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Placental Leucine Aminopeptidase Efficiently Generates Mature Antigenic Peptides In Vitro but in Patterns Distinct from Endoplasmic Reticulum Aminopeptidase 1

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All three members of the oxytocinase subfamily of M1 aminopeptidases, endoplasmic reticulum aminopeptidase 1 (ERAP1), ERAP2, and placental leucine aminopeptidase (PLAP), also known as insulin-regulated aminopeptidase, have been implicated in the generation of MHC class I-presented peptides. ERAP1 and 2 trim peptides in the endoplasmic reticulum for direct presentation, whereas PLAP has been recently implicated in cross-presentation. The best characterized member of the family, ERAP1, has unique enzymatic properties that fit well with its role in Ag processing. ERAP1 can trim a large variety of long peptide sequences and efficiently accumulate mature antigenic epitopes of 8–9 aa long. In this study, we evaluate the ability of PLAP to process antigenic peptide precursors in vitro and compare it with ERAP1. We find that, similar to ERAP1, PLAP can trim a variety of long peptide sequences efficiently and, in most cases, accumulates appreciable amounts of correct length mature antigenic epitope. Again, similar to ERAP1, PLAP continued trimming some of the epitopes tested and accumulated smaller products effectively destroying the epitope. However, the intermediate accumulation properties of ERAP1 and PLAP are distinct and epitope dependent, suggesting that these two enzymes may impose different selective pressures on epitope generation. Overall, although PLAP has the necessary enzymatic properties to participate in generating or destroying MHC class I-presented peptides, its trimming behavior is distinct from that of ERAP1, something that supports a separate role for these two enzymes in Ag processing. *The Journal of Immunology*, 2010, 185: 1584–1592.

The ability of cytotoxic T lymphocytes to recognize and eradicate diseased cells relies on the surface presentation of small peptide fragments bound onto specialized receptors of the MHC class I (MHC-I) (1). These antigenic peptides are the product of complex intracellular proteolytic pathways. MHC-I molecules present peptides 8–10 residues long that are either derived from the degradation of intracellular cytosolic proteins (classical pathway) or endocytosed extracellular proteins (cross-presentation pathway) (2, 3). During the last few years, an important role for aminopeptidases, enzymes that trim the N terminus of peptides or proteins, has been established in the final stage of the peptide processing pathway. Peptides generated by the proteasome and translocated into the endoplasmic reticulum (ER) by the TAP often carry N-terminal extensions compared to the mature epitopes (4). These epitope precursors have to

be trimmed by ER resident aminopeptidases so that they can bind onto nascent MHC-I molecules (5). ER aminopeptidase 1 (ERAP1, also named ERAAP and A-LAP) has been demonstrated to play a crucial role in trimming peptide precursors leading to the generation or destruction of mature antigenic peptides (6–13). ERAP1 has also been demonstrated to play important roles in the cross-presentation pathway (14). In the absence of ERAP1, many peptide epitopes are not appropriately processed and are therefore not presented on the cell surface (10). However, ERAP1 can also destroy some epitopes by further trimming them to smaller products that cannot bind onto MHC-I molecules (7, 8, 11, 15). ERAP1 can therefore either generate or destroy mature antigenic peptides. The epitope-destructive properties of ERAP1 appear to be physiologically relevant, by restricting the variety of epitopes generated and influencing the peptide pool available for presentation. It is currently well established that ERAP1 affects the generation of the antigenic peptide repertoire and in that context affects immunodominance in vivo (9, 10, 12, 13, 16). Although absent from mice, many other species also have a highly homologous ER aminopeptidase 2 (ERAP2, also named L-RAP) that can directly interact with ERAP1 and has been demonstrated to participate in antigenic peptide processing, albeit with a role that is secondary or accessory to ERAP1 (17).

ERAP1 and ERAP2 are zinc metallopeptidases that belong to the oxytocinase subfamily of M1 aminopeptidases (18). ERAP1 has unique biochemical properties that fit with its role in Ag presentation. In contrast to most aminopeptidases, it can trim large peptides efficiently and process a large number of peptide sequences (7, 15). Furthermore, it trims peptides in a sequence dependent manner and accumulates peptides 8–9 residues long, the optimal length for MHC-I binding. For this property, ERAP1 has been called a “molecular ruler” because it was considered to be the only aminopeptidase with a length-dependent trimming behavior (11, 15). The structural basis of the unusual enzymatic

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Abbreviations used in this paper: AMC, 7-amino-4-methylcoumarin; ER, endoplasmic reticulum; ERAP, ER aminopeptidase; ESI-MS, Electrospray mass spectral analysis; IRAP, insulin-regulated aminopeptidase; L-AMC, L-leucine-7-amido-4-methyl coumarin; MHC-I, MHC class I; PLAP, placental leucine aminopeptidase; shPLAP, soluble human PLAP; TFA, trifluoroacetic acid.

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properties of ERAP1 is not clear. However, it has been hypothesized that the peptide trimming properties of ERAP1 are a result of specific structural features of its substrate binding site (11, 15).

The third member of this subfamily is named placental leucine aminopeptidase (PLAP, also known as insulin-regulated aminopeptidase [IRAP]) (18, 19). PLAP shares with ERAP1 and ERAP2 a high degree of homology in both the Zn(II) catalytic center and the characteristic helical C-terminal domain (20). However, PLAP contains an N-terminal extension that presumably constitutes a transmembrane tail and is hypothesized to contribute to intracellular vesicle localization (18). PLAP has been found to colocalize with the glucose transporter GLUT4 in intracellular vesicles and degrade vasopressin as well as several other peptide hormones (21–23). PLAP has also been detected in intracellular vesicular trafficking pathways that are stimulated after antigenic stimuli, suggesting that PLAP may somehow be involved in the immune response (24). Interestingly, PLAP protein levels have been shown to be stimulated by IL-1 β (25). All these observations have hinted toward a role for PLAP in Ag presentation. This hypothesis was confirmed recently by the demonstration that PLAP participates in cross-presentation, especially in inflammatory monocyte-derived dendritic cells (26, 27).

The high degree of sequence conservation between ERAP1 and PLAP in combination with the fact that the two enzymes perform similar biological functions, raise the question as to whether the unique enzymatic properties of ERAP1 that make it suitable for Ag processing, are also shared by PLAP. In contrast, the involvement of PLAP in a separate location and pathway of Ag presentation than ERAP1 raises the question as to whether these two aminopeptidases have different enzymatic properties.

In an effort to evaluate the enzymatic properties of PLAP in the context of antigenic peptide processing, we expressed a soluble active form of PLAP in a mammalian cell line and used the purified protein in *in vitro* assays to test the ability of the enzyme to trim antigenic peptide precursors and generate the correct length mature epitopes in direct comparison with ERAP1. We focused our analysis on the accumulation of trimming intermediates, a feature that links the molecular and enzymatic properties of an aminopeptidase to its suitability to perform appropriate biological functions. Accumulation of correct length trimming intermediates that corresponded to antigenic epitopes has been previously thought to be a property unique to ERAP1. We tested the trimming of 14 different precursor peptides of different lengths and sequences that correspond to 10 different antigenic epitopes. PLAP was able to efficiently generate the antigenic epitope from 10 of the 14 precursors tested, whereas ERAP1 generated the epitope from 13 of the 14 precursors. However, in many cases (9/14) PLAP readily trimmed the mature epitope and generated smaller products, whereas ERAP1 was less effective in accumulating smaller intermediates (6/14 cases). Interestingly, the *in vitro* epitope generation properties of ERAP1 and PLAP varied depending on the epitope and precursor tested as did the overall trimming patterns and rates. Our findings suggest that PLAP shares key critical properties with ERAP1 that make it appropriate for intracellular Ag processing. However, the epitope-dependent trimming behavior of PLAP does not mirror that of ERAP1, suggesting the two related enzymes can impose different selective pressures in Ag presentation.

Materials and Methods

Materials

The 293F cells, 293Fectin, Freestyle 293 expression medium, and opti-MEM I transfection medium were bought from Invitrogen (Carlsbad, CA). All peptides were purchased from Genscript (Piscataway, NJ). Peptides were purified by reverse-phase HPLC and were >95% pure. MonoQ

column was from GE Healthcare (Piscataway, NJ). Ni-NTA agarose was purchased from Qiagen (Valencia, CA). L-leucine-7-amido-4-methyl coumarin (L-AMC) was from Sigma-Aldrich (St. Louis, MO). Trifluoroacetic acid (TFA) was purchased from Applichem (Darmstadt, Germany) and acetonitrile from Merck (Whitehouse Station, NJ).

Expression vector construction

A soluble version of human PLAP (shPLAP) was cloned between the EcoRI and XhoI sites of the multicloning site of the mammalian expression vector pcDNA6/myc-His. The final expression construct contains the gene for PLAP starting at amino acid position 155, lacking the extended N-terminal domain of the gene that contains the putative transmembrane region of the protein. The N terminus of the construct contained the signal sequence from the adenoviral E3/19K protein, so as to target the protein to the secretory pathway during expression.

PLAP expression and purification

The pcDNA6/myc-his construct containing the gene for shPLAP was used to transfect 293F cells grown in suspension culture at 8% CO₂ and 37°C. Transfection was performed using the 293Fectin transfection reagent according to the Freestyle 293 expression system (Invitrogen) instructions. Three days posttransfection, the cell supernatant was harvested, and expression of active enzyme confirmed by L-AMC assay and by Western blotting using an anti-5 \times His-HRP conjugate Ab (Qiagen). The cell supernatant was extensively dialyzed against 10 mM sodium phosphate buffer at pH 8.0 and the final composition adjusted to 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 5 mM imidazole. A total of 1 ml Ni-NTA agarose slurry was added to the dialysate and the mixture was gently stirred at 4°C for 1 h to allow for protein binding to the column. Elution was performed using the same buffer containing increasing concentrations of imidazole. Elution was followed by L-AMC aminopeptidase activity assay. The majority of the activity was eluted at 150 mM imidazole and the corresponding fractions were pooled and dialyzed in 10 mM Tris buffer, pH 8.0. The protein was further loaded onto a monoQ anion-exchange column attached onto a fast protein liquid chromatography system and eluted using a linear NaCl gradient. One major peak was evident from the chromatogram and also corresponded to the majority of L-AMC activity. The corresponding fractions were pooled, concentrated, and stored at -20°C in 20 mM Tris pH 8.0, 100 mM NaCl, 50% glycerol. Protein was quantified based on its absorbance at 280 nm using an extinction coefficient of 1.67 mg/ml OD₂₈₀, calculated based on the amino acid sequence of the expressed construct. Protein integrity was assessed by SDS-PAGE and Western blotting using the anti-5 \times His-HRP conjugate Ab. The protein was >98% pure based on SDS-PAGE.

ERAP1 expression and purification

Human recombinant ERAP1 was expressed in insect cells postinfection with baculovirus as previously described (11). The recombinant protein was harvested from the cell medium postinfection and was purified by a combination of Ni-NTA chromatography, anion-exchange chromatography, and size-exclusion chromatography again as previously described (11).

Measurement of aminopeptidase activity using the L-AMC fluorescent substrate

The aminopeptidase activity of human recombinant ERAP1 and PLAP during purification and storage was monitored, by following the change of solution fluorescence on incubation of the recombinant enzyme with the fluorogenic substrate L-AMC. A total of 140 μ l of a 100 μ M L-AMC solution containing 20 mM Tris pH 8.0 and 100 mM NaCl was incubated with either ERAP1 or PLAP and the fluorescence of the solution was monitored using a QuantaMaster4 (Photon Technology International, Birmingham, NJ) fluorimeter using an excitation wavelength of 380 nm and after the emission at 460 nm. The generated time-dependent change of the signal was calibrated using 7-amino-4-methylcoumarin (AMC) as a standard. The slope of the time course is directly proportional to the enzymatic activity.

Peptide trimming, followed by reverse-phase HPLC

The N-terminal trimming of model antigenic peptide precursors by either ERAP1 or PLAP was followed by separating the trimming products on a reverse-phase column attached to an HPLC system. For the trimming reactions, 100 μ M peptide in 20 μ l 50 mM Tris pH 8.0 and 100 mM NaCl was incubated with 1–2000 nM of either ERAP1 or PLAP at 37°C. In all cases, the peptide was in excess over the enzyme. At the end of the incubation, the reaction was stopped by adding TFA to a final concentration of 0.5% and storing the reaction at -20°C. The trimming products were analyzed

on a reverse-phase column (Chromolith, Merck) equilibrated with 0.05% TFA. Elution was performed using a linear concentration gradient of acetonitrile. Eluted peptides were typically detected by absorbance at 220 nm. Product peaks were identified either based on their elution time as defined by running control peptides or by spiking part of the reaction with a control peptide. Novel product peaks were collected during elution from the column and lyophilized for further analysis by Electrospray mass spectral analysis (ESI-MS) on an AQA Navigator (Finnigan, U.K.) mass spectrometer, to assist with peptide identification.

pH dependence of enzymatic activity of ERAP1 and PLAP

The rate of hydrolysis of the fluorogenic substrate L-AMC by either ERAP1 or PLAP was measured as described previously by substituting the appropriate buffer system to achieve the required pH value. The buffers used were the following: pH range 4–5.9 citrate; pH range 6.0–6.8 phosphates; pH range 6.9–7.9 HEPES; pH range 8.0–8.9 Tris; and pH range 9.0–9.8 glycine. All reaction solutions contained 50 mM buffer, 100 mM NaCl, and 100 μ M L-AMC.

Results

Expression and purification of an enzymatically active and soluble recombinant form of human PLAP

We used a mammalian cell line expression system to express a soluble version of human PLAP for in vitro assays, as described in the *Materials and Methods*. The pcDNA6/myc-his plasmid vector contained the human PLAP gene starting at 155 aa, lacking the PLAP N-terminal domain that is postulated to contain a transmembrane region that would attach the protein on the vesicular or plasma membrane of the cell. The designed construct more readily resembles the soluble version of PLAP that can be created after proteolytic digestion of the full-length protein (23). The expressed amino acid sequence encompasses the section of the enzyme, which shares up to 50% identity with full-length ERAP1 or ERAP2. Recombinant PLAP was purified to homogeneity by affinity purification (Ni-NTA column) and anion-exchange chromatography and was found to be active as an aminopeptidase (Fig. 1A). Compared with human recombinant ERAP1, PLAP was significantly more active toward the hydrolysis of the model fluorogenic substrate L-AMC, having a sp. act. of $233 \pm 14 \times 10^3$ mol AMC/mol enzyme \times min compared to $3.1 \pm 0.6 \times 10^3$ mol AMC/mol enzyme \times min for ERAP1.

Enzymatic activity of PLAP as a function of the pH value of the solution

Recently, Saveanu et al. (26) demonstrated PLAP affects cross-presentation in endosomal compartments. Because the pH of endosomal compartments becomes more acidic than the pH of the ER where ERAP1 primarily operates, we asked whether the optimal pH dependence of PLAP activity is different from the pH dependence for ERAP1. To test this, we measured L-AMC hydrolysis by ERAP1 or PLAP in varying pH solutions within the range of 4–10. Both aminopeptidases displayed maximum hydrolytic efficiency at pH 7.0 (Fig. 1B). However, PLAP retained significant hydrolytic activity over a wider range of pH compared with ERAP1. Specifically, PLAP was found to be very active at mildly acidic conditions and retained almost 70% of its activity at pH 6.0, compared with ERAP1 that retained \sim 30% of its maximum activity at that pH. In addition, PLAP retained 50% of its activity at pH 8.0. This property appears consistent with the proposed role of PLAP in endosomes that are gradually acidified after endocytosis or ones that are initially alkalized (28, 29). However, both aminopeptidases displayed no detectable activity under stronger acidic conditions (i.e., pH 5.0).

Accumulation of trimming intermediates by ERAP1 and PLAP

To evaluate the in vitro ability of PLAP to trim long peptides and accumulate intermediates that correspond to actual antigenic pep-

ptides, we used 14 model antigenic peptide precursors to 10 known antigenic epitopes. The peptides used were of varying size and sequence designed to test different aspects of the trimming capabilities of PLAP. All experiments were performed in direct comparison with trimming by ERAP1, an enzyme whose peptide processing properties are well established. We focused our analysis on the accumulation of trimming intermediates and more specifically the 8-mer or 9-mer mature antigenic epitope because up to now this has been considered to be a characteristic and unique property of ERAP1. Several tests were performed for each peptide, with varying amounts of enzyme in the range of 1 nM to 2 μ M, to identify all product peaks that accumulate during trimming. Accumulated product peaks were characterized by the use of peptide controls and mass spectrometry. In almost all trimming reactions examined, the use of similar concentrations of ERAP1 and PLAP was sufficient to trim the initial substrate and generate product peaks, indicating that PLAP is equally as effective as ERAP1 in trimming long peptides. In each case, characteristic chromatograms are depicted that highlight product accumulation.

Generation of the 8-mer peptide SIINFEKL from an extended precursor

We first used a model antigenic precursor of the well-characterized OVA antigenic epitope SIINFEKL that is presented by the MHC-I molecule H2-K^b (30). It has been previously demonstrated both

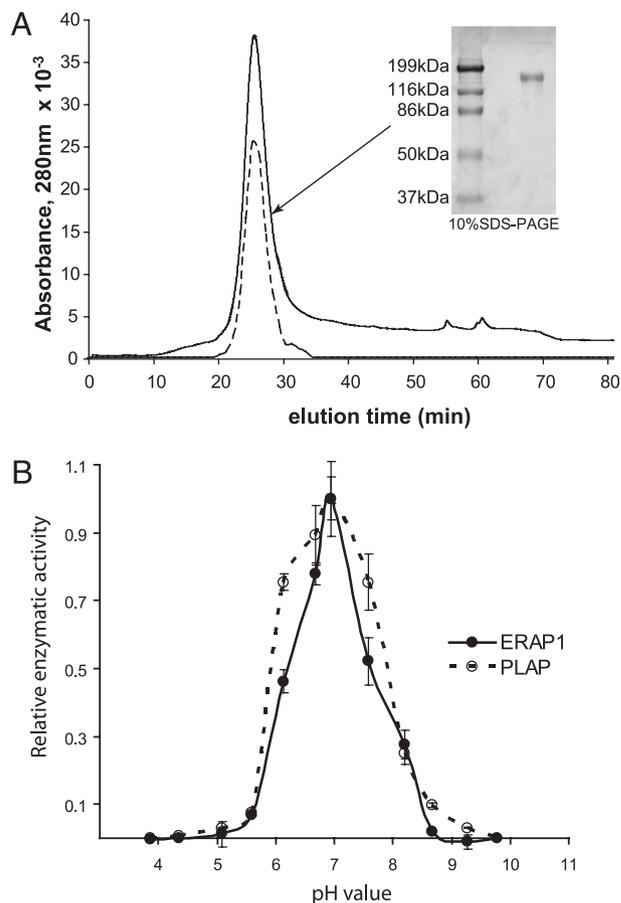


FIGURE 1. A, Elution chromatogram of shPLAP from the anion-exchange monoQ column. Solid line, absorbance at 280 nm; dashed line, L-AMC hydrolytic activity. A single major peak was detected. Fractions were analyzed on SDS-PAGE and showed a single band migrating at \sim 140 kDa. B, pH dependence of ERAP1 and PLAP enzymatic activity. Initial rates of hydrolysis of L-AMC were followed in the pH range 4–10. Relative rates are plotted for comparison. Error bars represent the SD of three measurements.

in vitro and in cell-based experiments, that ERAP1 generates the SIINFEKL peptide efficiently from N-terminally extended precursors (7, 8). In this study, we used a single amino acid extension, carrying a leucine residue, an optimal N-terminal residue for both ERAP1 and PLAP (31, 32). Consistent with previously reported results, ERAP1 trims LSIINFEKL efficiently, accumulating a single product that corresponds to the SIINFEKL peptide (Fig. 2A). PLAP is also able to trim the LSIINFEKL peptide efficiently and, similar to ERAP1, PLAP initially generates appreciable amounts of the SIINFEKL epitope. In contrast to ERAP1, however, PLAP continues trimming, generating smaller peptides and accumulating the 7-mer peptide with the sequence IINFEKL (Fig. 2B). Interestingly, both ERAP1 and PLAP were equally efficient in producing the mature epitope, having very similar specific rates of production (2.0 pmol/sec/ μ g for ERAP1 and 2.1 pmol/sec/ μ g for PLAP).

Generation of the 9-mer antigenic peptides from 10-mer precursors

Inspection of the naturally occurring epitopes deposited in the SYFPEITHI database reveals the majority of antigenic peptides are 9mers (33). Furthermore, analysis of antigenic peptide precursors generated by the immunoproteasome has suggested that the one of the most common N-terminal extensions is a single amino acid (4). As a result, the ability of an aminopeptidase to excise a single amino acid from a 10-mer antigenic peptide precursor and accumulate the 9-mer product is probably highly relevant to its suitability to efficiently generate antigenic epitopes in the cell. To evaluate the ability of PLAP to generate 9-mer model antigenic peptides from 10-mer precursors, we analyzed the trimming of seven model 10-mer

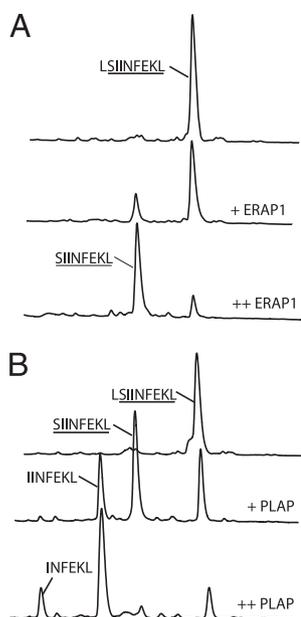


FIGURE 2. Trimming of the model epitope precursor LSIINFEKL by ERAP1 and PLAP. Characteristic chromatograms of digestion products are depicted. Enzyme concentrations were selected to achieve comparable amounts of substrate depletion for both ERAP1 and PLAP. *A*, A total of 100 μ M of the peptide was incubated with 20 nM or 400 nM ERAP1 for 1 h. *B*, A total of 100 μ M of the peptide was incubated with 60 nM PLAP for 1 h or 200 nM PLAP for 2 h. All incubations were at 37°C. The products of each digestion were analyzed on reverse-phase HPLC. Peaks were identified by running control peptides and by confirming their mass by ESI-MS. Digestion of LSIINFEKL by ERAP1 accumulated the 8-mer SIINFEKL that corresponds to the H2-K^b OVA epitope. Digestion of LSIINFEKL by PLAP accumulated the 8-mer epitope to a degree, but PLAP further trimmed the 8-mer to smaller products, accumulating large amounts of the 7-mer peptide IINFEKL.

peptides, five of which correspond to naturally occurring antigenic peptide precursors. Three of the peptides we tested (sequences IRLRPGGKKK, KSLYNTVATL, and KGYKDGNEYI) correspond to natural precursors of epitopes found to be cross-presented (34, 35). Fig. 3 demonstrates characteristic chromatograms from trimming reactions highlighting accumulated products. ERAP1 was able to efficiently generate the HLA-A*03 9-mer ligand RVYEKMALY and its variant RVYEKMALR from a 10-mer precursor, whereas PLAP did not accumulate the 9-mer but rather trimmed it further and accumulated 8-mer and 7-mer products (Fig. 3A, 3B). Both ERAP1 and PLAP efficiently accumulated the HLA-A*03 and HLA-B*27 epitopes RLRPGGKKK and SRHHAFSFR, respectively (Fig. 3C, 3D). Similarly, both ERAP1 and PLAP accumulated significant amounts of the HLA-A*02 epitope SLYNTVATL (Fig. 3E). This epitope was resistant to further trimming by PLAP but was degraded to accumulate the 7-mer product by ERAP1 (Fig. 3E). The HLA-B*27 epitope precursor DIRSSVQNKL was highly resistant to both enzymes and no detectable epitope was produced under reasonably achievable experimental conditions (Fig. 3F). However, incubation with larger amounts of ERAP1, led to accumulation of the 7-mer and 8-mer products, indicating that ERAP1 can destroy this epitope (Fig. 3F). Finally, both enzymes were able to accumulate the H-2-Kd epitope GYKDGNEYI as an intermediate, but continued to further trim it to accumulate the 6-mer product DGNEYI (Fig. 3G). The results for all these peptides are summarized in Table I, including the calculated rates of production of the mature antigenic epitopes.

Overall, both enzymes were reasonably successful in accumulating appreciable amounts of the 9-mer mature epitopes in vitro (six of the seven cases for ERAP1, Fig. 3A–E, 3G; four of the seven cases for PLAP, Fig. 3C–E, 3G). In addition, in four of seven cases, ERAP1 trimmed the epitope further and accumulated smaller products 6–8 aa long effectively destroying the epitope (Fig. 3C, 3E–G). Similarly, PLAP accumulated smaller fragments in four of seven cases (Fig. 3A–C, 3G). Epitope generation rates varied over a range of three orders of magnitude but were very often comparable between ERAP1 and PLAP (Table I). Interestingly, for both enzymes, the ability to generate epitopes depended on the epitope sequence examined. Overall, ERAP1 and PLAP showed significant differences in which of the tested epitopes they were able to accumulate or continue trimming to smaller products, indicating that the two enzymes have significant differences in their substrate preferences.

Generation of the 9-mer antigenic peptide from longer extended precursors

Some antigenic peptide precursors transported into the ER are longer peptides up to 16 aa long (4, 36, 37). Trimming from ER resident aminopeptidases would therefore be a multistep process that may necessitate coordinated trimming steps from more than one aminopeptidase. Recently, such a mechanism has been proposed for ERAP2 (17). ERAP1 has been shown to be able to efficiently generate mature antigenic peptides from long N-terminally extended precursors (7, 8, 15). To evaluate the ability of PLAP to perform this function, we studied the trimming of six antigenic peptide precursors based on three antigenic epitopes. The following peptides were tested: 1) the KRFEGLTQR epitope, a ligand for HLA-B27 and a potential link between predisposition to the autoimmune disease ankylosing spondylitis and this MHC allele (38–40); 2) the ATPDPTLTY epitope that is a HLA-A*03 ligand and a tumor-related Ag derived from human integrin α 6 chain (41); and 3) the cross-presented GPGRAFVTI peptide that can bind onto the H-2-L^d allele and is derived from the env protein of the HIV 1 IIIB (42, 43).

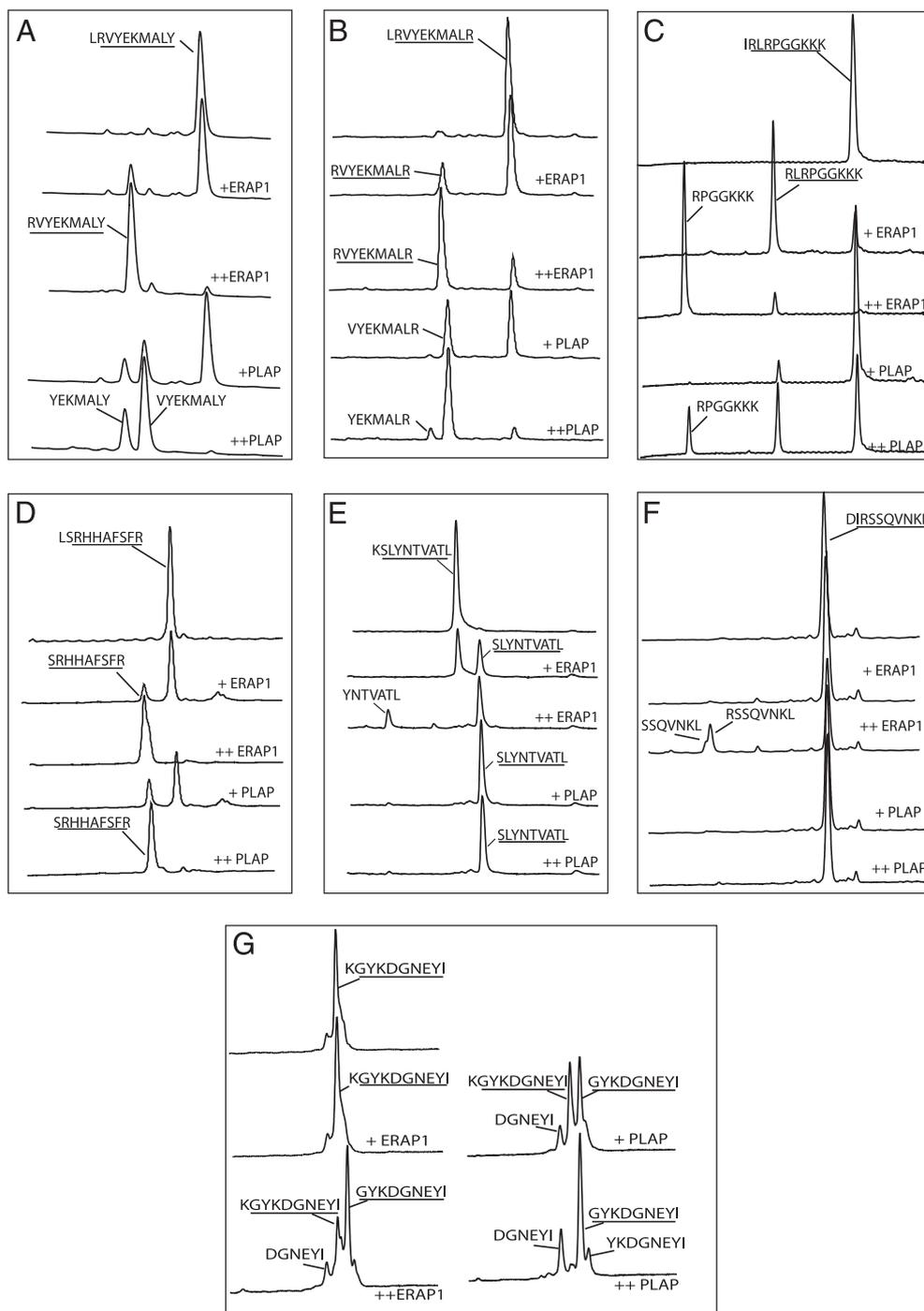


FIGURE 3. Trimming of the 10-mer epitope precursors by ERAP1 and PLAP. The initial concentration for all peptides was 100 μ M. The enzyme concentration and incubation time for displayed chromatograms are listed below. The products of the digestion were analyzed on reverse-phase HPLC. Peaks were identified by running control peptides and by confirming their mass by ESI-MS. The mature epitopes are underlined. *A*, The LRVYEKMALY precursor was incubated with 40 nM or 200 nM ERAP1 and 40 nM or 200 nM PLAP for 1 h at 37°C. *B*, The LRVYEKMALR precursor was incubated with 4 nM or 20 nM ERAP1 and 40 nM or 100 nM PLAP for 1 h at 37°C. *C*, The IRLRPPGGKKK precursor was incubated with 90 nM or 270 nM ERAP1 and 90 nM or 270 nM PLAP for 2 h at 37°C. *D*, The LSRHHAFSFR precursor was incubated with 3 nM or 10 nM ERAP1 and 3 nM or 10 nM PLAP for 1 h at 37°C. *E*, The KSLYNTVATL precursor was incubated with 90 nM or 270 nM ERAP1 and 90 nM or 270 nM PLAP for 2 h at 37°C. *F*, The DIRSSQVNKL precursor was incubated with 90 nM or 810 nM ERAP1 and 90 nM or 810 nM PLAP for 2 h at 37°C. *G*, The KGYKDGNEYI precursor was incubated with 90 nM or 810 nM ERAP1 and 90 nM or 810 nM PLAP for 2 h at 37°C.

We first tested the ability of ERAP1 and PLAP to generate the KRFEGLTQR epitope from a 14-mer N-terminally extended precursor with the sequence LNYTYKRFEGLTQR derived from the natural protein precursor sequence. Generation of the 9-mer epitope requires the sequential removal of five N-terminal amino acids. Fig. 4 shows representative chromatograms of the trimming products after

incubation of the 14-mer precursors with ERAP1 and PLAP, respectively. ERAP1 trimmed the peptide *in vitro* efficiently and generated appreciable amounts of KRFEGLTQR. However, ERAP1 did not accumulate the 9-mer peptide exclusively but continued trimming down to the 7-mer product FEGLTQR. This is consistent with previous observations that ERAP1 can generate but can also destroy

Table I. Summary of results of trimming reactions

Figure No.	Antigenic Peptide Precursor	Antigenic Epitope	HLA	Epitope Accumulation		Smaller Peptide Accumulation		Epitope Production Rate ^a (pmol/sec/μg)	
				ERAP1	PLAP	ERAP1	PLAP	ERAP1	PLAP
2	LSIINFEKL	SIINFEKL	H2-Kb	✓	✓		✓	2.0	2.1
3A	LRVYEKMALY	RVYEKMALY	HLA-A*03	✓	ND ^b		✓	1.0	ND
3B	LRVYEKMALR	RVYEKMALR	HLA-A*03	✓	ND		✓	21	ND
3C	IRLRPGGKKK	RLRPGGKKK	HLA-A*03	✓	✓	✓	✓	0.90	0.26
3D	LSRHHAFFSR	SRHHAFFSR	HLA-B*2705	✓	✓		✓	16	30
3E	KSLYNTVATL	SLYNTVATL	HLA-A*0201	✓	✓		✓	0.53	2.4
3F	DIRSSVQNKL	IRSSVQNKL	HLA-B*2705	ND	ND		✓	ND	ND
3G	KGYKDGNEYI	GYKDGNEYI	H-2-Kd	✓	✓		✓	0.10	0.66
4	LNYTYKRFEGLTQR	KRFEGLTQR	HLA-B*2704	✓	ND	✓	✓	0.034	ND
5A	AKLIATFPDTLTY	ATFPDTLTY	HLA-A*03	✓	✓		✓	—	—
5B	AEKIATFPDTLTY	ATFPDTLTY	HLA-A*03	✓	✓		✓	—	—
5C	AKLIATFPDTLTK	ATFPDTLTK	HLA-A*03	✓	✓		✓	—	—
6A	KIRIQRGSGRAFVTI	GSGRAFVTI	H-2-Ld	✓	✓		✓	—	—
6B	KIRIQRGSGRAFVTI	GSGRAFVTI	H-2-Ld	✓	✓	✓		—	—

Checkmarks indicate detection of trimming products that correspond either to the mature epitope or to smaller peptides. In the last two columns, the specific rates of epitope generation are shown. Rates were calculated only for single residue removal, because the trimming of multiple residues from longer precursors displays complex kinetics that cannot be analyzed from single-peptide experiments.

^aAverage error in rate calculation is 20%. Rates were not calculated for peptide precursors with multiple intermediates.

^bEpitope production not detectable.

some epitopes depending on their sequence (8, 11). In contrast to ERAP1, PLAP trimmed the 14-mer peptide but was found to accumulate only a 13-mer and the 7-mer intermediate. No 9-mer epitope was detectable in any of our tests indicating that in this case PLAP was unable to accumulate the 9-mer and instead only destroyed the antigenic peptide by trimming it further (Fig. 4).

We next evaluated the *in vitro* generation of the antigenic peptide ATFPDTLTY using three 13-mer model precursor peptides that differ either in the preceding sequence or the C-terminal residue of the naturally occurring precursor sequence (Fig. 5). In all three cases, ERAP1 efficiently trimmed the peptide, accumulating as the primary intermediate the 9-mer epitope. PLAP was also able to trim the peptide and accumulate some of the 9-mer epitope but proceeded to trim the 9-mer down to a 7-mer product that was not

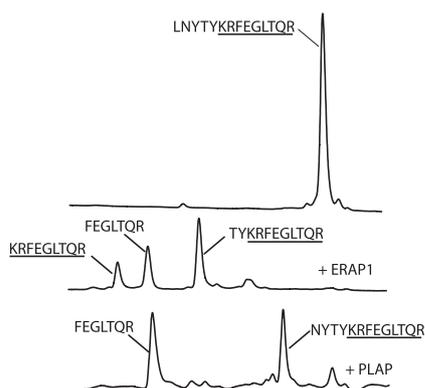


FIGURE 4. Trimming of the model epitope precursor LNYTYKRFEGLTQR by ERAP1 and PLAP. A total of 100 μM of the peptide was incubated with 300 nM ERAP1 or 150 nM PLAP for 2 h and the products of the digestion were analyzed on reverse-phase HPLC. Peaks were identified by running control peptides and by confirming their mass by ESI-MS. Digestion of LNYTYKRFEGLTQR by ERAP1 accumulated limited amounts of the 9-mer peptide with the sequence KRFEGLTQR that corresponds to an HLA-B27 ligand. ERAP1 trimming did not lead to exclusive accumulation of this product but rather continued to generate the 7-mer FEGLTQR. Digestion by PLAP did not generate any detectable amounts of the 9-mer intermediate but rather led to the accumulation of the 7-mer peptide FEGLTQR.

further hydrolyzed. Interestingly, the nature of the preceding sequence or the C-terminal residue did not seem to affect the qualitative outcome of the reaction, although it did affect the kinetics of the reaction as judged from the different amounts of enzyme necessary to generate the epitope.

We finally evaluated the trimming of the precursor sequence KIRIQR on the N terminus of the epitope GSGRAFVTI (Fig. 6A). Consistent with previous reports (17, 26), both PLAP and ERAP1 were able to efficiently generate the mature epitope. However, because in this epitope sequence the second residue is a proline, this sequence is expected to be a very poor substrate for many aminopeptidases of this family and as a result accumulation of the 9-mer peptide may not be restricted to these specific aminopeptidases. To test whether ERAP1 and PLAP can still generate the correct 9-mer even in the absence of the Pro residue, we tested trimming of the same precursor sequence attached onto the model epitope GSGRAFVTI in which the proline residue is substituted by a serine (Fig. 6B). ERAP1 was still able to accumulate large amounts of the 9-mer product but then proceeded to trim it further to smaller products. In contrast, PLAP was able to produce the 9-mer epitope almost exclusively and no smaller products were detectable (Fig. 6B)

Effect of pH in intermediate accumulation

To test whether the pH of the solution can affect the ability of the enzyme to accumulate specific trimming intermediates, we performed digestions of the peptides LSIINFEKL and LRVYEKMALY (used in Figs. 2, 3) by PLAP in pH 6.0. We found that PLAP trimmed both peptides and accumulated intermediates in an indistinguishable manner to trimming in pH 8.0, indicating that the pH value of the solution does not affect the ability of the enzyme to generate these antigenic epitopes (data not shown).

Discussion

A model aminopeptidase that shows little to no specificity for peptidic substrates should process all trimming intermediates sequentially and accumulate them to a minimal degree. Deviations from this model trimming behavior can be attributed to substrate preferences and particularly the difficulty of the enzyme to process a specific intermediate, leading to that intermediate's accumulation.

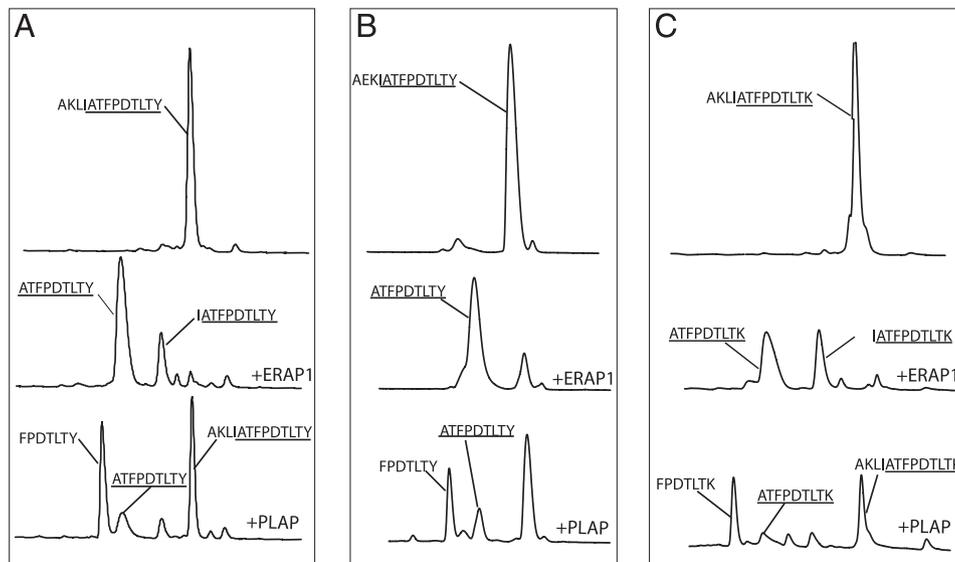


FIGURE 5. Trimming of the model epitope precursors to antigenic peptide ATFDPDLTY and variant ATFDPDLTK by ERAP1 and PLAP. Characteristic chromatograms highlight trimming intermediate accumulation are depicted. *Top chromatograms*, control reaction (no enzyme); *middle chromatograms*, digestion by ERAP1; *bottom chromatograms*, digestion by PLAP. A, A total of 100 μ M of the peptide AKLIATFPDLTY was incubated with 30 nM ERAP1 or 100 nM PLAP for 3 h. B, A total of 100 μ M of the peptide AEKIATFPDLTY was incubated with 250 nM ERAP1 or 400 nM PLAP for 3 h and 16 h, respectively. C, A total of 100 μ M of the peptide AKLIATFPDLTK was incubated with 250 nM ERAP1 or 150 nM PLAP for 3 h. Digestion products were analyzed on reverse-phase HPLC. Peaks were identified by running control peptides and by confirming their mass by ESI-MS. In all cases, peptides were successfully digested by both enzymes and ERAP1 accumulated the 9-mer intermediate that corresponds to the antigenic epitope. PLAP accumulated limited amounts of the 9-mer and proceeded trimming to generate the 7-mer product.

Intermediate accumulation can be indicative of the suitability of a particular aminopeptidase to perform a specific biological function by trimming specific substrates or preferentially accumulating specific products. The ability of ERAP1 to: first, trim efficiently a large variety of long peptides and second, accumulate intermediates that are 8 or 9 aa long has been considered to be a hallmark of the role of the enzyme in Ag processing. These properties of ERAP1 have been hypothesized to be derived from an extended peptide-binding site adjacent to the catalytic site of the enzyme (11, 15). This site can accommodate long peptides, forming a large network of atomic interactions that affect the trimming rate of the substrate. The high level of sequence homology between ERAP1 and PLAP suggests the possibility that PLAP retains at least some degree of the substrate recognition properties of ERAP1. Consistent with this hypothesis, we demonstrate in this study that PLAP, similarly to ERAP1, can trim many different peptide sequences efficiently and accumulate intermediates that correspond to mature antigenic epitopes *in vitro*. In that context, PLAP has the minimum required enzymatic properties to process antigenic peptide precursors inside the cell.

Recently, the mouse homolog of PLAP, IRAP, was demonstrated to play a critical role in the cross-presentation pathway (26). We demonstrate in this study that PLAP is active in a slightly wider range of pH compared with the sharp pH dependence of ERAP1. This is consistent with PLAP operating in endosomal or phagosomal compartments that have a wider range of possible pH values compared with the tightly regulated ER pH. Indeed, phagosomes can initially alkalize, reaching values of pH 8 promotes cross-presentation, but later acidify (28, 29, 44–46). This acidification is significantly slowed down in dendritic cells compared with other cell types, with the pH of phagosomes remaining in the range of 7–7.5 for several hours (29, 47–50). Because we find this is the optimal pH range for PLAP, this suggests that PLAP is an appropriate aminopeptidase to perform Ag processing in cross-presentation vesicles.

According to our observations, both ERAP1 and PLAP appear suitable to generate correct length antigenic epitopes from longer precursors but also to destroy some of them by further trimming to smaller products that would not be expected to be able to bind onto MHC-I molecules. Although it is difficult to determine whether the enzyme and peptide concentrations present in the cell during Ag

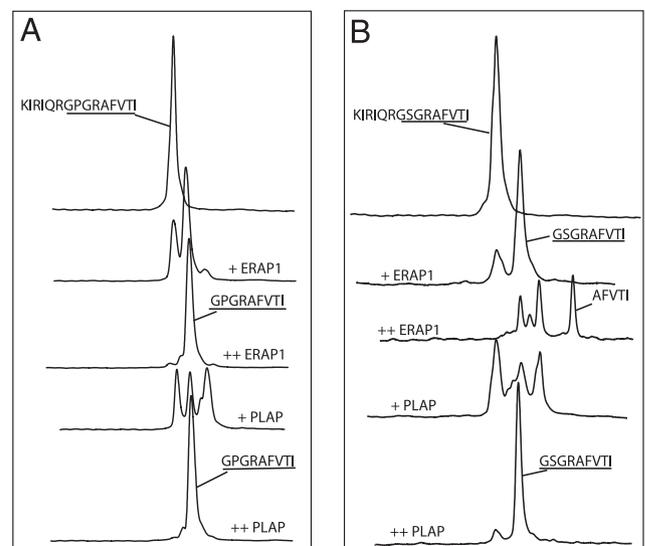


FIGURE 6. Trimming of precursors to the antigenic peptide GPGRAFVTI and its variant GSGRAFVTI by ERAP1 and PLAP. A, A total of 100 μ M peptide KIRIQRGPGRAFVTI was incubated with 90 nM or 810 nM ERAP1 and 90 nM or 810 nM PLAP for 2 h at 37°C. B, A total of 100 μ M peptide KIRIQRGSRAFVTI was incubated with 270 nM ERAP1 or PLAP for 2 h and with 1500 nM ERAP1 or PLAP for 4 h at 37°C. The products of each digestion were analyzed on reverse-phase HPLC. Peaks were identified by running control peptides and by confirming their mass by ESI-MS.

processing are sufficient to destroy epitopes, this *in vitro* behavior shows that such a destruction is a possibility. Indeed, ERAP1 has been demonstrated to be able to destroy many epitopes *in vivo* (8, 10). Moreover, this complex behavior appears to be both epitope and enzyme dependent suggesting that sequence determinants in the precursor examined can affect how well the epitope will be produced. As seen in Table I, ERAP1 and PLAP followed different patterns in their ability to generate intermediates that correspond or do not correspond to antigenic epitopes. This was more pronounced in the case of accumulation of smaller products from further trimming of the antigenic epitope, in which PLAP was slightly more effective than ERAP1. Differences in these trimming patterns are likely the result of differences in the specificity of these related enzymes. We have previously demonstrated that ERAP1 trims peptides depending on their internal sequence and it is reasonable to assume that PLAP shares this property but displays different preferences for amino acid side chains in the peptide sequence.

During our analysis, even when the mature antigenic peptide was detected to be produced, the degree of its accumulation was found to vary significantly. To which degree, the antigenic peptide accumulation during a trimming reaction and/or its susceptibility to further trimming are important parameters for Ag presentation *in vivo*, is currently unknown. On the one hand, it is possible that the degree to which the mature epitope accumulates determines how much will be bound to and then presented by MHC-I molecules. On the other hand, it is possible that even very little epitope accumulation may be sufficient for Ag presentation *in vivo* if it is rapidly bound by MHC-I molecules. This is because MHC-bound peptides are protected from aminopeptidase degradation as their N terminus is tightly bound by the MHC and is therefore not accessible to peptidases (51, 52). In this context, even minimal epitope accumulation *in vitro* may signify efficient *in vivo* generation of certain epitopes by either ERAP1 or PLAP because MHC-I molecules may bind and protect a fraction of the epitopes as they are generated and before they can be further hydrolyzed. Recently, trimming rates by ERAP1 has been found to correlate with Ag presentation efficiency on cultured cells, suggesting that the fundamental trimming properties of this aminopeptidase are directly relevant to its biological function and it is therefore reasonable to hypothesize that the same applies for PLAP (53).

The combination of the generating and destructive properties of ERAP1 *in vivo* has led to the description of the enzyme as an antigenic peptide editor, an enzyme that affects the fate of potential antigenic peptides. Consistent with this idea, *in vivo* experiments in mouse models have demonstrated that the presence of ERAP1 can alter immunodominance, leading to complex and hard to predict changes in the immune response (9, 10, 13). In this study, we demonstrate that PLAP shares the basic properties of ERAP1 in both generating and destroying antigenic epitopes. Recent work has implicated PLAP in cross-presentation in certain cells (26, 27). The trimming patterns that affect epitope generation, however, appear to be distinct between ERAP1 and PLAP suggesting that if PLAP can also affect Ag presentation and immunodominance *in vivo*, it may do so differently compared with ERAP1. Why it would be necessary to have distinct trimming behaviors of specialized Ag-processing aminopeptidases in separate antigenic processing pathways is not clear at the moment. It is possible the two aminopeptidases encounter different pools of antigenic peptides and have adapted accordingly. Alternatively, it is conceivable the antigenic peptide selection requirements in separate processing pathways in the cell are different and require corresponding trimming behaviors. Finally, the presence of two trimming patterns within the cell may be advantageous in terms of increasing

the variety of epitopes that can be processed, therefore contributing to the efficiency of the processing machinery toward the very large number of antigenic precursor sequences that are encountered. In contrast, if CD8 T cells are generated to unique cross-presented peptides, then the effector cells that would be generated would be unable to recognize target cells that are presenting peptides generated through direct presentation. Although such responses would not be effective in purging abnormal parenchymal (non-cross-presenting) cells, they might still be useful in recognizing dendritic cells or macrophages harboring pathogens in their phagosomes. A more comprehensive investigation of the trimming properties of all three members of the oxytocinase subfamily of aminopeptidases (ERAP1, ERAP2, and PLAP) may be necessary to help us understand why the cellular Ag generation machinery uses two distinct trimming activities to achieve what seems to be the same biological function.

In summary, our study suggests that the key enzymatic properties that make ERAP1 especially fit for intracellular Ag processing are also shared, to a large degree, by the homologous PLAP. However, the epitope-dependent trimming patterns of PLAP do not mirror that of ERAP1, suggesting that the two enzymes may impose different selective pressures on Ag generation.

Disclosures

The authors have no financial conflicts of interest.

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