cells, only the mature epitopes are presented on H-2Kb and detected by 25.D1.16 (19, 21). This assay gave highly reproducible results between replicate groups and independent experiments. This analysis revealed that N-terminal flanking 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, lysine, tyrosine, and methionine, seem to be removed efficiently from precursors to generate the mature S-L peptide. 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 to cleave the various Ub XSS-L constructs imprecisely or at different rates, this could contribute to the differential presentation of the various Ub XSS-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 XSS-L in another way. For these experiments, we generated a subset of minigenes that consisted of a Kozac consensus sequence followed by an initiating methionine, and the sequence AXXX-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 XSS-L (Fig. 1C). The concordance of the data from the Ub XSS-L and MAXXX-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-SL, Ub XXS-SL, or MAXXS-SL (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-SL and MAXXS-SL (C) or Ub XS-SL (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 ≥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 (R² = 0.82) (Fig. 2D). The magnitude of ERAP1 dependence in MEFs was greater than in HeLa cells, ranging from ~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) (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...
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.

**X/XXS-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 XSS-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 XSS-Ls are poor substrates for ERAP1 and must be converted into XS-L in the cytosol (XSS-L → XS-L) for ERAP1-dependent removal of X (XS-L → S-L) or that following the ERAP1-dependent generation of XS-L in the ER (XS-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/WW/EE/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 XSS-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.

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 cytosol 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

![FIGURE 5. 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 XSS-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 XS-L or ss XSS-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.](http://www.jimmunol.org/)
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 biocronstrically 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 different 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 inhabitable 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 peptide 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 MHC–peptide 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.
SPECIFICITY OF CYTOSOLIC AND ER AMINOPEPTIDASES

References


