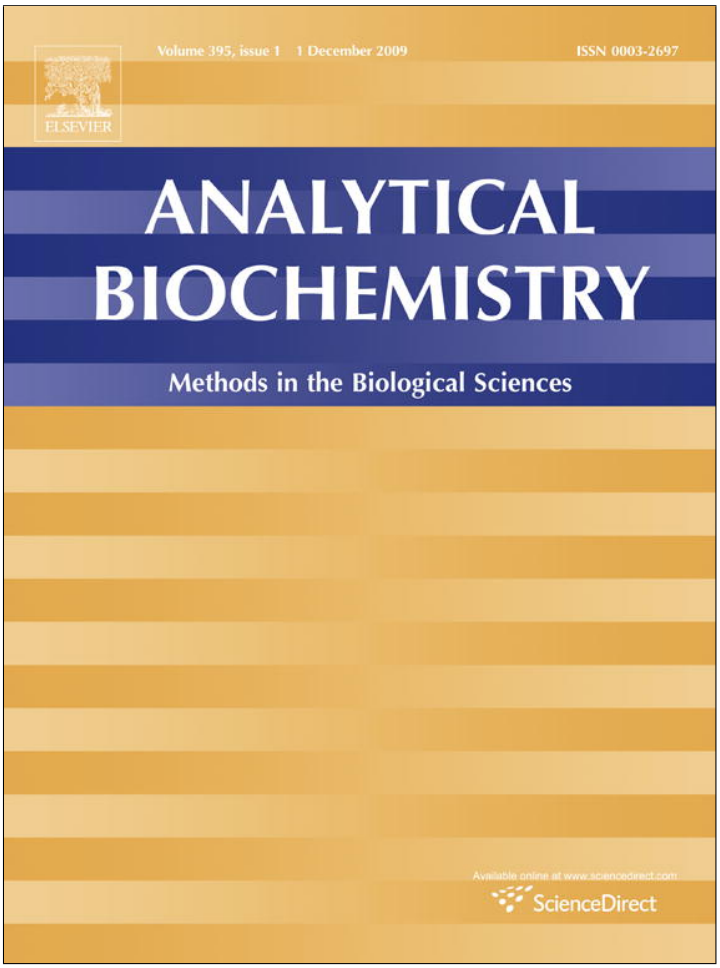

Analytical Biochemistry 395 (2009) 33–40

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A continuous fluorigenic assay for the measurement of the activity of endoplasmic reticulum aminopeptidase 1: Competition kinetics as a tool for enzyme specificity investigation

Irini Evnouchidou [a], Marcelo J. Berardi [b], Efstratios Stratikos [a,*]

[a] Protein Chemistry Laboratory, IRRP, National Centre for Scientific Research "Demokritos," 15310 Athens, Greece
[b] Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA

ARTICLE INFO

Article history:
Received 30 May 2009
Available online 26 July 2009

Keywords:
Enzyme
Assay
Peptide
Fluorescence
Aminopeptidase
Kinetics
Competition
Specificity
Processing
Presentation
Immunity

ABSTRACT

Endoplasmic reticulum aminopeptidase 1 (ERAP1) is a recently discovered enzyme that plays critical roles in antigen presentation and the immune response. Unlike other aminopeptidases, ERAP1 displays strong sequence preferences for residues distal to the peptide–substrate's N terminus. This unusual substrate specificity necessitates the development of new assays that are appropriate for the study of such aminopeptidases. Here we describe a continuous fluorigenic assay suitable for the analysis of the enzymatic properties of ERAP1. In this assay, signal is generated by the excision of an internally quenched N-terminal tryptophan residue from a 10mer peptide by the aminopeptidase, resulting in the enhancement of tryptophan fluorescence in the solution. This method overcomes the limitations of previously used fluorigenic and high-performance liquid chromatography (HPLC)-based assays and is appropriate for small molecule inhibitor screening as well as for rapid substrate specificity analysis by kinetic competition experiments. Such efficient peptidic fluorigenic substrates like the ones described here should greatly simplify specificity analysis and inhibitor discovery for ERAP1 and similar aminopeptidases.

The recently discovered endoplasmic reticulum aminopeptidase 1 (ERAP1; also named ERAAP for endoplasmic reticulum aminopeptidase associated with antigen presentation)[1] is an M1 metalloproteinase that belongs to the oxytocinase subfamily [1]. ERAP1 has been shown to play a critical role in antigen presentation and the immune response in cell-based and mouse models by generating mature antigenic peptides from N-terminally extended precursors [2–9]. ERAP1 has several unique properties (for an aminopeptidase) that fit well with its role in antigen processing; it trims a large variety of long peptide sequences efficiently, and its activity drops dramatically for peptides shorter than eight or nine amino acids, the length of most antigenic epitopes [10]. Although ERAP1 has been classified as a leucine aminopeptidase based on its ability to efficiently trim only leucine-7-amido-4-methylcoumarin (L-AMC)—and, to a lesser extent, methionine-AMC (M-AMC)—it has been demonstrated that it can efficiently excise virtually any amino acid in the context of a larger peptide [7,10–12]. We recently demonstrated that ERAP1's broad N-terminal specificity is due to strong preferences for residues downstream in the peptide–substrate sequence and that these preferences probably arise from an extended peptide binding site adjacent to the catalytic center of the enzyme [12]. Due to these unique properties of ERAP1, existing enzymatic assays are not sufficient for the full characterization of its enzymatic properties.

Inhibitors for ERAP1 have potential therapeutic interest due to the unique biological effects of the enzyme. ERAP1 activity has been linked to disease progression and predisposition to various diseases such as autoimmunity, diabetes, and cancer [13–19]. The small, nonspecific, metalloenzyme inhibitor leucinethiol has been shown to inhibit ERAP1 in vitro and induce antigen presentation changes in cell-based assays, similar to the ones observed in ERAP1 knockdown experiments [4]. However, no potent and specific inhibitor for ERAP1 has been identified yet. The development of a highly specific ERAP1 inhibitor may be challenging because of the high structural and mechanistic conservation of the catalytic site of aminopeptidases. However, the unusual specificity of ERAP1, and specifically ERAP1's extended substrate recognition

* Corresponding author. Address: Protein Chemistry Laboratory, IRRP, National Centre for Scientific Research "Demokritos," 15310 Agia Paraskevi, Athens, Greece. Fax: +30 210 6543526.
E-mail address: stratos@rrp.demokritos.gr (E. Stratikos).

[1] *Abbreviations used:* ERAP1, endoplasmic reticulum aminopeptidase 1; L-AMC, leucine-7-amido-4-methylcoumarin; M-AMC, methionine-AMC; HTS, high-throughput screening; AMC, 7-amino-4-methylcoumarin; NA, naphthylamide; HPLC, high-performance liquid chromatography; DNP, dinitrophenyl; DTT, dithiothreitol; TFA, trifluoroacetic acid; Ni–NTA, nickel–nitrilotriacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.07.032

site, may provide novel design or screening opportunities for the development of such inhibitors. However, to exploit this unique specificity in the context of high-throughput screening (HTS) for small molecule inhibitors, it is crucial to use a substrate as close to the natural enzyme substrate as possible so that the screen covers the full array of molecular interactions between the peptide and the extended enzyme binding site.

Unlike ERAP1, typical aminopeptidases trim peptides with preferences primarily for the substrate's N terminus, and as a result efficient chromogenic or fluorigenic methods have been developed for assaying this type of specificity. Typical assays usually use small model dipeptidic substrates that, when proteolyzed, release compounds such as 7-amino-4-methylcoumarin (AMC) and naphthylamide (NA) in the solution, leading to fluorescence or absorbance enhancement that can be followed kinetically [20–22]. Aminopeptidase substrates used for the characterization of ERAP1 are usually of the type X-AMC or X-NA, where X is any amino acid [11,23–25]. These assays are rapid and accurate, allowing the efficient characterization of the N-terminal specificity of the studied aminopeptidase. However, they are limited in that they probe only the S1 specificity pocket of the enzyme and, therefore, do not assay other important interactions between the enzyme and its natural substrates.

The substrate preferences of ERAP1 have been investigated before by separating the products of enzymatic digestion of model peptide–substrates on a C18 reverse-phase column attached onto a high-performance liquid chromatography (HPLC) system [7,10,12,25]. HPLC analysis allows the separation and quantification of peptidic fragments but has several serious drawbacks. First, peak separation is often problematic, especially when the excised amino acid is hydrophilic. Second, the reaction is followed in a noncontinuous fashion, meaning that the reaction must be sampled at specific time intervals and chemically stopped and that the sample must be analyzed on the HPLC device. This makes the determination of trimming rates cumbersome and slow. Third, detection of product peaks is limited at the start of the reaction due to the overwhelming presence of the substrate peak, making calculation of initial rates difficult or nearly impossible. For these reasons, the development of a new method for the measurement of the enzymatic activity of ERAP1 and other similar aminopeptidases is necessary.

Here we describe the development of a continuous fluorigenic method that is well-suited for the analysis of the activity of aminopeptidases that display sequence preferences beyond the N terminus of the substrate peptide such as ERAP1. This method relies on the excision of a single N-terminal tryptophan residue from the peptide by the aminopeptidase. The tryptophan fluorescence is normally quenched on the peptide due to the close proximity of a dinitrophenyl (DNP) group attached on a cysteine residue in the peptide. When the proteolytic activity of the aminopeptidase releases the N-terminal tryptophan to the solution, the tryptophan is no longer quenched and solution fluorescence is enhanced. This method combines the advantages of the existing fluorigenic methods for the detection of aminopeptidase activity—continuous measurement, high sensitivity, and the ability to follow the reaction at initial conditions—with the use of a long-peptide substrate that is closer to the enzyme's natural substrates. We demonstrate the applicability of this method to the study of the internal sequence specificity of ERAP1 by measuring trimming rates for three peptides with internal sequence variations. Measured rates vary over three orders of magnitude and are consistent with the preference of ERAP1 for positively charged residues as demonstrated previously [12]. Furthermore, we show that this method is appropriate for the screening of potential small molecule inhibitors and for the rapid analysis of substrate specificity through competition experiments with unlabeled peptides. This fluorigenic assay should greatly facilitate further studies of ERAP1 and related aminopeptidases as well as large-scale screens for inhibitor discovery.

Materials and methods

Reagents

DNP C2 maleimide was obtained from Anaspec (San Jose, CA, USA). Dithiothreitol (DTT) and trifluoroacetic acid (TFA) were purchased from Applichem (Darmstadt, Germany). L-AMC was obtained from Sigma–Aldrich. Acetonitrile was obtained from Merck.

Peptide synthesis

Peptides WRVYEKMALKC, WRVYEKCALK, WEVYEKCALK, and WEVYEKCALE were synthesized by GenScript (Piscataway, NJ, USA). All peptides were purified by reverse-phase HPLC and were more than 98% pure.

Protein expression and purification

Human recombinant ERAP1 was expressed in insect cells driven by a baculoviral vector as described previously [12]. The active enzyme was secreted into the cell medium and purified to homogeneity by a combination of nickel–nitrilotriacetic acid (Ni–NTA) chromatography, anion exchange chromatography (monoQ column, Pharmacia), and size exclusion chromatography (S200 column, Pharmacia). Protein purity was assayed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and was found to be more than 98%. Enzyme activity was measured by L-AMC hydrolysis.

Peptide labeling

Peptides were labeled with DNP by allowing the free cysteine residue on the peptide to react with DNP maleimide. Then 200 μM of the peptide in 10 mM sodium phosphate buffer (pH 8.0) was incubated for 15 min with 200 μM freshly dissolved DTT to break any disulfide dimers. An 800-μM final concentration of DNP C2 maleimide was then added, and the reaction was allowed to proceed for 1 h at room temperature. After that, the labeled peptide was purified by reverse-phase HPLC and successful labeling was confirmed by measuring the absorbance at 280 and 340 nm to confirm a 1:1 labeling ratio.

Peptide quantification

The nonlabeled peptides were quantified by their absorbance at 280 nm using a calculated extinction coefficient based on their sequence. The extinction coefficient for each peptide was calculated, based on tryptophan, tyrosine, and cysteine content of the peptide, to be 6990 M^{-1} cm^{-1}. Labeled peptides were quantitated based on absorbance at 280 and 340 nm. An extinction coefficient of 17,500 M^{-1} cm^{-1} was used for DNP at 340 nm. The contribution of DNP absorbance at 280 nm was experimentally found to correspond to 25% of the absorbance at 340 nm, and the labeled peptide absorbance at 280 nm was corrected to account for this contribution. The corrected absorbance at 280 nm was used to calculate peptide concentration.

Fluorescence measurements

Fluorescence measurements were performed on a QuantaMaster 4 spectrofluorimeter (Photon Technology International, Birmingham, NJ, USA) and on a Tecan SpectraFluor plate reader.

Fluorescence spectra were collected by irradiating at 280 or 295 nm using a slit width of 4 mm and by scanning from 310 to 420 nm at a rate of 1 nm/s with a slit of 8 mm. Kinetics was followed by excitation at 295 nm and emission at 350 nm using 2-s integration and 30-s acquisition intervals.

Enzymatic reactions

Enzymatic reactions were performed at either room temperature or 37 °C. In all reactions, the substrate concentration was at least 100-fold in excess over the enzyme concentration. Typically, 10 to 1000 nM ERAP1 was mixed with 1 to 100 μM peptide and the reaction was followed by fluorescence. For HPLC analysis, the reaction was stopped by the addition of TFA to a final concentration of 0.3% and kept at –20 °C until analysis.

HPLC analysis

Analysis of the peptide trimming products was done as described previously [12]. Briefly, reactions stopped by the addition of TFA were analyzed by reverse-phase HPLC on a Chromolith analytical column (Merck) using a linear gradient of 5 to 50% acetonitrile and following the absorbance at either 280 or 340 nm.

Competition by unlabeled peptide substrates

To study the effect of the presence of a competing substrate on the hydrolysis of the fluorigenic peptide WEVYEKC^{DNP}ALK, the fluorigenic peptide (10 μM) was mixed with increasing amounts of the competing peptide LVAFKARKF (2–90 μM) and its hydrolysis by ERAP1 (80 nM) was followed by the enhancement of tryptophan fluorescence. The fluorescence increase was found to be linear under these reaction conditions, and the slope of the least-squares fit was plotted versus the competitor peptide concentration and normalized to percentage activity recovered. The data points were fit to the following equation that describes single site substrate competition kinetics. This equation was derived as follows. For two substrates, S_1 and S_2, competing for the same enzyme,

$$E + S_1 \leftrightarrow ES_1 \rightarrow EP_1 \rightarrow E + P_1 + S_2 \leftrightarrow ES_2 \rightarrow EP_2 \rightarrow E + P_2.$$

The following equations apply, by definition, a mass balance:

$$K_{M,1} = \frac{E \cdot S_1}{ES_1} \text{ and } K_{M,2} = \frac{E \cdot S_2}{ES_2} \text{ and } E_t = E + ES_1 + ES_2,$$

where E_t is the total enzyme concentration and $K_{M,1}$ and $K_{M,2}$ are the Michaelis constants for the respective substrates. By substituting the value of E,

$$\begin{aligned} K_{M,1} \cdot ES_1 &= E \cdot S_1 = (E_t - ES_1 - ES_2) \cdot S_1 \\ &= E_t \cdot S_1 - ES_1 \cdot S_1 - ES_2 \cdot S_1 \end{aligned} \quad (1)$$

By substituting the value of $ES_2 \cdot S_1 = \left(E \cdot \frac{S_2}{K_{M,2}}\right) \cdot S_1$ into Eq. (1), we get

$$K_{M,1} \cdot ES_1 = E_t \cdot S_1 - ES_1 \cdot S_1 - E\frac{S_2}{K_{M,2}} \cdot S_1 \quad (2)$$

However, $E = K_{M,1} \cdot \frac{ES_1}{S_1}$, and substituting the value of E in Eq. (2), we get

$$K_{M,1} \cdot ES_1 = E_t \cdot S_1 = ES_1 \cdot S_1 - K_{M,1} \cdot \frac{ES_1}{S_1} \cdot \frac{S_2}{K_{M,2}} \cdot S_1 \quad (3)$$

Solving Eq. (3) for ES_1, we get

$$ES_1 = \frac{E_t \cdot S_1}{K_{M,1} + S_1 + \frac{K_{M,1}}{K_{M,2}} \cdot S_2} \quad (4)$$

Because the reaction rate $v1 = k_{cat,1} \cdot ES_1$,

$$v_1 = k_{cat,1} \cdot ES_1 = k_{cat,1} \frac{E_t \cdot S_1}{K_{M,1} + S_1 + \frac{K_{M,1}}{K_{M,2}} \cdot S_2} \quad (5)$$

or

$$\frac{1}{v_1} = \left(\frac{1}{k_{cat,1} \cdot E_t}\right) \cdot \left(1 + \frac{K_{M,1}}{S_1} \cdot \left(1 + \frac{S_2}{K_{M,2}}\right)\right) \quad (6)$$

$$\iff v_1 = \frac{V_{max}}{\left(1 + \frac{K_{M,1}}{S_1} \cdot \left(1 + \frac{S_2}{K_{M,2}}\right)\right)} \quad (7)$$

Competition titration data were fit to the model of Eq. (7) using Origin 5.0 software as follows: v_1 was the reaction rate as calculated by the slope of the fluorescence signal change versus time (dependent variable), S_2 was the competing peptide concentration (independent variable), V_{max} was the maximal velocity of the reaction, S_1 was the fluorigenic peptide concentration, $K_{M,2}$ was the competing peptide's K_M, and $K_{M,1}$ was the fluorigenic peptide's K_M. S_1 was set to a fixed value depending on the experimental conditions. To facilitate multiparameter fitting, V_{max} and $K_{M,1}$ were first estimated by HPLC experiments. Using Eq. (7), it was possible to calculate the $K_{M,2}$ value for the competing peptide with accuracy.

Results and discussion

Two synthetic peptides with the sequences WRVYEKCALK and WRVYEKMALKC based on the naturally occurring epitope RVYEKMALK (which has been found to bind onto the MHC class I allele HLA-A*03) were synthesized and labeled with DNP as described in Materials and Methods. The fluorescence spectra of the labeled and unlabeled peptides were recorded on excitation at 295 nm. To reduce any potential negative steric interactions between the introduced label and the catalytic center of the enzyme, the label was introduced at locations far from the peptide's N terminus and specifically at positions 7 and 11 of the peptide. Labeling the cysteine residue of the peptide WRVYEK**C**ALK (label at position 7) produced a marked reduction in the tryptophan fluorescence spectrum of the peptide, as would be expected because of DNP quenching (Fig. 1, top panel). Introduction of DNP onto the cysteine of peptide WRVYEKMALK**C** (label at position 11) did not result in any appreciable quenching, presumably due to the large distance between the label and the N-terminal tryptophan (Fig. 1, bottom panel). As a result, position 7 of the peptide was selected as appropriate for efficient quenching of the N-terminal tryptophan.

The basic principle behind the enzymatic assay appears schematically in Fig. 2. The N-terminal tryptophan residue of the labeled 10-mer peptide WRVYEKC^{DNP}ALK is quenched by the close proximity of the DNP group. Excision of the tryptophan by the aminopeptidase leads to unquenching and solution fluorescence enhancement. To test this principle, the labeled peptide WRVYEKC^{DNP}ALK was incubated with highly purified recombinant ERAP1 and the tryptophan fluorescence spectra were recorded at set intervals (Fig. 3A). We observed a time-dependent enhancement of the fluorescence corresponding to the excision of W from the peptide by the action of the enzyme. This time-dependent fluorescence enhancement was linear for the duration of this experiment and could be corrected by a tryptophan fluorescence standard curve to correspond to the free tryptophan in the solution (Fig. 3B). To demonstrate that the reaction followed by fluorescence was indeed the trimming of the peptide, we analyzed the reaction products by reverse-phase HPLC. We observed a decrease of the parent peak that coincided with the appearance

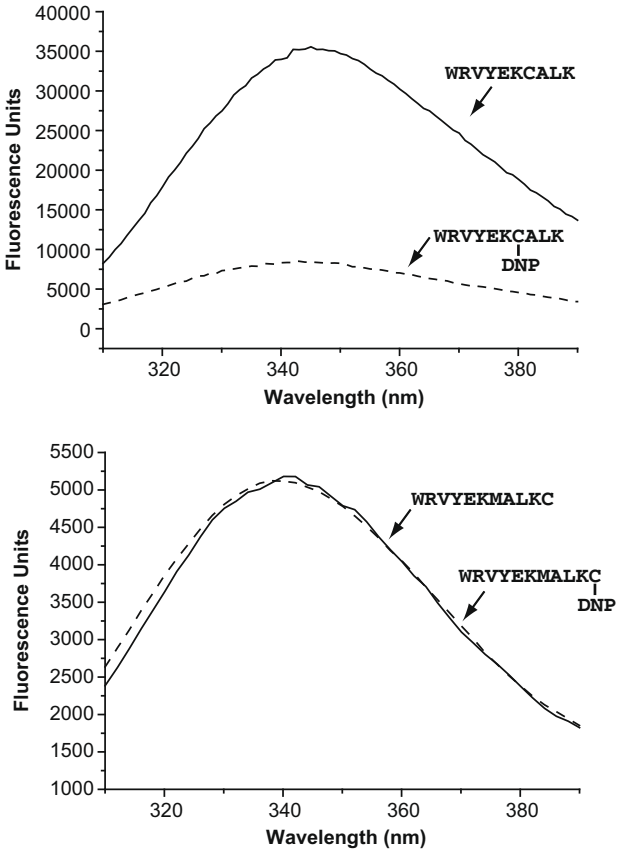

Fig. 1. Fluorescence spectra of labeled and unlabeled peptides. Excitation was done at 295 nm, and spectra were recorded from 310 to 400 nm. Top Panel, tryptophan fluorescence of the peptide with sequence WRVYEKCALK (solid line) and of the DNP-labeled peptide (dashed line). The addition of the DNP label on the cysteine 6 residues away from the tryptophan, significantly reduces fluorescence. Bottom Panel, tryptophan fluorescence of the peptide with sequence WRVYEKMALKC (solid line) and of the DNP-labeled peptide (dashed line). No significant quenching is evident when the DNP is attached to the cysteine 10 residues away from the tryptophan.

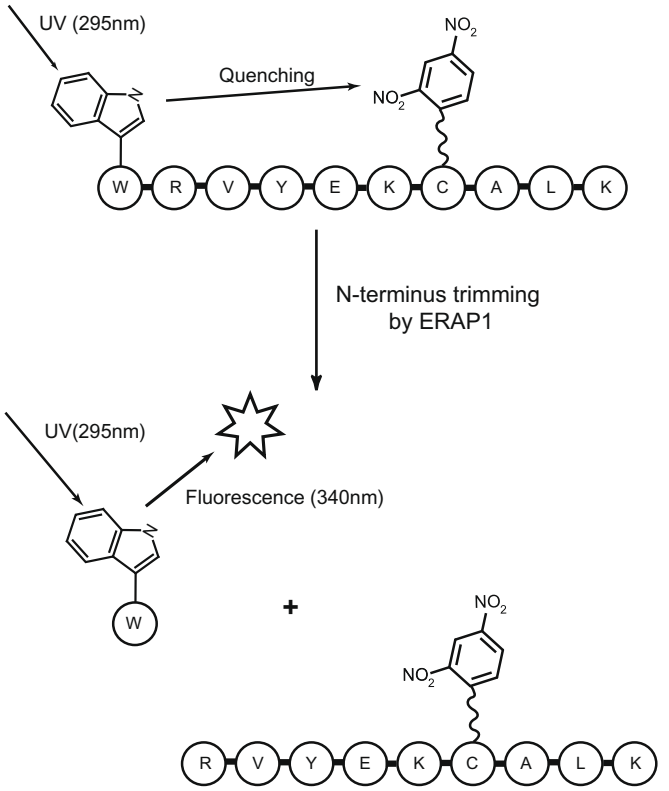

Fig. 2. Method principle. The fluorescence of the N-terminal tryptophan residue of the labeled peptide is quenched by the DNP adduct on the cysteine residue six amino acids away. When the N-terminal tryptophan is excised by the hydrolytic activity of ERAP1, the fluorescence of the solution is enhanced.

of smaller peaks corresponding to smaller peptide fragments (Fig. 3C).

To test the generality of this method on other peptide sequences as well as the accuracy of the method in investigating sequence-related effects, we designed two additional peptides based on the peptide WRVYEKC^{DNP}ALK (net charge = 2). We showed before that ERAP1 has strong sequence preferences, in particular for positively charged amino acids. Therefore, it would be expected that the trimming kinetics would be affected by charge reversals within the sequence of the peptide. To test this hypothesis, two more peptides were synthesized and labeled, specifically W**E**VYEKC^{DNP}ALK (net charge = 0) and W**E**VYEKC^{DNP}AL**E** (net charge = −2), in which the positively charged residues at position 2 and 10 of the peptide are replaced in order by a negatively charged residue (residue substitutions are marked in bold). Trimming of the three peptides by ERAP1 was followed by tryptophan fluorescence (Figs. 4A–C). By calibrating with a tryptophan standard curve, it was possible to calculate the specific enzymatic activity for each peptide (Fig. 4D). Peptide WRVYEKC^{DNP}ALK was trimmed at a rate of 0.279 ± 0.026 mol substrate/mol enzyme/s, where peptide W**E**VYEKC^{DNP}ALK was trimmed 37-fold slower, at a rate of 0.0076 ± 0.0019 mol substrate/mol enzyme/s, a dramatic reduction consistent with previous findings regarding specificity for position 2 [12]. Peptide W**E**VYEKC^{DNP}AL**E** was trimmed 8-fold slower and at a rate of 0.0010 ± 0.0005 mol substrate/mol enzyme/s, consistent again with the overall charge preference of ERAP1. These results define the minimum window of rates that are easily measurable by this method to span three orders of magnitude.

To investigate whether the 10mer fluorigenic peptide substrate is appropriate for HTS of small molecules for ERAP1 inhibitor discovery, we incubated the peptide WEVYEKC^{DNP}ALK with ERAP1 in the presence of increasing concentrations of the general metalloproteinase inhibitors leucinethiol and amastatin. Both molecules previously have been shown to inhibit, albeit with low efficacy and specificity, ERAP1 in L-AMC assays [11,26]. Both molecules were able to inhibit ERAP1 trimming of the fluorigenic peptide in a concentration-dependent manner, indicating that this method is appropriate for ERAP1 inhibitor screening (Fig. 5). Using the data from Fig. 5 that demonstrated more than 80% inhibition by leucinethiol, we calculated the *Z* factors for HTS assay validation as defined by Zhang and coworkers [27]. The *Z* factor for the L-AMC assay was found to be 0.2, and the *Z* factor for the assay using the WEVYEKC^{DNP}ALK peptide was found to be 0.5. These results essentially classify our method as an excellent assay for HTS of putative ERAP1 inhibitors.

To investigate whether this method can be used to simplify the investigation of ERAP1 specificity, we incubated the labeled peptide WEVYEKC^{DNP}ALK with ERAP1 in the presence of 10-fold higher concentration competitor peptides with the sequences LVAFKARKF and LTAEEAVET. As we demonstrated before, peptide LVAFKARKF is a very good ERAP1 substrate and peptide LTAEEAVET is a very poor one [12]. Consistent with this finding, peptide LVAFKARKF was able to fully inhibit the fluorigenic reaction, whereas peptide LTAEEAVET competed only partially (Fig. 6A). This result demonstrates that it is possible to quickly and easily discern between good and bad ERAP1 substrates using this fluorigenic method.

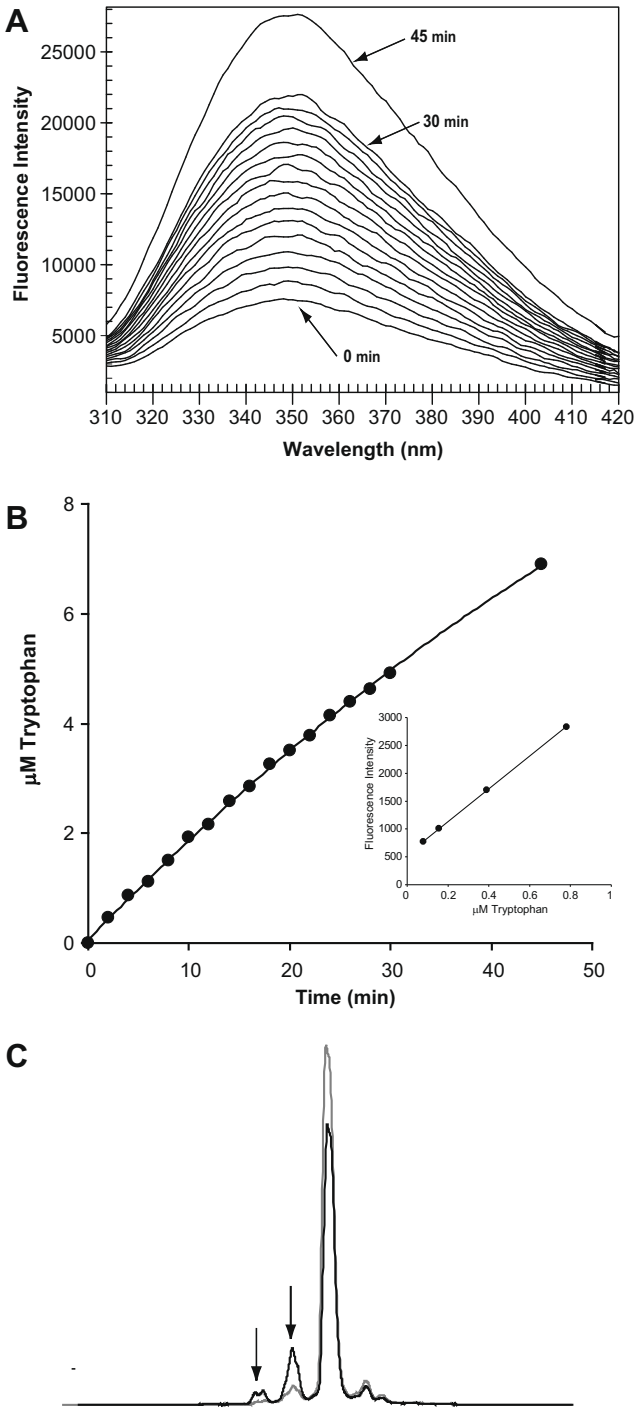

Fig. 3. (A) Fluorescence spectra were recorded every 2 min as the labeled peptide WRVYEKCALK was incubated with ERAP1. (B) The amount of tryptophan produced during the reaction was plotted as a function of time. The fluorescence signal was converted to tryptophan concentration using a tryptophan fluorescence standard curve (inset). (C) HPLC analysis of trimming products of labeled peptide WRVYEKCALK by ERAP1. The gray line is for the peptide incubated in the absence of ERAP1, and the black line is for the peptide incubated in the presence of ERAP1. Arrows indicate smaller peptidic product peaks.

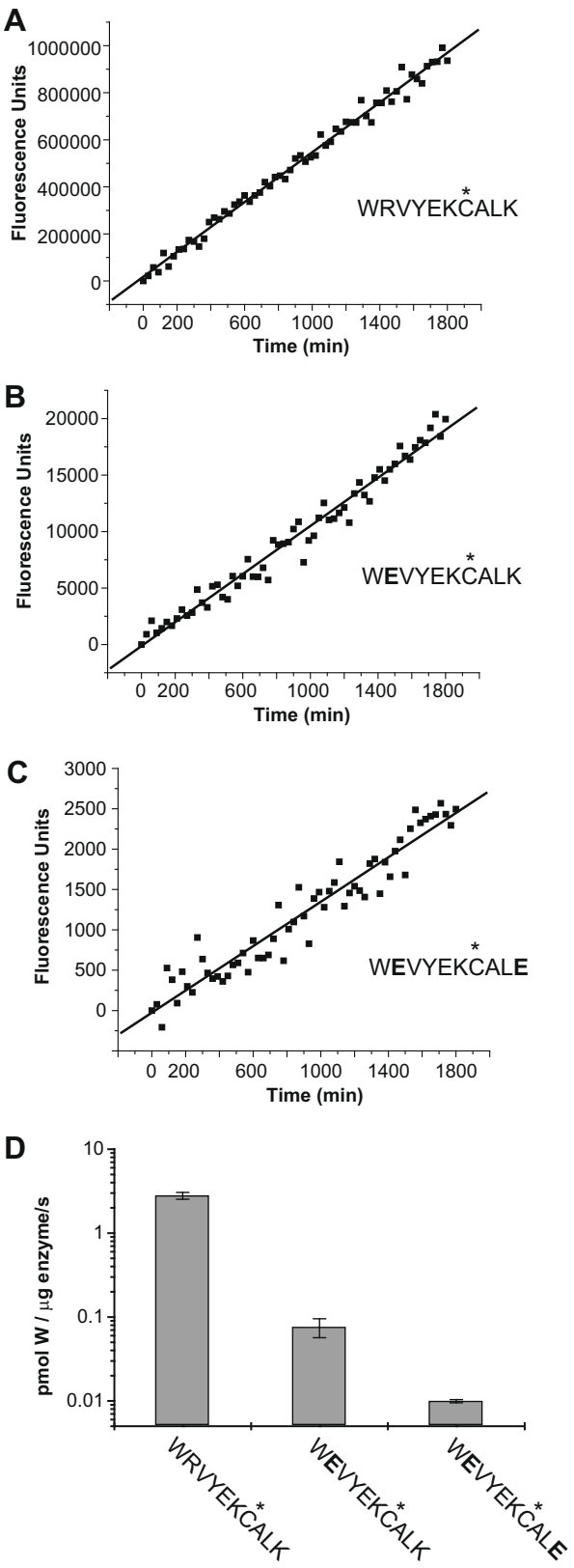

Fig. 4. (A–C) Time course of tryptophan fluorescence enhancement on trimming of peptides WRVYEKCALK (A), WEVYEKCALK (B), and WEVYEKCALE (C). The position of the DNP label is marked by an asterisk. Lines represent a least-squares linear fit to the experimental data. The slope of the fit is taken to correlate with the trimming rate of the peptide. (D) Relative rates of N-terminal tryptophan trimming by ERAP1. The *y* axis is logarithmic. Note the dramatic decrease in trimming rate as the positively charged residues at positions 2 and 10 are substituted by negatively charged residues.

Finally, we used competition kinetics to calculate K_M values for the competitor peptide LVAFKARKF (Figs. 6B and 6C). Here 10 μM of labeled peptide WEVYEKC^{DNP}ALK or WRVYEKC^{DNP}ALK was incubated with 0.8 μg of ERAP1 in the presence of increasing amounts of the competitor peptide LVAFKARKF, and the fluorescence signal was followed over time and used to calculate the

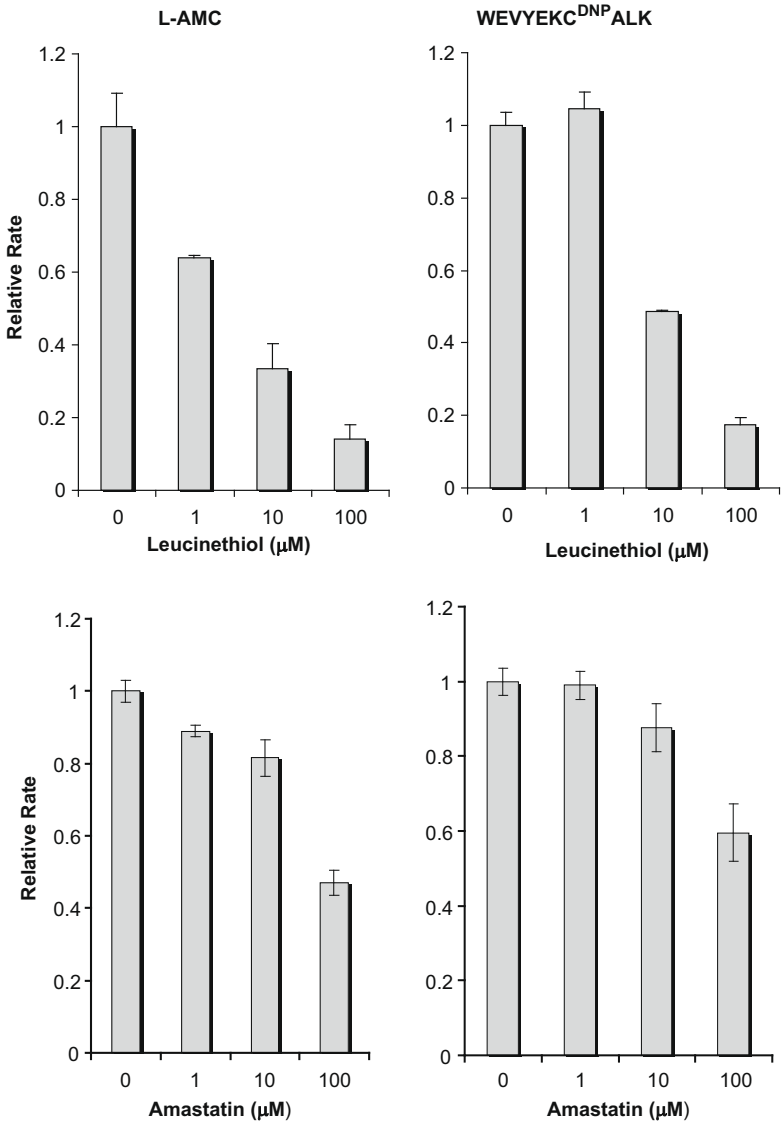

Fig. 5. Trimming of the fluorigenic substrate L-AMC (left panels) and fluorigenic peptide WEVYEKC^{DNP}ALK (right panels) by ERAP1 is inhibited by the aminopeptidase inhibitors leucinethiol (top) and amastatin (bottom) in a dose-dependent manner.

trimming rate of the labeled peptide. The resulting rates were plotted versus competitor concentration, and the data were fit to a substrate competition model as described in Materials and Methods. According to Eq. (7), the trimming rate of the labeled peptide is dependent on the concentration and the K_M of the competitor but is independent of the k_{cat} of the competitor. To facilitate multiparameter fitting, the V_{max} and K_M values for the fluorigenic peptides were first estimated by independent HPLC experiments (not shown). The calculated parameters are shown in Table 1. Because the same competitor peptide (LVAFKARKF) was used in both experiments in Figs. 6B and 6C, it would be expected that the calculated K_M value for the competitor would be identical. Indeed, within experimental error, identical K_M values of approximately 9 μM were calculated for peptide LVAFKARKF from both experiments, validating our approach. This K_M value is significantly lower than K_M values previously measured for ERAP1 peptide–substrates, consistent with the high trimming rate for this optimized substrate [10].

In summary, we have described a new enzymatic method for the analysis of the activity and specificity of ERAP1, an important enzyme with critical roles in the immune response that has received a considerable amount of attention from the scientific community recently. Our method combines the advantages of small fluorigenic substrates with the use of large, more natural-like peptidic substrates and successfully addresses the shortcomings of methods previously used for the study of ERAP1. The new assay is appropriate for screening small molecules as potential inhibitors with the added advantage of probing molecular interactions throughout the extended ERAP1 binding site. We demonstrated that this method is appropriate for the study of aminopeptidase specificity either directly or indirectly by competition kinetics using a simple two-substrate kinetic model for the determination of Michaelis constants. Overall, this method should greatly facilitate further study of ERAP1 as well as other aminopeptidases that display preferences for the substrate's internal sequence.

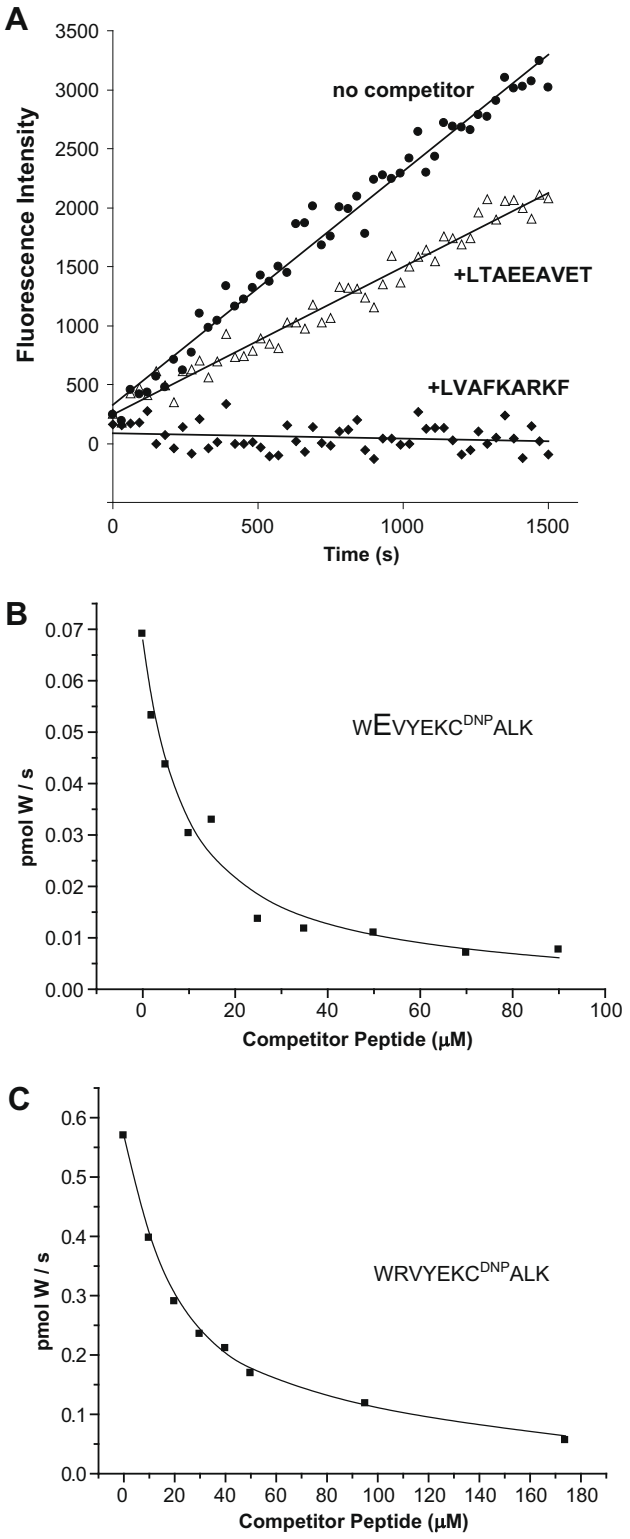

Fig. 6. (A) Trimming of DNP-labeled peptide WEVYEKC^{DNP}ALK (10 μM) by ERAP1 is inhibited by the addition of competitor peptides (100 μM) to different degrees. Peptide LTAEEAVET that previously has been shown to be a poor ERAP1 substrate shows little inhibition, whereas peptide LVAFKARKF that previously has been shown to be a very good ERAP1 substrate produces complete inhibition. (B,C) Titration of competitor peptide LVAFKARKF leads to a gradual loss of trimming of peptides WEVYEKC^{DNP}ALK (B) and WRVYEKC^{DNP}ALK (C) by ERAP1. The solid line represents the modeling of the experimental data to a competition kinetic model as described in Materials and Methods.

Table 1
Calculated enzymatic parameters from competition experiments in Fig. 6

Substrate	V_{max} (pmol/s)	K_M (μM)	Competitor K_M (μM)
WEVYEKC^{DNP}ALK	2.3 ± 0.2	328 ± 15	8.7 ± 1.1
WRVYEKC^{DNP}ALK	1.03 ± 0.3	7.5 ± 0.3	9.4 ± 0.5

Acknowledgment

Funding for this work was provided by the Sixth Framework Programme of the European Union (Marie Curie International Reintegration grant 017157 to E.S.).

References

[1] M. Tsujimoto, A. Hattori, The oxytocinase subfamily of M1 aminopeptidases, Biochim. Biophys. Acta 1751 (2005) 9–18.
[2] T. Serwold, F. Gonzalez, J. Kim, R. Jacob, N. Shastri, ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum, Nature 419 (2002) 480–483.
[3] G.E. Hammer, F. Gonzalez, M. Champsaur, D. Cado, N. Shastri, The aminopeptidase ERAAP shapes the peptide repertoire displayed by major histocompatibility complex class I molecules, Nat. Immunol. 7 (2006) 103–112.
[4] G.E. Hammer, F. Gonzalez, E. James, H. Nolla, N. Shastri, In the absence of aminopeptidase ERAAP, MHC class I molecules present many unstable and highly immunogenic peptides, Nat. Immunol. 8 (2007) 101–108.
[5] I.A. York, M.A. Brehm, S. Zendzian, C.F. Towne, K.L. Rock, Endoplasmic reticulum aminopeptidase 1 (ERAP1) trims MHC class I-presented peptides in vivo and plays an important role in immunodominance, Proc. Natl. Acad. Sci. USA 103 (2006) 9202–9207.
[6] I.A. York, S.C. Chang, T. Saric, J.A. Keys, J.M. Favreau, A.L. Goldberg, K.L. Rock, The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8–9 residues, Nat. Immunol. 3 (2002) 1177–1184.
[7] T. Saric, S.C. Chang, A. Hattori, I.A. York, S. Markant, K.L. Rock, M. Tsujimoto, A.L. Goldberg, An IFN-γ-induced aminopeptidase in the ER, ERAP1, trims precursors to MHC class I-presented peptides, Nat. Immunol. 3 (2002) 1169–1176.
[8] J. Yan, V.V. Parekh, Y. Mendez-Fernandez, D. Olivares-Villagomez, S. Dragovic, T. Hill, D.C. Roopenian, S. Joyce, L. Van Kaer, In vivo role of ER-associated peptidase activity in tailoring peptides for presentation by MHC class Ia and class Ib molecules, J. Exp. Med. 203 (2006) 647–659.
[9] E. Firat, L. Saveanu, P. Aichele, P. Staeheli, J. Huai, S. Gaedicke, A. Nil, G. Besin, B. Kanzler, P. van Endert, G. Niedermann, The role of endoplasmic reticulum-associated aminopeptidase 1 in immunity to infection and in cross-presentation, J. Immunol. 178 (2007) 2241–2248.
[10] S.C. Chang, F. Momburg, N. Bhutani, A.L. Goldberg, The ER aminopeptidase, ERAP1, trims precursors to lengths of MHC class I peptides by a "molecular ruler" mechanism, Proc. Natl. Acad. Sci. USA 102 (2005) 17107–17112.
[11] A. Hattori, K. Kitatani, H. Matsumoto, S. Miyazawa, T. Rogi, N. Tsuruoka, S. Mizutani, Y. Natori, M. Tsujimoto, Characterization of recombinant human adipocyte-derived leucine aminopeptidase expressed in Chinese hamster ovary cells, J. Biochem. (Tokyo) 128 (2000) 755–762.
[12] I. Evnouchidou, F. Momburg, A. Papakyriakou, A. Chroni, L. Leondiadis, S.C. Chang, A.L. Goldberg, E. Stratikos, The internal sequence of the peptide–substrate determines its N-terminus trimming by ERAP1, PLoS ONE 3 (2008) e3658.
[13] D. Fruci, P. Giacomini, M.R. Nicotra, M. Forloni, R. Fraioli, L. Saveanu, P. van Endert, P.G. Natali, Altered expression of endoplasmic reticulum aminopeptidases ERAP1 and ERAP2 in transformed non-lymphoid human tissues, J. Cell. Physiol. 216 (2008) 742–749.
[14] D. Fruci, S. Ferracuti, M.Z. Limongi, V. Cunsolo, E. Giorda, R. Fraioli, L. Sibilio, O. Carroll, A. Hattori, P.M. van Endert, P. Giacomini, Expression of endoplasmic reticulum aminopeptidases in EBV-B cell lines from healthy donors and in leukemia/lymphoma, carcinoma, and melanoma cell lines, J. Immunol. 176 (2006) 4869–4879.
[15] E. Fung, D.J. Smyth, J.M. Howson, J.D. Cooper, N.M. Walker, H. Stevens, L.S. Wicker, J.A. Todd, Analysis of 17 autoimmune disease-associated variants in type 1 diabetes identifies 6q23/TNFAIP3 as a susceptibility locus, Genes Immun. 10 (2009) 188–191.
[16] P.R. Burton, D.G. Clayton, L.R. Cardon, N. Craddock, P. Deloukas, A. Duncanson, Et al., Association scan of 14, 500 nonsynonymous SNPs in four diseases identifies autoimmunity variants, Nat. Genet. 39 (2007) 1329–1337.
[17] B. Seliger, U. Ritz, S. Ferrone, Molecular mechanisms of HLA class I antigen abnormalities following viral infection and transformation, Intl. J. Cancer 118 (2006) 129–138.

[18] A.M. Mehta, E.S. Jordanova, T. van Wezel, H.W. Uh, W.E. Corver, K.M. Kwappenberg, W. Verduijn, G.G. Kenter, S.H. van der Burg, G.J. Fleuren, Genetic variation of antigen processing machinery components and association with cervical carcinoma, Genes Chromosomes Cancer 46 (2007) 577–586.
[19] A.M. Mehta, E.S. Jordanova, G.G. Kenter, S. Ferrone, G.J. Fleuren, Association of antigen processing machinery and HLA class I defects with clinicopathological outcome in cervical carcinoma, Cancer Immunol. Immunother. 57 (2008) 197–206.
[20] S. Kawabata, T. Miura, T. Morita, H. Kato, K. Fujikawa, S. Iwanaga, K. Takada, T. Kimura, S. Sakakibara, Highly sensitive peptide-4-methylcoumaryl-7-amide substrates for blood-clotting proteases and trypsin, Eur. J. Biochem. 172 (1988) 17–25.
[21] R. Lottenberg, U. Christensen, C.M. Jackson, P.L. Coleman, Assay of coagulation proteases using peptide chromogenic and fluorogenic substrates, Methods Enzymol. 80 (1981) 341–361.
[22] A.F. Kisselev, A.L. Goldberg, Monitoring activity and inhibition of 26S proteasomes with fluorogenic peptide substrates, Methods Enzymol. 398 (2005) 364–378.
[23] A. Hattori, H. Matsumoto, S. Mizutani, M. Tsujimoto, Molecular cloning of adipocyte-derived leucine aminopeptidase highly related to placental leucine aminopeptidase/oxytocinase, J. Biochem. (Tokyo) 125 (1999) 931–938.
[24] Y. Kanaoka, T. Takahashi, H. Nakayama, A new fluorogenic substrate for aminopeptidase, Chem. Pharm. Bull. (Tokyo) 25 (1977) 362–363.
[25] Y. Goto, A. Hattori, Y. Ishii, M. Tsujimoto, Reduced activity of the hypertension-associated Lys528Arg mutant of human adipocyte-derived leucine aminopeptidase (A-LAP)/ER-aminopeptidase-1, FEBS Lett. 580 (2006) 1833–1838.
[26] T. Tanioka, A. Hattori, S. Masuda, Y. Nomura, H. Nakayama, S. Mizutani, M. Tsujimoto, Human leukocyte-derived arginine aminopeptidase: The third member of the oxytocinase subfamily of aminopeptidases, J. Biol. Chem. 278 (2003) 32275–32283.
[27] J.H. Zhang, T.D. Chung, K.R. Oldenburg, A simple statistical parameter for use in evaluation and validation of high throughput screening assays, J. Biomol. Screen. 4 (1999) 67–73.