Requirement of the Proteasome for the Trimming of Signal Peptide-derived Epitopes Presented by the Nonclassical Major Histocompatibility Complex Class I Molecule HLA-E*

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The nonclassical major histocompatibility complex class I molecule HLA-E acts as a ligand for CD94/NKG2 receptors on the surface of natural killer cells and a subset of T cells. HLA-E presents closely related nonameric peptide epitopes derived from the highly conserved signal sequences of classical major histocompatibility complex class I molecules as well as HLA-G. Their generation requires cleavage of the signal sequence by signal peptidease followed by the intramembrane-cleaving aspartic protease, signal peptidease. In this study, we have assessed the subsequent proteolytic requirements leading to generation of the nonameric HLA-E peptide epitopes. We show that proteasome activity is required for further processing of the peptide generated by signal peptidease. This constitutes the first example of capture of a naturally derived short peptide by the proteasome, producing a class I peptide ligand.

Peptide presentation by major histocompatibility complex (MHC) class I molecules allows the display of intracellular protein fragments to the immune system. Several parameters control this process including the ability of peptide to bind to MHC class I molecules, precursor protein expression and stability, processing efficiency, and rate of peptide transport into the endoplasmic reticulum (ER).

Most peptides presented by MHC class I molecules originate from proteins fragmented in the cytosol by the ubiquitin–26 S proteasome pathway (1). Generally, the antigenic peptides produced by the proteasome are either of the correct size or extended at their N termini. Further trimming primarily by aminopeptidases occurs in the cytosol (2) or in the ER lumen (3–5). The peptides are transported into the ER lumen by the transporter associated with antigen processing (TAP) and are finally loaded onto newly synthesized MHC class I molecules, which traffic to the cell surface. In addition, a small fraction of MHC class I epitopes was reported to be generated independently of the proteasome (6–8) and may be produced by the cytosolic subtilisin-like tripeptidyl peptidase II (9, 10).

The nonclassical MHC class I molecule HLA-E is specialized in the presentation of peptides derived from MHC class I signal sequences, reporting their expression level to natural killer (NK) cells (11, 12). HLA-E interacts with CD94/NKG2A or CD94/NKG2C receptors, resulting in inhibition or activation of NK cell function, respectively (13–15). More recently, HLA-E has also been implicated in the presentation of self-peptides and bacterial antigens to T cells, although the role of these T cells in immune responses remains to be established (16–19).

We have previously demonstrated that the generation of the MHC signal peptide-derived epitopes binding to HLA-E does not follow the "conventional" antigen-processing pathway described above. During biosynthesis of the MHC class I precursor and its translocation into the ER lumen, the signal sequence is cleaved by signal peptidease and subsequently processed by signal peptide peptidease (SPP) (20, 21). This second cut occurs in the hydrophobic membrane-spanning region, allowing the release of an ~14 residue-long N-terminal signal peptide fragment into the cytosol. Peptide entry into the cytosol is consistent with the TAP dependency of HLA-E expression. The epitope-containing fragment is then transported into the ER lumen for loading onto newly synthesized HLA-E (20).

In the present study, we investigated the subsequent steps needed to generate the nonameric peptide binding to HLA-E. We show that following cleavage by SPP, proteasomal activity is required to trim the extended peptide fragment liberated into the cytosol. This is consistent with our previous finding that C-terminal trimming increases the efficiency of TAP transport and is required for peptide binding to HLA-E (20). Disruption of the trimming propensity in a few alleles including HLA-A*2901 prevents the generation of the nonameric peptide.

EXPERIMENTAL PROCEDURES

Inhibitors—Lactacystin, clasto-lactacystin β-lactone, epoxomicin, MG132, EST ([2S,3S]-trans-epoxyoctanoyl-α-lysyeaamido-3-methylbutane ethyl ester), and calpain inhibitor I were all obtained from CN Biosciences. Leucinethiol, bestatin, and 1,10-phenanthroline were obtained from Sigma. (Z-LL)2-ketone was synthesized as described previously (22). Ala-Ala-Phe-chloromethylketone was obtained from Affinity
Research. All of the inhibitors were maintained in stock solutions in Me₂SO with the exception of lactacystin, which was dissolved in water, and 1,10-phenanthroline and leucinethiol, which were dissolved in methanol, the latter of which was reduced prior to use in the presence of 50 mM dithiothreitol for 30 min at 4 °C.

Plasmid Construction—pcDNA3 containing the full-length cDNA coding for HLA-B*3508 (pcDNA3-B3508wt) was a kind gift from Emmanuel Zorn (Harvard Medical School, Boston, MA). To generate the mutant (HLA-B*3508mt), base 11 was exchanged by the QuikChange site-directed mutagenesis kit (Stratagene) using the sense primer 5′-ATGGCGGTCTATGGGCCCATGCCCAGTGGTGAAG-3′, resulting in pcDNA3-B3508mt. pcDNA1/Amp containing the full-length cDNA coding for HLA-A*2901wt was a kind gift from Pedro Romero (Ludwig Institute for Cancer Research, Lausanne, Switzerland). The BamHI/XhoI fragment was transferred into pcDNA3 (Invitrogen) to produce pcDNA3A*-2901. To generate the mutant (HLA-A*2901mt), base 41 was exchanged by the above method using the sense primer 5′-CTGCTACTCTGCCCCCG124CTGCCCCTGACC-3′ and the antisense primer 5′-GGCCAGGGCCATGACCCGCATGGTGAAG-3′, resulting in pcDNA3-B3508mt. pcDNA1/Amp containing the full-length cDNA coding for m7G(5′)ppp(5′)G CAP analog (New England Biolabs). In vitro transcription and translation were carried out as described previously (20). The SPP assay with CHAPS-solubilized microsomal membrane proteins and the S-35-labeled substrate peptide p-PrP40-50 (20) was performed as described previously (22). Proteins and peptides were analyzed by SDS-PAGE using Tris-Bicine gels (15% T, 8% C, 8 M urea), and radiolabeled proteins were visualized by a PhosphorImager (STORM 860, Amersham Biosciences, IQMac version 1.2) (22, 24).

Production of Stably Transfected Cell Lines—To prepare stably transfected cell lines, LCL721.221 (721.221) cells were electroporated with 30 μg of the respective plasmid DNA at 270 V with a capacitance of 1500 microfarads (25). Stable transfected clones were obtained after 3 weeks in culture with medium supplemented with 1 mg/ml G418-disulfate (Melford Laboratories Ltd). Surface expression of HLA-A*0301, HLA-A*2901mt, and HLA-E cell surface expression was monitored using GAP-A3 and DT9 antibodies, respectively (11). The secondary antibody used was phycoerythrin-labeled anti-mouse F(ab)'2 (DAKO). Anti-trinitrophenol antibodies were used as isotype controls.

Acid Treatment and Flow Cytometry—1.4 × 10⁶ 721.221-A*0301 cells or 721.221-A*2901mt cells were resuspended in 300 μl of media with appropriate supplements in the presence or absence of increasing concentrations of inhibitors. Cells were incubated for 2 h at 37 °C, washed once in phosphate-buffered saline, and resuspended in 175 μl of “acid buffer” (a 1:1 mixture of 0.263 M citric acid and 0.132 M NaH₂PO₄, pH 2.5) for 50 s. The cell suspensions were then neutralized by the addition of RPMI 1640 medium. After centrifugation, cells were resuspended in 300 μl of media and incubated for an additional 3 h at 37 °C in the presence or absence of inhibitors. Aliquots of cells were taken after the 2-h preincubation stage, the acid treatment step, and the final 3-h incubation. Toxicity of inhibitors was established by trypan blue staining both after the initial 2-h incubation and again after the final 3-h incubation following acid wash. None of the inhibitors was toxic at the given concentrations.

RESULTS

SPP-mediated Intramembrane Proteolysis Is Required but Not Sufficient to Generate the MHC Signal Peptide-derived Epitope Bound to HLA-E—We previously reported that the generation of the HLA-E binding epitope from the signal sequence of MHC class I molecules requires proteolysis by SPP, promoting the release of an epitope-containing fragment into the cytosol (20). To further characterize the sequence of events leading to the generation of the HLA-E binding nonamer, we analyzed the effect of single point mutations at the putative SPP cleavage site. For this purpose, we took advantage of naturally occurring polymorphisms within MHC signal peptides. Helix-breaking serine and glycine residues within the central hydrophobic region of MHC signal peptides were shown to be required for intramembrane cleavage by SPP (20) (Fig. 1A). The most common polymorphism at these residues (positions (P) 14–15) is found within two-thirds of HLA-B alleles in which the P14 serine is replaced by a tryptophan. Interestingly, this polymorphism is in linkage disequilibrium with a threonine at P2 of the nonameric peptide (P4 of the signal peptide) reported to be responsible for inefficient stabilization of HLA-E at the cell surface (12, 13, 26).

To study the effect of the serine to tryptophan substitution at P14, we stably transfected the HLA-A, HLA-B, HLA-C, and HLA-G-negative cell line 721.221 with the cDNA of HLA-
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B*3508 (B*3508wt) or a mutant of HLA-B*3508 (B*3508mt) that possessed a methionine at P2 of the HLA-E binding epitope (Fig. 1A). HLA-E cell surface expression as measured by flow cytometry using the monoclonal anti-HLA-E/C antibody DT9 (11) was only observed on cells expressing HLA-B*3508mt (Fig. 1B). HLA-E cell surface expression could not be detected on cells expressing HLA-B*3508wt (Fig. 1B). These results demonstrate that a serine to tryptophan substitution at P14 of the signal sequence does not affect the generation of the HLA-E-binding peptide (23). Importantly, these data show that the lack of surface HLA-E expression on cells transfected with most HLA-B alleles is due solely to the presence of a threonine at P2 of the HLA-E binding peptide rather than inefficient processing as suggested in a previous report (37).

The second polymorphism we studied was the substitution of the serine at P14 to a leucine, as found in some HLA-A alleles including HLA-A*2901 (Fig. 1A). Interestingly, we could not detect any cell surface HLA-E expression upon transfection of HLA-A*2901wt into 721.221 cells despite the presence of the HLA-E-binding peptide within the signal sequence (Fig. 1B). To confirm that the leucine was responsible for the lack of HLA-E cell surface expression, we reintroduced a serine at P14 (HLA-A*2901mt). When transfected into 721.221, HLA-A*2901mt induced cell surface expression of HLA-E, consistent with a critical role for the P14 leucine in disrupting the generation of the HLA-E-binding epitope (Fig. 1B).

We then investigated whether the P14 leucine was affecting cleavage by SPP or a subsequent event. For this purpose, we used a previously established cell-free in vitro translation/translocation assay (20). mRNA coding for the 24-residue-long signal sequence of HLA-A*2901wt and HLA-A*2901mt plus an additional 100 residues from the α1 domain were translated in reticulocyte lysate in the presence of ER-derived rough microsomes (Fig. 2A). An analysis of [35S]methionine-labeled translation products showed that both signal sequences were cleaved by signal peptidase as the HLA-A/100 products were generated (Fig. 2A, lanes 2, 3, 6, and 7). Interestingly, in both HLA-A*2901wt and HLA-A*2901mt, the full-length signal peptide was not detected, suggesting that it had been further processed. On addition of the SPP inhibitor (Z-LL)2-ketone (22, 28), the signal peptides were recovered, demonstrating that SPP not onlycleaves the HLA-A*2901mt signal peptide containing the two helix-breaking residues, serine and glycine, but also when the serine is replaced by a leucine as in HLA-A*2901wt. This result is consistent with our previous study that defined requirements for SPP cleavage using mutated preprolactin signal sequences (23).

(Z-LL)c-ketone also affected re-expression of HLA-E in a cellular assay. Re-expression of HLA-E after a mild acid treatment, which removed cell surface MHC class I, was substantially impeded on 721.221-HLA-A*2901mt (Fig. 2B) and 721.221-HLA-A*0301 (data not shown) in the presence of 10 and 20 μM (Z-LL)c-ketone. No toxicity was observed as measured by death rate in the presence or absence of inhibitor (data not shown). Taken together, these results indicate that HLA-A*2901 and some additional HLA-A alleles with identical signal sequences are unable to induce cell surface HLA-E, despite both the presence of the HLA-E binding peptide within the signal sequence, and SPP cleavage releasing an epitope-containing fragment into the cytosol. This finding underlines additional requirements for the generation of HLA-E binding epitopes such as further trimming, sensitivity to peptide degradation, or constraints imposed by the TAP transporter.

Requirement of the Proteasome for Trimming of MHC Class I Signal Peptide Fragments Containing the HLA-E Binding Peptide—SPP releases a peptide fragment that requires trimming at both the N and C termini to generate the nonamer binding to HLA-E. To investigate which proteases are required for trimming, we tested the effect of a series of protease inhibitors on HLA-E cell surface expression. For this purpose, we monitored HLA-E re-expression at the cell surface of 721.221-HLA-A*0301 following a mild acid treatment and in the presence of increasing concentrations of inhibitors. Leucinamide and 1,10-phenanthroline, which were recently reported to inhibit the ER aminopeptidase 1 (3–5), did not affect HLA-E cell surface expression at the concentrations previously shown to inhibit ER aminopeptidase 1 (Table I). This finding suggests that N-terminal trimming by ER aminopeptidase 1 in the ER may not be required to generate the HLA-E binding peptide from MHC class I signal sequences.

Interestingly, only proteasome inhibitors impeded HLA-E re-expression (Table I and Fig. 3A). Epoxomicin, which is thought to be the most specific proteasome inhibitor available commercially and primarily affects the chymotrypsin-like activity, blocked HLA-E re-expression at a concentration as low as 0.5 μM. Lactacystin and its active component, clasto-lactacystin β-lactone, are also potent inhibitors of the proteasome, although lactacystin is able to inhibit tripeptidyl peptidase II at concentrations higher than those used in this study. Again, both inhibitors blocked HLA-E re-expression. In contrast,
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**Table I**

| Inhibitor         | Specificity                                      | Concentration tested | Surface HLA-E expression in presence of inhibitor |
|-------------------|--------------------------------------------------|----------------------|--------------------------------------------------|
| Lactacystin       | Proteasome chymotryptic and tryptic activities   | 10–50                | −                                                |
| Clasto-lactacystin β-lactone | Proteasome chymotryptic and tryptic activities   | 5–10                 | −                                                |
| Epoxomicin        | Proteasome chymotryptic, tryptic and peptidylglutamyl peptide/hydrolyzing activities | 0.5–2               | −                                                |
| Calpain inhibitor I | Proteasome, calpains I and II, cathepsins B, and L, SPP | 350                  | −                                                |
| MG132             | Proteasome, SPP                                  | 5–10                 | −                                                |
| (Z-LL)₂-ketone    | SPP                                              | 10–20                | −                                                |
| Leucinethiol      | Metalloproteases, aminopeptidases B and M        | 10–30²               | +                                                |
| AAF-CMK           | Serine proteases, TPPII                          | 25–100               | +                                                |
| EST                | Cysteine proteases                               | 20–100               | +                                                |
| 1,10-Phenanthroline | Metalloproteases and caspase-1                   | 100–250              | +                                                |
| Bestatin          | Metallo-aminopeptidases                          | 10–200               | +                                                |

* Denotes toxicity observed at given concentration. Leucinethiol was reduced with dithiothreitol.

HLA-A*0301 re-expression was not significantly affected, consistent with previously published findings (8, 29).

To ensure that inhibition of HLA-E re-expression was not a result of SPP blockade, we tested whether the proteasome inhibitors could inhibit SPP activity using the *in vitro* assay with detergent-solubilized ER membrane proteins. Cleavage of the standard SPP substrate preprolactin signal peptide p-PrP(B/T) (23) was blocked by 1 μM (Z-LL)₂-ketone or 10 μM MG132 but was unaffected by lactacystin and epoxomicin at concentrations affecting HLA-E re-expression (Fig. 3B). Conversely, proteasome activity was not inhibited by (Z-LL)₂-ketone at the concentration used in our assay as previously shown by competition experiments (22). Therefore, these results implicated the proteasome in the generation of the MHC signal peptide-derived nonamer binding to HLA-E. The finding that (Z-LL)₂-ketone blocks re-expression of HLA-E in a cellular assay (Fig. 2B) argues against proteasome-mediated epitope generation from defective MHC class I ribosomal products (30). Altogether, these results indicate that the proteasome is involved in trimming of the epitope-containing fragment released into the cytosol after cleavage by SPP. The finding that the serine to leucine substitution at P14 in HLA-A*2901 blocked HLA-E expression without affecting SPP cleavage is consistent with disrupted proteasomal trimming.

**DISCUSSION**

The 26 S proteasome is the major proteolytic complex in the cytosol involved in the generation of peptides from intracellular proteins. Most of these peptides are further degraded into amino acids by endopeptidases and exopeptidases (1, 31). However, a minority escape this fate and are presented at the cell surface by MHC class I molecules. The proteasome generally produces peptides of the correct size or extended at the N termini (32). Trimming of the N-terminal extensions may occur in the cytosol involving peptidases such as leucine aminopeptidase, bleomycin hydrolase, and puromycin-sensitive aminopeptidase (2) and may occur in the ER lumen involving ER aminopeptidase 1 (3–5). In this report, we show that HLA-E epitope generation requires the proteasome but at a different step, namely in trimming the extended epitope liberated into the cytosol following class I signal peptide cleavage by SPP. This result was unexpected to our knowledge, as the proteasome has not yet been implicated in the trimming of such short peptides *in vivo*. In addition, it was previously reported that in mice, cell surface expression of the functional homologue of HLA-E, Qu-1, was unaffected by lactacystin (33). Discrepancy between humans and mice may be explained by a differential processing pathway because the signal sequences are not homologous.

A role for the proteasome in trimming the peptide liberated by SPP seems logical considering its localization. Proteasomes are found in the cytosol and the nucleus (34) with a significant proportion found in proximity to the ER (35). This co-localization would favor capture of the peptide following cleavage by SPP. The involvement of the proteasome in processing of the MHC signal peptide fragment is also consistent with previous studies, demonstrating its ability to generate antigenic peptides with correct C termini. Similar to other class I peptide ligands, the MHC signal peptide fragment with a correct C terminus is more efficiently transported by TAP and binds HLA-E with a higher affinity (20). SPP generates a peptide extended at the C-terminal end (20), and therefore, trimming at the C terminus is required, which is likely to involve the proteasome. Interestingly, a mutation at P14 in the HLA-A*2901 disrupted the generation of the HLA-E binding peptide. It is unclear at present whether the epitope in HLA-A*2901 signal sequence is destroyed by the proteasome, for example, or whether trimming fails to generate the correct C terminus required for efficient transport into the ER lumen or binding to HLA-E. However, the data presented here indicate that the P14 leucine disrupts proteasomal trimming.

Our findings emphasize that proteasomal trimming constitutes a vital step in the generation of HLA-E epitopes as equally important as the preceding cleavage by SPP and the ability of the peptide to bind to HLA-E and interact with CD94/NKG2 receptors. Therefore, HLA-E may signal the presence of unhealthy cells to NK cells, not only when cell surface MHC class I expression is defective but also when proteasomal degradation is suboptimal. Proteasome inhibitors are currently in clinical trials for the treatment of various cancers. In light of our results, it is likely that HLA-E cell surface expression will be disrupted. This may provide a host advantage over cancer cells by favoring NK-mediated recognition and lysis of tumor cells. Conversely, disrupted HLA-E expression on surrounding healthy cells may be deleterious by enhancing toxicity.

The fate of antigenic peptides released by the proteasome is not known, but studies have suggested that they could associate with heat shock proteins (36) or other chaperones (37) and thus escape destruction by cytosolic proteases. It has also been proposed that the proteasome discharges its peptides directly into TAP (38). However, a recent study (31) demonstrates that peptides are distributed throughout the cytoplasm and the nucleus and diffuse freely before being degraded or encounter-
ing TAP. We propose that the capture of the extended MHC signal peptide fragment by the proteasome prevents its degradation and allows the generation of a peptide, which is then efficiently transported by TAP into the ER lumen for binding to HLA-E. Entering the proteasome may therefore be one mechanism that antigenic peptides use to escape degradation in the cytosol. This would be consistent with the study of Reits et al. (31) showing that, in vivo, aminopeptidases are responsible for the degradation of short peptides instead of carboxypeptidase and endopeptidase such as the proteasome.

In conclusion, we show that processing of MHC class I signal sequences to generate the HLA-E binding epitope is a multistep process involving at least three proteases: signal peptidase, SPP, and the proteasome. Interestingly, SPP is not required for the processing of the human cytomegalovirus glycoprotein UL40 signal sequence that was shown to upregulate HLA-E in infected cells (39). This is because of the presence of positively charged residues in the C-terminal region of the UL40 signal sequence that inhibit SPP cleavage (23). Further studies are underway to identify the proteases responsible for the generation of the virally derived peptide, which must be delivered directly into the ER lumen. Unique features of the UL40 signal sequence, such as an unusually long N-terminal region and the presence of the peptide in the middle of the signal peptide, are likely to play a role in directing the peptide to the ER.

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