H-2Ld Class I Molecule Protects an HIV N-extended Epitope From in Vitro Trimming by Endoplasmic Reticulum Aminopeptidase Associated With Antigen Processing

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H-2L^d CLASS I MOLECULE PROTECTS AN HIV N-EXTENDED EPITOPE FROM *IN VITRO* TRIMMING BY ERAAP

Susana Infantes [*,¶], Yolanda Samino [†], Elena Lorente [*,¶], Mercedes Jiménez [*], Ruth García [*,¶], Margarita Del Val [†,‡], and Daniel López [*,¶]

[*] Unidad de Proteómica, [¶] Unidad de Procesamiento Antigénico, and [†] Unidad de Inmunología Viral. Centro Nacional de Microbiología. Instituto de Salud Carlos III. 28220 Majadahonda (Madrid), Spain. [‡] Centro de Biología Molecular Severo Ochoa, CSIC/Universidad Autónoma de Madrid, 28049 Madrid, Spain.

Running Title: MHC protects to ERAAP trimming

Address correspondence and reprint requests to Dr. Daniel López. Unidad de Proteómica, Centro Nacional de Microbiología. Instituto de Salud Carlos III. 28220 Majadahonda (Madrid), Spain. Telephone number 34 91 822 37 08, FAX number 34 91 509 79 19, E-mail address dlopez@isciii.es.

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Non standard abbreviations

ERAAP, mouse ER aminopeptidase associated with antigen processing;

ERAP1, human ER aminopeptidase 1;

ERAP2, human ER aminopeptidase 2;

ESI-IT MS, Electrospray ionization-ion trap mass spectrometry;

E/S Ratio, Enzyme/substrate ratio;

ABSTRACT

In the classical major histocompatibility complex (MHC) class I antigen presentation pathway, antigenic peptides derived from viral proteins by multiple proteolytic cleavages are transported to the endoplasmic reticulum lumen and then are exposed to aminopeptidase activity. In the present study, a long MHC class I natural ligand recognized by cytotoxic T lymphocytes was used to study the kinetics of degradation by aminopeptidase. The *in vitro* data indicate that this N-extended peptide is efficiently trimmed to a 9mer, unless its binding to the MHC molecules protects the full-length peptide.

INTRODUCTION

In the cytosol, newly synthesized viral proteins are proteolytically processed prior to MHC class I heavy chain-β_2m-peptide complex formation in the lumen of the endoplasmic reticulum (ER) (1). In addition to the proteasome, a number of alternative proteases that contribute to endogenous antigen processing have been identified (reviewed in (2)). Usually, MHC class I molecules bind peptides of 8-10 residues by interaction of the peptide anchor residues with the pockets of the binding groove of the presenting molecule (3), (4). Some peptides can be directly produced in their final form, whereas others are generated as precursors (5). These precursor peptides must generally have the correct C-terminus of the final antigenic peptides (6). In contrast, the extraordinarily diverse pool of substrates for TAP transport do not yet have precise N-termini and therefore require editing and customization before they yield the final peptide-MHC complexes. The ER-resident enzyme that trims the N-terminally extended residues of peptide precursors to their final length has been identified as the ER aminopeptidase associated with antigen processing (ERAAP) in mice (7) and ER aminopeptidase 1 (ERAP1) (8), (9) and aminopeptidase 2 (ERAP2) (10) in humans.

In a classical paper from the early 1990s, Falk et al. (11) proposed six different and mutually exclusive models to explain the interactions among precursor peptides, MHC molecules, and an unknown trimming peptidase, later named ERAAP. Currently, the mechanism of ERAAP function is still a controversial issue, with two models supported by different studies. First, the "molecular ruler" mechanism (12) proposes the binding of both N- and C-

terminal residues of a 9-16 aa substrate to the enzyme. The lateral chain of the C-terminus residue would interact with a hydrophobic pocket away from the active site. The N-terminal residues are accessible to the active site, and the enzyme trims longer precursors in a nonprocessive manner, after which MHC class I molecules can bind the epitopes. A later study (13), indicates that ERAP1 also recognizes internal sequence of the peptide. The second or “template” model (14) implies that peptides with a proper MHC I motif but retaining the too-long N-terminus would bind to MHC molecules and would then be trimmed by ERAAP; in this way, the superfluous amino acids at the N-terminus would be cut away to generate the minimal epitope.

A previous study investigated natural peptides endogenously processed from the HIV gp160 glycoprotein in living cells that express murine H-2^d class I molecules. Unexpectedly, the natural situation was more complex than a single type of peptide/MHC class I complex. A nested set of three abundant and equally antigenic peptides that differed in the N-terminus was found. The shortest natural ligand derived from gp160 was a 9-mer with canonical anchor motifs for L^d (15); the amino-terminal extended ligand 10-mer had all anchor motifs for D^d binding (16), (17). Both peptides were present in infected cells in similar amounts. The third peptidic species corresponded to a 15-mer peptide with an amino-terminal extension of six residues that probably protruded out of the L^d binding groove (18), and was recognized by CTL with an antigenicity only slightly lower than that of the optimal 9-mer but was more abundant in infected cells than this minimal 9-mer epitope.

To test the “template” and the "molecular ruler" models, analysis of mouse aminopeptidase activity with the longest to date N-extending MHC class I

natural ligand recognized by CTL was carried out. Efficient trimming of the soluble peptide substrate to nonamer length was observed; the nonamer has the canonical anchor motif for binding to L^d and is the minimal natural ligand for the mouse H-2L^d molecule. In contrast, the six amino-terminal residues that most likely extended out of the MHC class I binding groove when the peptide-L^d complex was folded were not accessible to ERAAP activity.

MATERIALS AND METHODS

Cell Lines and Antibodies (Ab)

For stability assays, the TAP-deficient human lymphoblastoid T2 cells transfected with L^d was employed (19). This cell line was cultured in RPMI 1640 supplemented with 10% fetal bovine serum and $5x10^{-5}$ M β-mercaptoethanol. The Ab used in this study was 30-5-7S, which recognizes L^d bound to peptides (20).

Synthetic peptides

Peptides were synthesized in a peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA) and purified by Ion-Exchange-HPLC and/or RP-HPLC (purity > 99%, for an example see Supplementary Fig. 2, t = 0 h) (18). Identities were confirmed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. All peptide sequences are derived from the sequence ^{313}KIRIQRGPGRAFVTI^{327} of the HIV-1 strain IIIB envelope glycoprotein. Peptides were named according to the first residue, the length, and the last residue. Thus, G9I refers to the nonamer of sequence ^{319}GPGRAFVTI^{327}. The monosubstituted Ala analogues of HIV-1 peptides were named according to the substituted residue (Ala → Pro) and their position inside the HIV-1 peptide. Thus, A2-G9I refers to the nonamer of sequence G<u>A</u>GRAFVTI. The single-letter aa code is used.

Peptide-H-2L^d complex

Purified synthetic peptides K15I or R13I, L^d MHC class I heavy chain, and β2microglobulin were folded as previously described (21) followed by purification by the NIH Tetramer Core Facility (Emory University, Atlanta, GA, USA). In addition, an aliquot of these complexes were denatured by addition of trifluoroacetic acid to a final concentration of 0.1%, and the peptide identity was confirmed by both MALDI-TOF and electrospray-ion trap mass spectrometry.

Digestion with recombinant ERAAP enzyme

Recombinant purified mouse ERAAP enzyme (R&D Systems, Minneapolis, MN, USA) was incubated for the indicated periods at 37°C with purified synthetic peptide and/or H-2L^d-peptide complexes at the indicated molar enzyme/substrate ratios in 50 mM Tris, pH 7.4, and 1 mM DTT. The addition of trifluoroacetic acid to a final concentration of 0.1% stopped the digestions and denatured the proteins.

MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry was performed using a Reflex IV instrument (Brucker-Franzen Analytik, Bremen, Germany) operating in the positive ion reflection mode. The samples were dried, resuspended in 1 μl of 0.1% trifluoroacetic acid in a 2:1 solution of water:acetonitrile, and mixed with 1 μl of saturated α-cyanohydroxycinnamic acid matrix in the same solution. One microliter of the mixture was dried and subjected to analysis.

The intensity peaks obtained in each time point for all peptides were added up and this sum taken as 100% for each time point and depicted. Data shown are representative of 2-4 different experiments.

Electrospray-ion trap mass spectrometry

Aliquots of total digestions were dried and dissolved in 10 μl of 0.1% trifluoroacetic acid in water and sequenced by quadrupole ion trap microHPLC (Biobasic C18 column 150x0.18 mm, Thermo Electron, San Jose, CA, USA) electrospray MS/MS in a Deca XP LCQ mass spectrometer (Thermo Electron). The eluents used were as follows: A, 0.5% acetic acid in water; and B, 80% acetonitrile containing 0.5% acetic acid. The gradient was 0-40% B in 24 min, 40-100% in 5 min, with a flow rate of 1.5 μl/min. The MS/MS mode focused to each hypothetical parental peptide with an isolation width (m/z) of 1.5 Da was used (22), (23).

MHC/peptide stability assay

TAP-deficient T2 cells transfected with L^d were cultured at 26°C, and 14 h later they were washed and incubated for 2 h at 26°C with 500 μM of the different synthetic peptides, as described (24), (23). After washing, the cells were further incubated at 37°C. Aliquots removed at 60 min were stained with mAb 30-5-7S followed by flow cytometry. Cells incubated without peptide had peak fluorescence intensities close to background staining with second Ab alone. Fluorescence Index (FI) was calculated at each time point as the ratio of peak channel fluorescence of the sample to that of the control incubated without peptide. Data shown are the mean of 3 independent experiments.

RESULTS AND DISCUSSION

ERAAP efficiently trims the natural soluble substrate

In a previous study (18), a striking example of a nested set of three highly antigenic and similarly abundant natural MHC class I ligands of 15-, 10-, and 9-aa in length and derived from a single HIV gp160 epitope was identified. To demonstrate that these three peptides are bound with identical register to H-2L^d molecules MHC/peptide complex stability assays using TAP-deficient cells transfected with L^d molecule were carried out. The exchange for alanine of the proline residue that serves as L^d canonical anchor motif abolished interaction with the MHC of the N-extended K15I and R10I peptides, as well as of G9I (Supplementary Fig. 1). This indicates that they bind to L^d molecule using the canonical anchors, and thus both K15I and R10I peptides protrude with an N-terminus extending out of the L^d peptide groove. These data confirm our previous report about the nature of these interactions (18).

To study susceptibility to aminopeptidase activity, recombinant ERAAP was incubated with K15I synthetic peptide, the same substrate detected in living murine cells. Subsequently, cleavage products generated after enzymatic digestion were analyzed by mass spectrometry at different times. At time 0 or in the absence of enzyme, this system allowed for the detection of molecular species with the same monoisotopic m/z as the K15I substrate (Supplementary Fig. 2, upper panel). Additionally, an ion 62 Daltons greater than the m/z of K15I (Supplementary Fig. 2, asterisk) and compatible with a molecular Cu^+ adduct was detected. Na^+ or K^+ adducts were also found in some experiments

(Supplementary Fig. 2, 10m panel, asterisk). Minority peaks with K15I m/z less 16 or 36 Daltons were frequently found (Supplementary Fig. 2, arrow), probably due to the neutral loss in the peptides under desorption/ionization conditions. After only 5 seconds in the presence of the enzyme, partial cleavage of the substrate was observed via detection of trimmed I14I and R13I products (Supplementary Fig. 2). In only 5 minutes, all of the substrate was converted into the R13I peptide, with small traces of R10I peptide starting to be detectable. After 10 minutes, traces of the shorter G9I peptide were also found. Finally, after 20 minutes of enzymatic reaction, significant detection of both G9I and R10I products was revealed. No signals were found at m/z values corresponding to I12I and Q11I peptides.

To verify the species detected by MALDI-TOF experiments and to confirm their respective sequences, MS/MS analysis was performed via microHPLC with on-line detection by ESI-IT MS/MS. In this separation-detection system, the substrate and the four trimmed peptidic products (I14I, R13I, R10I, and G9I) were studied. The interpretation of the MS/MS fragmentation spectra allowed the unequivocal identification of all five peptides with the corresponding molecular ions detected by MALDI-TOF analysis (data not shown).

The results shown in Supplementary Fig. 2 and other intermediate points are summarized and depicted in Fig. 1A for further clarification. The K15I substrate was quickly processed by mouse ERAAP to 14- and 13-aa products. The N-terminal Arg of the 13mer peptide was rather resistant to trimming because the R13I peptide was virtually the only molecular species detected between 2.5 and 15 min. The short R10I and G9I peptides appeared over 5-10 min of the reaction and were relevant in the reaction pool at 20 min. I12I and Q11I

intermediates were not detected. Thus, these two peptide intermediates must be processed quickly after R13I trimming.

To study this enzymatic activity in more detail, similar experiments were performed with decreased enzyme concentration and increased reaction time. As shown Fig. 1B, the K15I substrate was detected for a longer period of time. The I14I and R13I peptides were present for shorter periods of time, as in Supplementary Fig. 2. R13I was the major product detected. After approximately 1 hour of reaction, both R10I and G9I were present, and finally all of the substrate was trimmed to the G9I product. In summary, even with a slower reaction, peptide intermediates between R13I and R10I were not detected, indicating a fast conversion to R10I. This data is in agreement with the findings in infected living cells, where neither one can be detected (25), (18).

Peptides shorter than G9I were not detected in the above experiments. To confirm this point, enzymatic reactions with larger amounts of ERAAP were performed. A pattern of appearance of trimmed products similar to that in previous experiments was observed (Fig. 1C) with one only difference: because of the increased reaction speed, all of the substrate was practically trimmed to the G9I product after 15 min. The reaction showed no change after the 30 min time point, and peptides shorter than the G9I nonamer were not detected. This finding is in agreement with a previous report (26) where this enzyme efficiently trims N-terminal residues of antigenic precursors unless they are flanked by proline and causes the accumulation of X-P-X_n peptides. Thus, mouse ERAAP is not able to trim the Gly-Pro bond of the G9I peptide (GPGRAFVTI).

To exclude the possibility that lack of detection of intermediate and shorter peptides was due to different ionization efficiencies rather than to lack of

trimming products, titrations of peptides between 7 and 15 residues in length were analyzed by MALDI-TOF mass spectrometry. In the range studied (between 100 pmol and 1 ηmol), no differences were found among these 9 N-extended peptides (data not shown). This is likely because they make up an N-extended nested set of peptides with an identical C-end region; thus their ionization properties should be similar.

In summary, mouse ERAAP efficiently trims the soluble K15I peptide to the minimal G9I peptide, a natural D^d and L^d ligand in infected cells (25), (18).

Previous studies showed that the enzymatic activity of ERAAP in microsomes or living cells trims peptide precursors in a short time period (minutes) (27), (28), (29). In contrast, *in vitro* ERAAP was more inefficient because longer incubations are required with the recombinant or purified enzyme (in the order of hours) (7). This is also true for human ERAP enzymes trimming the same K15I precursor (10). The present study demonstrated significant ERAAP trimming of the K15I peptide in a very short time (seconds) and total substrate degradation in a few minutes. Some peptides can thus be trimmed *in vitro* with physiologic efficiency by ERAAP.

ERAP1 is the human homologue of mouse ERAAP. Additionally, in humans but not mice, a second aminopeptidase, ERAP2, also trims certain precursors to MHC class I-presented antigenic peptides (30). The homology between the two human enzymes is less (~50% identity) than that between human ERAP1 and mouse ERAAP. ERAAP reproduced the activity of ERAP1 on the 15-mer substrate (10), in agreement with their high degree of homology.

H-$2L^d$ molecule protects against *in vitro* trimming by aminopeptidase

Some authors have reported that MHC molecules may play a key role in contributing to generate the final peptide-MHC I complex (14). To test whether K15I could be trimmed while associated with the MHC class I presenting molecule, folded H-2L^{d}-K15I complexes were used as a possible substrate for ERAAP activity. As shown in Table 1 (upper panel), destruction of K15I peptide was not observed, and no trimming products were detected with high E/S ratios (ten fold higher than the quantity used in the experiment depicted in Fig. 2B). Similar results were obtained with lower E/S ratios (data not shown).

To verify the activity of ERAAP, similar experiments with the K15I soluble peptide were carried out in parallel (Table 1, second panel). In this case, with an E/S ratio five fold less than that in the complexed substrate condition, substantial K15I disappearance and conversely emergence of aminoterminal cleavage products were detected.

To rule out the possibility that absence of ERAAP trimming was the result of a nonspecific effect of some unknown component of the peptide-MHC complex preparation, a mix of soluble and complexed substrates was incubated in the presence of this mouse enzyme. As shown in Table 1 (third panel), efficient trimming of the K15I soluble peptide was found, and residual K15I amounts were detected (~3%) that were compatible with the total quantity of L^{d}-K15I complex incubated in the reaction mix. In addition, to release K15I from the complex, in order to verify its integrity, L^{d}-K15I complex was denatured at pH=2, later equilibrated at pH=7 and this substrate was incubated with ERAAP. Similar trimming rate to the one observed with K15I soluble peptide was found (Table I, fourth panel). Also, ERAAP can trim soluble but not Ld-complexed

R13I peptide (Table I, lower panels). Collectively, these data excluded any general non-specific effect on ERAAP activity by the L^d-K15I complex preparation.

Considering that (1) ERAAP trimming is very efficient with the soluble 15-mer epitope and (2) yields the shorter R10I and overcoat G9I peptides, but (3) cannot trim the six N-terminally extended residues of K15I peptide *in vitro* when the peptide-MHC class I complex is fully folded, the two likely explanations for our previous (25), (18) and present results are the following. The K15I substrate is efficiently transported by murine TAP, as suggested by our previous study of transport to the ER (18). In the ER lumen, most of the substrate is quickly and efficiently folded within the H-2L^d molecules and thus protected from ERAAP activity. The remaining K15I molecules might be accessed by ERAAP and trimmed to R10I. A fraction of this peptide might be protected by either D^d or L^d molecules, and the rest might be trimmed to the final product G9I, which can also efficiently stabilize both MHC molecules. Alternatively, the three natural MHC class I ligands could be generated independently in the cytosol and transported to the ER lumen. There, in order to be protected from ERAAP trimming, both N-extended ligands would bind quickly to MHC class I molecules as their affinity is similar to that of the minimal epitope (25), (18). This second explanation is supported by the identification, in the natural L^d peptide mixtures resulting from the endogenous processing in infected cells, of only G9I, R10I and K15I natural ligands in contrast with the similar MHC binding affinities of nested set of synthetic peptides previously tested (G9I, R10I, Q11I, I12I, R13I and K15I) (18).

In summary, our data with a single epitope show that the H-2L^d molecule protects the HIV N-extended epitope from trimming by ERAAP. This indicates that ERAAP cannot trim the H-2L^d ligand when the peptide-MHC complex is fully folded, not complying with to the template model. Nevertheless, this model might still apply to this particular epitope if ERAAP would only trim the substrate bound to MHC when it is in the peptide-loading complex. But the existence of R10I natural ligand (that is not fully trimmed to minimal H-2L^d ligand, G9I) detected in infected living cells does not favour this possibility (18).

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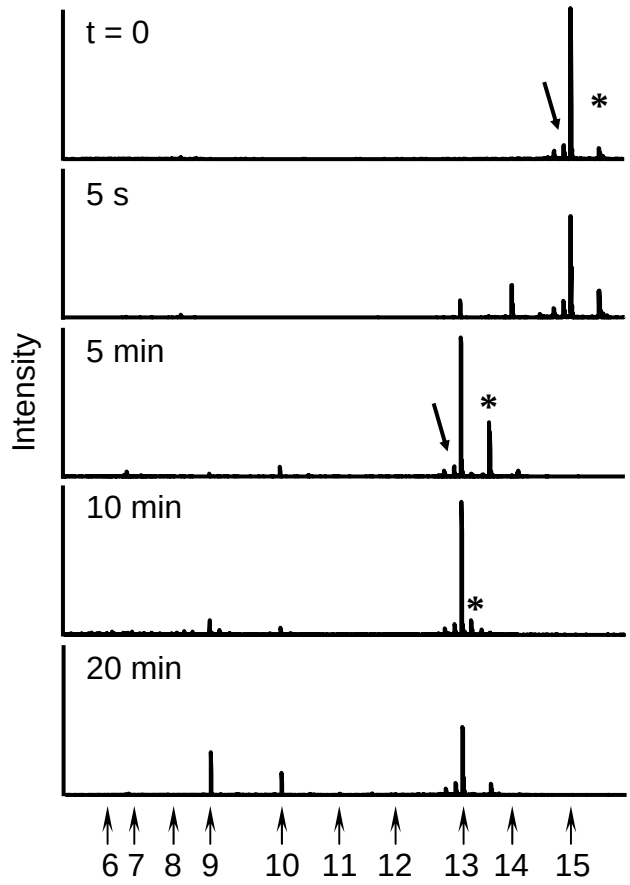
FIGURE LEGENDS

Figure 1

Area charts of digestions performed with several E/S ratios.

Soluble K15I synthetic peptide was digested with purified ERAAP. The intensity peaks obtained by MALDI-TOF analysis for all peptides in each time point were added up and this sum taken as 100% for each time point and depicted. The different products detected are named in their respective region. The E/S ratio used in each experiment is indicated at the top of the respective panel.



Infantes et al. Figure 1

Table 1

Comparison of trimming by ERAAP of soluble and/or MHC class I complexed K15I peptide

Time (min)	G9I	R10I	R13I	I14I	K15I	Substrate [a]	L^d-K15I / sK15I [b]	Enzyme / Substrate [c]
0	ND [d]	ND	ND	ND	**100**	L^d-K15I	-	1:60
15	ND	ND	ND	ND	**100**			
30	ND	ND	ND	ND	**100**			
60	ND	ND	ND	ND	**100**			
90	ND	ND	ND	ND	**100**			
0	ND	ND	ND	ND	**100**	sK15I	-	1:300
0.08	ND	ND	**40**	**22**	**38**			
5	**4**	**11**	**45**	**17**	**23**			
15	**15**	**11**	**46**	**14**	**14**			
0	ND	ND	ND	ND	**100**	L^d-K15I + sK15I	4:96	1:300
0.08	ND	ND	**36**	**26**	**38**			
5	**5**	**8**	**84**	ND	**3**			
15	**41**	**18**	**38**	ND	**3**			
0	ND	ND	ND	ND	**100**	dL^d-K15I	-	1:300
0.08	ND	ND	**33**	**24**	**43**			
5	**5**	**30**	**49**	**13**	**3**			

[a] The substrates used were L^d-complexed K15I (L^d-K15I), denatured L^d-complexed K15I (dL^d-K15I) and soluble peptide (sK15I).
[b] K15I-L^d/sK15I molar ratio.
[c] Enzyme/substrate molar ratio.
[d] ND, Not detected including Q11I and I12I peptides.