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The Final N-Terminal Trimming of a Subaminoterminal Proline-Containing HLA Class I-Restricted Antigenic Peptide in the Cytosol Is Mediated by Two Peptidases

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The proteasome produces MHC class I-restricted antigenic peptides carrying N-terminal extensions, which are trimmed by other peptidases in the cytosol or within the endoplasmic reticulum. In this study, we show that the N-terminal editing of an antigenic peptide with a predicted low TAP affinity can occur in the cytosol. Using proteomics, we identified two cytosolic peptidases, tripeptidyl peptidase II and puromycin-sensitive aminopeptidase, that trimmed the N-terminal extensions of the precursors produced by the proteasome, and led to a transient enrichment of the final antigenic peptide. These peptidases acted either sequentially or redundantly, depending on the extension remaining at the N terminus of the peptides released from the proteasome. Inhibition of these peptidases abolished the CTL-mediated recognition of Ag-expressing cells. Although we observed some proteolytic activity in fractions enriched in endoplasmic reticulum, it could not compensate for the loss of tripeptidyl peptidase II/puromycin-sensitive aminopeptidase activities.

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5 Abbreviations used in this paper: ER, endoplasmic reticulum; AMC, 7-amido-4-methylcoumarin; CMK, chloromethylketone; 2-D, two-dimensional; EBNA, EBV-encoded nuclear Ag; EGFP, enhanced GFP; GFP, green fluorescent protein; HA, hemagglutinin; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MS, mass spectrometry; PSA, puromycin-sensitive aminopeptidase; RCC, renal cell carcinoma; RT, room temperature; TFA, trifluoroacetic acid; TPP II, tripeptidyl peptidase II; Ub, ubiquitin; VSV, vesicular stomatitis virus.

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N-terminal extended precursors and that the final N terminus is generated by peptidases localized within the ER (7, 21).

Contrary to this prediction, our results indicate that the antigenic peptide RU134−42, which contains P at second position, can be produced in the cytosol, before TAP-mediated transport. By using a substrate-based assay, we identified two cytosolic peptidases that trim the N terminus of the RU134−42 precursors produced by the proteasome. These two peptidases, tripeptidyl peptidase II (TPII) and puromycin-sensitive aminopeptidase (PSA), act on the N-terminally extended precursors to produce, and transiently enrich for, the exact N terminus of the antigenic peptide. We also searched for proteolytic activities in membranes enriched in ER. Although we observed a detectable proteolytic activity against one of the proteasomal products, RU131−42, it could apparently not rescue the loss of CTL recognition of tumor cells resulting from the inhibition of proteasome and TPII/PSA activities. Our data suggest that the production of RU134−42/HLA-B51 is a cytosolic process, involving the proteasome to generate the exact C terminus of the antigenic peptide and TPII and PSA to trim the N-terminal extensions produced by the proteasome.

Materials and Methods
Protein purification
For the identification of TPII, BB64-renal cell carcinoma (RCC) (6.3 × 10⁷ cells) were mechanically disrupted by douncing in a Dounce homogenizer. Sucrose was immediately added to the homogenate to a final concentration of 250 mM. Debris was immediately removed by centrifugation at 13,000 × g for 15 min at 4°C. The supernatant was transferred to a stainless steel tubes and subjected to ultracentrifugation at 80,000 × g for 45 min at 4°C. Proteasomes were removed from the clear supernatant (complete cytosol) by affinity purification, using the mAb anti-proteasome MCP21 (22). Proteasome-depleted cytosol was loaded onto a high performance ion-exchange Source 15Q PE 4.6/100 Sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ) at a flow rate of 1 ml/min in buffer A (20 mM Tris-HCl, pH 7.6), washed with five-column volumes of buffer A, and pre-eluted with 50% buffer B (20 mM Tris-HCl, pH 7.6, 1 M NaCl). The adsorbed material was then eluted, using a linear gradient of 30–70% buffer B, in 35 fractions of 1 ml each. An aliquot of each fraction (50 μl) was incubated with 4 nmol peptide RU131−42 (TGSTAVPYGSKFH YDTRLQ, in one-letter code, in which the underlined sequence corresponds to the final C-terminal defined antigenic peptide) for 1 h at 37°C. The reaction was stopped by adding trifluoroacetic acid (TFA) to a final concentration of 1%. The samples were then analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS), as previously described (22). The positive fractions (fractions 10–19) were mechanically disrupted by douncing in a Dounce homogenizer, before lyophilization, and 20 μl streptavidin-coated agarose beads (Pierce, Rockford, IL). The plasmid PSA-vesicular stomatitis virus (VSV), directing the synthesis of PSA carrying a C-terminal Ab epitope from the vesicular stomatitis virus (a generous gift of A. Fontana, University Hospital, Zürich, Switzerland), was transiently transfected into 4 × 10⁷ HEK293 cells using Fugene (Roche, Basel, Switzerland) and the manufacturer-supplied protocol. Two to four hours posttransfection, cells were lysed in 1% Triton X-100, and PSA-VSV was immunoprecipitated using 5 μg anti-VSV tag mAb (Fluka, Buchs, Switzerland) and 20 μl protein G-Sepharose slurry (Pierce).

The limited number of cells used in this assay, the purity of the isolated peptides could not be ascertained. However, the specificities of both Abs have been described by others (23, 24), and the proteolytic activities of the TPII and PSA preparations could be completely blocked by butabindide and puromycin, respectively (data not shown). The immunoprecipitated material was washed four times and was used to digest 4 nmol peptides of interest. At the end of the digestion, the supernatant was collected and the digestion products were analyzed by MS.

Preparative 2-D gel electrophoresis
The protein pellets obtained from the above-mentioned fractions 10–14 were resuspended in 600 μl loading buffer (40 mM Tris-HCl, pH 8.0, 8 M urea, 4% CHAPS (3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonate), 65 mM dithioerythritol, 0.01% bromphenol blue), loaded onto 18-cm-long, 1-mm-thick, pH 3–10 gradient strips (Amersham Pharmacia Biotech, Piscataway, NJ), and separated overnight by electrophoresis. During the initial 3 h, the voltage was linearly increased from 300 to 3500 V, followed by 3 h at 3500 V, to reach the final voltage of 5000 V. After separation in the first dimension, the strips were equilibrated in 50 mM Tris-HCl, pH 8.4, 6 M urea, 30% glycerol, 2% SDS, and 2% dithioerythritol for 12 min. Thiol groups were subsequently blocked with 2.5% iodoacetamide. Separation in the second dimension was conducted using a vertical gradient slab gel with a modified Laemmlsi-SDS discontinuous system (10% acrylamide-piperazine diacryyl gel) and run at 200 V for ~5 h. Gels were silver stained according to a protocol compatible with MS (25).

Destaining, in-gel protein digestion, extraction, and purification
Each visible spot of the 2-D gel was cut and lyophilized in a sterile Eppendorf tube. The silver stain was removed by covering the gel piece with 30 mM K-ferricyanide and 100 mM Na-thiosulfate (1:1, v/v), shaking for some minutes, and observing the destaining (26). Each gel piece was then washed with water three times, covered with 0.2 M ammonium bicarbonate, and incubated for at least 20 min at room temperature (RT). The ammonium bicarbonate was then removed, replaced with 100 mM acetonitrile, and washed three times. Each gel piece was dried, and the digestion was started by adding trypsin (Promega; 0.5 μg/gel piece) in 0.2 M ammonium bicarbonate and kept on ice for 15–20 min. More buffer was added in small steps to allow a slow uptake of the protease. The digestion was conducted overnight at 37°C. The reaction was stopped by adding TFA to a final concentration of 1%, and the sample was sonicated for 10 min. The supernatant was saved, 0.1% TFA/60% acetonitrile was added to cover each gel piece, and the tube was incubated for at least 30 min at 37°C. This extract was combined with the previous supernatant, and extraction was repeated. A final extraction was performed, using 100% acetonitrile. All extracts were then combined, and the volume was reduced by vacuum centrifugation to about one-third of the original volume. The samples were desalted by passing them though Zip-Tips (C18, Millipore, Bedford, MA). The samples were lyophilized and resuspended in 3 μl 0.1% TFA/H₂O. One microliter of the sample was mixed (1:1, v/v) with matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA) and spotted on the target of the mass spectrometer.

Treatment of the bands excised from the Coomassie-stained gel was identical, except that the K-ferricyanide/Na-thiosulfate treatment was omitted.

Synthetic peptide digestion assays
A total of 4 nmol peptide was used in digestion assays with cytosol, its derived fractions, and purified peptidases. The digestions were allowed to proceed for the time indicated in the figures, and the reactions were stopped by adding 2% TFA. After lyophilization, the samples were analyzed by MS, as previously described (22). In digestion performed in the presence of peptidase inhibitors, the inhibitors were added to the cytosol and incubated for 15 min at RT prior to the addition of peptide. The concentration of butabindide (a kind gift of J.-C. Schwartz, Institut National de la Santé et
MALDI-TOF mass-spectroscopic analysis and database search

For protein identification, all analyses were performed using a Perseptive Biosystems Voyager-DESTR MALDI-TOF Voyager-DE/RF or a Voyager-DE-STR mass spectrometer (Framingham, MA) operated in the delayed extraction and reflector mode. The search program ProFound, developed by The Rockefeller University Mass Spectrometry Laboratory and New York University (New York, NY), was used for database searches (27). Peptides were selected in the mass range of 800–4000 Da. Spectra were calibrated using a matrix and trypic autoligation ion peaks as internal standards.

For regular peptide digestion assays, the settings of the instrument were as reported (22).

Cell fractionation

BB64-RCC (2 × 10⁶ cells) were mechanically disrupted in hypotonic buffer, and the membranes were immediately equilibrated with 250 mM sucrose. Cell debris were pelleted at 13,000 × g for 10 min. The supernatant was subjected to high speed centrifugation (80,000 × g) for 45 min at 4°C. Incomplete cytosol (6 ml) was obtained by the removal of the protease inhibitors with 1 ml. Twenty microliters of incomplete cytosol, 4 ml of the volume of the detergent-rich phase was readjusted to the original volume by centrifugation at 12,000 × g for 10 min and the phase separation was repeated. After two washing cycles, the detergent-poor fraction and containing hydrophilic luminal proteins, was transferred into a new tube, and the remaining detergent-rich lower phase (fraction B), containing membrane-anchored proteins, was washed by adding flat-bottom microwells and transfected with 1.5 μl Lipofectamine, 20 ng plasmid pcDNA3 containing the HLA-B*5101 cDNA, and 12.5 ng of either pcDNA3.1TOPO plasmid (Invitrogen) containing the RR1 full-length cDNA, pEGFP/Ub plasmid containing the cDNA-encoding RR141–47, or pEGFP/Ub-encoding RR141–47. A total of 100 ng pBjIneo construct encoding the herpes simplex-derived TAP inhibitor ICP47 molecule (a kind gift of H. G. Rammmensee, Tübingen, Germany) was added to this mix in one-half of the wells. After 2 h, the transfected cells were incubated with 10² anti-RR141–47/HLA-B51 CTL 381/84, along with 25 U/ml IL-2. The amount of TNF-α secreted in the supernatant was assessed 24 h later by ELISA (Endogen, Woburn, MA).

For metabolic labeling, 2 × 10⁶ HEK293 cells were transfected with 4 μg plasmid using the Fugene reagent (Roche) and following the manufacturer’s protocol. Sixteen hours posttransfection, the cells were washed for 45 min in Met/Cys-free DMEM medium (ICN Biomedicals, Aurora, OH) at 37°C. The cells were metabolically labeled for 20 min at 37°C in fresh Met/Cys-free medium containing 150 μCi [³⁵S]Met/Cys (Pro-mix; Amersham Pharmacia Biotech). Cells were washed once and lysed in 1 ml lysis buffer containing 1% Triton X-100 and 30 mM iodoacetamide to prevent postlysis deubiquitylation. Unsolubilized material was removed by centrifugation, and the supernatant was incubated with saturating amounts of the mAb anti-hemagglutinin (HA) epitope (Berkeley Antibody, Richmond, CA) and 20 μl protein G-Sepharose. The immunoprecipitates were washed and treated, as described earlier (22), subjected to SDS-12% PAGE, followed by autoradiography.

Treatment of target cells with inhibitors of Ag processing

BB64-RCC cells were acid treated as follows to discard peptides from the surface HLA molecules. One million cells were incubated for exactly 30 s at 37°C in 500 μl 300 mM glycine buffer (pH 2.5), supplemented with 1% sterile BSA, and then washed several times with culture medium, Eight thousand cells seeded in microplates were incubated for 14 h with 50 μM lactacystin (Calbiochem, La Jolla, CA) or 100 μM AAF-CMK in culture medium. After 30 min, the cells were pulsed with the RR1 antigenic peptide VPYGSFKHV at a final concentration of 10 μM, washed, and incubated with 3000 CTL 381/84 and 25 U/ml IL-2. The ability of the treated cells to stimulate the CTL was assessed by measuring the production of TNF by the CTL after 20 h of incubation. The TNF (α and β) content of the supernatants was evaluated by testing their cytotoxic effect on WEHI-164 clone 13 cells (29).

Results

TPP II mediates the initial trimming of RR141–47 precursors, but fails to produce the final N terminus of the antigenic peptide

Earlier work, using the precursor peptide RR141–47, demonstrated that the exact C terminus of the antigenic peptide was directly produced by purified standard proteasome, but that the N terminus always carried additional 3 (RR138–41) and 5 (RR132–42) aa (16) (data not shown). This suggested that other peptidases might be necessary to trim the N-terminal extensions to the final nonamer RR141–47. We therefore sought to identify the cytosolic peptidases involved in the N-terminal editing of the RR141–42 epitope precursors. Because we were not able to determine the relative abundance of each of the two fragments produced by the proteasome due to their coelution from the HPLC columns (data not shown), we investigated the editing of both species.

We first focused our attention on the precursor RR141–47, which carries an N-terminal extension of 5 aa, and whose C-terminal processing was studied in our previous work (Fig. 1) (16). To identify the peptidase(s) involved in the N-terminal trimming of
this precursor, we adopted the following strategy: proteasome-depleted cytosol isolated from the renal carcinoma cell line BB64-RCC was fractionated by ion-exchange chromatography. The presence of a proteolytic activity was assayed by incubating an aliquot of each fraction with the peptide RU1 29–47 and by subsequent analysis by MS. The 5-aa extension at the C terminus of the antigenic peptide sequence was included in the precursor so as to monitor for the possibility that a peptidase other than the proteasome may directly generate the CTL-defined epitope. No fraction was found to generate the exact N terminus of the antigenic peptide. Rather, fractions 10–14, eluting at ~420–460 mM NaCl, contained an activity that resulted in the trimming of the first 3 N-terminal aa (Fig. 2, A and B). No proteolytic activity on the C-terminal extension of the antigenic peptide was detected in any fraction (data not shown). Fractions 10–14 were pooled and separated by 2-D gel electrophoresis. After silver staining of the gel, visible spots (218) were manually excised, and 77 of those were subjected to in-gel trypsin digestion. The tryptic peptide fragments were extracted from the gel and analyzed by MS. The m.w. values of the extracted peptides were introduced into the program ProFound (http://129.85.19.192/profound_bin/WebProFound.exe) and used for peptide mass fingerprinting. Based on the pattern of its tryptic fragments and its migrating properties in the 2-D gel, one spot was unambiguously identified as TPP II. No other peptidases were identified among the analyzed spots. TPP II is a very large homomultimeric peptidase (molecular mass 5000–9000 kDa), with subunit molecular mass 138 kDa, that removes tripeptides from the N terminus of peptides and also displays endoproteolytic activities (30, 31). Based on indirect evidence, TPP II has been suggested to participate in the MHC class I Ag-processing pathway, as it may partially compensate for the generation of MHC class I ligands, in situations in which proteasomes are pharmacologically inactivated (32). To confirm that the proteolytic activity producing the fragment RU1 32–47 could be ascribed to TPP II, we purified TPP II by immunoadsorption and assayed its activities on the precursor RU1 29–47 (Fig. 2C). After numerous unsuccessful attempts to purify active rTPP II from prokaryotic as well as eukaryotic expression systems, we developed a new purification scheme that yielded active TPP II from 5 × 10^7 cells. As demonstrated by the MS analysis, the peak profile of the digested peptide, using immunoadsorbed TPP II, was identical with the one detected after incubation of the same precursor with cytosolic fraction 12 (Fig. 2, compare B and C). Because the final N terminus of the antigenic peptide was not detected, two possibilities were envisaged: either the generation of the final N terminus was sequential and required another peptidase for the removal of the last 2 aa, or the N-terminal processing occurred entirely within membranes, which were absent from this preparation.

Puromycin-sensitive aminopeptidase generates the exact N terminus of RU1 34–42

To address the first hypothesis, we investigated the possibility that a second cytosolic peptidase might be responsible for the final trimming of the N-terminal extension. To exclude the possibility of contamination from peptidases located in subcellular compartments, proteasome-depleted cytosol isolated from erythrocytes (these cells do not contain internal membranes (33)) was subjected to fractionation. The proteolytic activities of the fractions obtained after separation on Q-Sepharose were tested using the fluorogenic peptide AAF-AMC (Fig. 3A). Two major peaks of activity were detected in fractions 29–33 and 38–45. The activity of the first peak was able to digest the N terminus of RU1 32–47, resulting in the enrichment of a fragment corresponding to RU1 34–47, which displays the exact N terminus of the antigenic peptide (Fig. 3A, inset). In light of this result, fraction 30, the proteolytically most active fraction, was precipitated by TCA, separated on SDS-PAGE, and stained by Coomassie blue. Each of the 12 visible protein bands was excised from the gel, digested by trypsin, and further processed as above. The tryptic peptides obtained from a protein band with apparent molecular mass 100 kDa led to the identification of the peptidase puromycin-sensitive aminopeptidase (PSA). This peptidase, originally found in brain tissues (see Ref. 34 and references therein), has recently been identified as playing

![Figure 1](image1.png)

**FIGURE 1.** Sequence of the RU1 peptides used in this study. The numbers above the sequence refer to the relevant positions within the full-length protein. The antigenic peptide is underlined, and the filled box corresponds to the constant sequence 34–47 shared by the different peptides. The open box corresponds to the sequence 34–47. TGSTA and STA are the two N-terminal extensions produced by the proteasome.

![Figure 2](image2.png)

**FIGURE 2.** Isolation and characterization of TPP II. A, Proteasome-depleted cytosol from the renal carcinoma cell line BB64-RCC was separated by ion-exchange chromatography and eluted, in 1-ml fractions, using a linear gradient from 300 to 650 mM NaCl (dotted line). Protein content was monitored for the possibility that a peptidase other than the proteasome may directly generate the CTL-defined epitope. No fraction was found to generate the exact N terminus of the antigenic peptide. Rather, fractions 10–14, eluting at ~420–460 mM NaCl, contained an activity that resulted in the trimming of the first 3 N-terminal aa (Fig. 2, A and B). No proteolytic activity on the C-terminal extension of the antigenic peptide was detected in any fraction (data not shown). Fractions 10–14 were pooled and separated by 2-D gel electrophoresis. After silver staining of the gel, visible spots (218) were manually excised, and 77 of those were subjected to in-gel trypsin digestion. The tryptic peptide fragments were extracted from the gel and analyzed by MS. The m.w. values of the extracted peptides were introduced into the program ProFound (http://129.85.19.192/profound_bin/WebProFound.exe) and used for peptide mass fingerprinting. Based on the pattern of its tryptic fragments and its migrating properties in the 2-D gel, one spot was unambiguously identified as TPP II. No other peptidases were identified among the analyzed spots. TPP II is a very large homomultimeric peptidase (molecular mass 5000–9000 kDa), with subunit molecular mass 138 kDa, that removes tripeptides from the N terminus of peptides and also displays endoproteolytic activities (30, 31). Based on indirect evidence, TPP II has been suggested to participate in the MHC class I Ag-processing pathway, as it may partially compensate for the generation of MHC class I ligands, in situations in which proteasomes are pharmacologically inactivated (32). To confirm that the proteolytic activity producing the fragment RU1 32–47 could be ascribed to TPP II, we purified TPP II by immunoadsorption and assayed its activities on the precursor RU1 29–47 (Fig. 2C). After numerous unsuccessful attempts to purify active rTPP II from prokaryotic as well as eukaryotic expression systems, we developed a new purification scheme that yielded active TPP II from 5 × 10^7 cells. As demonstrated by the MS analysis, the peak profile of the digested peptide, using immunoadsorbed TPP II, was identical with the one detected after incubation of the same precursor with cytosolic fraction 12 (Fig. 2, compare B and C). Because the final N terminus of the antigenic peptide was not detected, two possibilities were envisaged: either the generation of the final N terminus was sequential and required another peptidase for the removal of the last 2 aa, or the N-terminal processing occurred entirely within membranes, which were absent from this preparation.

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a role in the N-terminal trimming of another antigenic peptide precursor (13). To ascertain the role of PSA in the final N-terminal trimming of RU134–42, we transfected and immunoadsorbed VSV-tagged PSA in the human embryonic kidney cell line HEK293. Using the same technique developed for the purification of active TPP II, immunoadsorbed and proteolytically active PSA was used to digest the precursor peptide RU132–47. Analysis of the digested material by MS revealed that, after 2-h incubation at 37°C, the peak corresponding to the original peptide RU132–47 had completely disappeared and a single peak corresponding to peptide RU134–47 could be detected (Fig. 3B). This fragment corresponds to a species displaying the exact N terminus of the antigenic peptide (Fig. 1). In contrast, the precursor peptide RU130–47 was barely degraded by purified PSA during the same time frame (Fig. 3B).

The fractions obtained after separation on Q-Sepharose were also incubated with RU130–47, and the proteolytic activity detected in fractions 38–45 was compatible with the one of TPP II. However, we did not manage to unambiguously identify the peptidase responsible for this activity. We concluded from these in vitro experiments that PSA trims the N-terminal extension of RU132–47 to yield the exact N terminus of the antigenic peptide. Although PSA is a peptidase with broad specificity (34), it is noteworthy that no fragment shorter than RU134–47 could be detected, indicating that PSA cannot completely degrade the peptide precursor, thereby leading to an enrichment of a peptide with the exact N terminus.

**TTP II and PSA can both edit the N terminus of peptide RU131–42 to its final size**

Because neither TPP II nor PSA alone could efficiently trim RU130–47 to the exact N terminus of the CTL-defined epitope, we tested whether this was also the case for the second N-terminally extended precursor produced by the proteasome, RU131–42 (Fig. 1). Contrary to the first RU1 precursor, we used in this study a precursor that already displayed the exact C terminus of the antigenic peptide. Using the same assay as described above, we incubated the precursor peptide either with immunoadsorbed TPP II (Fig. 4A) or PSA (Fig. 4B). Analysis of the digested products by MS revealed that both peptidases were capable of trimming the N-terminal extension of the precursor peptide to a fragment corresponding to the exact antigenic peptide. The dichotomy between the processing of RU132–47 and RU131–42 led us to conclude that the sequential trimming of the first one by TPP II and PSA may be caused by the presence of particular amino acids that resist cleavage by PSA (see Discussion).

**The N-terminal trimming of RU134–42 precursors is sensitive to specific TPP II and PSA inhibitors**

To determine the contribution of the two identified cytosolic peptidases to the N-terminal trimming of RU134–42 precursors in unfractonated cytosol, we performed a series of digestions of the

FIGURE 3. Isolation and characterization of PSA. A, The proteolytic activity of the fractions obtained after ion-exchange chromatography was tested using 100 μM AAF-AMC and monitoring the increased fluorescence, in arbitrary units (A.U.), emitted by the released fluorogenic group AMC (excitation/emission of 380/440 nm). Fluorescence released after incubation of AAF-AMC with unfractonated cytosol (T) or in buffer (−) is indicated on the right. Inset, Depicts the proteolytic activity of fraction 31, using RU132–47 as precursor. This activity leads to the production of peptide RU134–47. The filled box is as before. B, Peptides RU132–47 and RU139–47 were incubated with purified PSA-VSV for 0 or 2 h and subsequently analyzed by MS. After 2 h, RU132–47 was completely converted into a fragment lacking the 2 N-terminal aa (middle panel). This fragment matches the one shown in the inset of A and corresponds to a fragment displaying the exact N terminus of the antigenic peptide. In contrast, RU139–47 was barely degraded (right panel). The peak labeled −1 corresponds to a fragment lacking 1 N-terminal aa. See Materials and Methods for details.

FIGURE 4. Both TPP II and PSA can trim RU131–42. A, Purified human TPP II was incubated with peptide RU131–43 for 2 h at 37°C, and the digested material was analyzed by MS. The open box corresponds to the sequence VPYGSFKHV, displaying the final N and C terminus of the antigenic peptide. B, Same as A, except that the precursor peptide was digested with purified PSA-VSV.
Various peptide precursors in the presence or absence of specific TTP II and PSA inhibitors (Fig. 5). The peptides RU129–47, RU131–42, and RU132–47 (Fig. 1) were incubated with proteasome-depleted cytosol for 10 min at 37°C (Fig. 5, A–C). The reaction was stopped by the addition of 2% TFA and analyzed by MS. The same digestions were also performed in the presence of butabindide, a specific TTP II inhibitor (Fig. 5, D–F) (35); AAF-CMK, an inhibitor of TTP II, PSA, and bleomycin hydrolase (G–I) (13, 30); and puromycin, a specific PSA inhibitor (J–L) (34). In the case of RU129–47, a fragment displaying the final N terminus of the antigenic peptide was readily detectable after incubation with untreated cytosol (Fig. 5A). No such fragment could be detected when the peptide was incubated with cytosol pretreated with butabindide or AAF-CMK (Fig. 5, D and G, respectively). Interestingly, not only was the fragment with the exact N terminus absent, but the intermediate corresponding to RU132–47 was not detectable either. The result obtained after treatment with butabindide confirms and extends the findings shown in Figs. 2 and 3, namely that the processing of the N-terminal extension of RU129–47 in unfractionated cytosol is a sequential process that requires the activity of TTP II. Finally, puromycin-treated cytosol led to an accumulation of the intermediate RU132–47 by blocking the second proteolytic event that normally leads to the generation of the final N terminus (Fig. 5J).

Peptide RU132–47, which corresponds to the fragment produced after the digestion of the longer precursor by TTP II, was also incubated with cytosol. As expected, the fragment displaying the exact N terminus of the antigenic peptide was efficiently produced in untreated cytosol (Fig. 5C) and was insensitive to butabindide and only partially sensitive to AAF-CMK (F and I, respectively). However, no digestion was observed when the cytosol was pretreated with puromycin (Fig. 5L), confirming the essential role of PSA in the final trimming of this intermediate. Finally, we analyzed the trimming of the precursor RU131–42. As already predicted from the result shown in Fig. 4, a fragment corresponding to the final size of the antigenic peptide was produced both in untreated cytosol and in cytosol treated with inhibitors (Fig. 5, B, E, H, and K). We conclude that this precursor can be edited simultaneously either by TTP II or PSA, but the contribution of yet another peptidase on the editing of this particular peptide cannot be ruled out, as peaks corresponding to fragments lacking 1 and 2 N-terminal aa could be detected in all conditions.

The N-terminal processing of RU134–42 precursors occurs predominantly in the cytosol

Several reports have suggested that the N-terminal trimming of antigenic peptides may occur within the ER. Although we presented evidence that the processing of RU134–42 precursors could occur in the cytosol (Fig. 5), we nevertheless tested whether the trimming could also take place within ER membranes. We purified membranes from the BB64-RCC cell line, separated integral membrane proteins from soluble luminal proteins by Triton X-114 extraction (see Materials and Methods for details), and tested the proteolytic
activity of each of these fractions using the three peptide precursors. As before, we could clearly detect proteolytic cleavage of all precursors in the cytosolic fraction, resulting in the formation of a fragment displaying the exact N terminus of the antigenic peptide (Fig. 6, A–C). Very little proteolytic activity was detected in the fraction A, containing soluble luminal proteins (Fig. 6, D–F), and no activity was detected in the fraction B, containing membrane-embedded proteins (Fig. 6, G–I). Proteolytic activity present in the three fractions was also independently monitored by the release of AMC from the fluorogenic tripeptide AAF-AMC (Fig. 6J). The cytosolic extract was proteolytically active against AAF-AMC, and these activities could be partially blocked either by butabindide or puromycin, indicating that: 1) TPP II and PSA are present in this fraction, and 2) other peptidases are also active against this fluorogenic peptide. The presence of the two peptidases was further confirmed by Western blot analysis (Fig. 6K). Some proteolytic activity could also be detected in fraction A. However, this activity was totally resistant to puromycin (confirming the absence of PSA from this fraction), but was completely blocked by butabindide, indicating the presence of contaminating TPP II in this fraction. This was independently confirmed by Western blot analysis using an anti-TPP II Ab (Fig. 6K). The presence of TPP II could be responsible for the small peak of RU134-42 by HLA-B51, we treated BB64-RCC with specific inhibitors and tested their recognition by CTL. Cells treated with the proteasome inhibitor lactacystin were poorly recognized by specific CTL, as indicated by the low level of TNF produced by the CTL clone (Fig. 7). Recognition was partially restored when the antigenic peptide was added exogenously. In comparison, untreated cells were efficiently recognized by the same CTL clone, confirming that the proteasome played an essential role in the presentation of this CTL-defined epitope. We also tested whether the recognition of RU134-42/HLA-B51 was influenced by the presence of the inhibitor AAF-CMK. As with lactacystin, cells treated with AAF-CMK were poorly recognized by specific CTL. Again, exogenously added peptide led to a partial restoration of the recognition of BB64-RCC cells. The lack of recognition resulting from the AAF-CMK treatment could not be ascribed to an inhibition of proteasome because, as tested by us and reported by others, proteasomal activities were not influenced by AAF-CMK (13, 30) (data not shown). In our hands, 100 μM lactacystin did not inhibit

The presentation of RU134-42/HLA-B51 is blocked by lactacystin and AAF-CMK

In an attempt to correlating our results obtained in an acellular system with the cellular pathway leading to the presentation of RU134-42 by HLA-B51, we treated BB64-RCC with specific inhibitors and tested their recognition by CTL. Cells treated with the proteasome inhibitor lactacystin were poorly recognized by specific CTL, as indicated by the low level of TNF produced by the CTL clone (Fig. 7). Recognition was partially restored when the antigenic peptide was added exogenously. In comparison, untreated cells were efficiently recognized by the same CTL clone, confirming that the proteasome played an essential role in the presentation of this CTL-defined epitope. We also tested whether the recognition of RU134-42/HLA-B51 was influenced by the presence of the inhibitor AAF-CMK. As with lactacystin, cells treated with AAF-CMK were poorly recognized by specific CTL. Again, exogenously added peptide led to a partial restoration of the recognition of BB64-RCC cells. The lack of recognition resulting from the AAF-CMK treatment could not be ascribed to an inhibition of proteasome because, as tested by us and reported by others, proteasomal activities were not influenced by AAF-CMK (13, 30) (data not shown). In our hands, 100 μM lactacystin did not inhibit
the activity of TPP II (data not shown). Taken together, these results indicate that, in cells, the proteasome most likely does not directly produce the final antigenic peptide RU1_{34–42}, but that additional peptidases, like TPP II and/or PSA, are necessary to generate the antigenic peptide. We were unable to test the effect of butabindine on the presentation of RU1_{34–42} by this drug does not cross the cell membrane (data not shown). Puromycin could not be tested either, due to its excessive cell toxicity (data not shown).

Cells transfected with a minigene encoding the minimal antigenic peptide RU1_{34–42}/HLA-B51 are efficiently recognized by specific CTL

The antigenic peptide RU1_{34–42} contains a subaminoterminal P (Fig. 1) and is thus predicted to be poorly transported by TAP in its final form. However, we have shown that the production of the final N terminus can occur in the cytosol. To test whether the N-terminally trimmed antigenic peptide can be transported into the ER, the human embryonic kidney cells HEK293 were transiently transfected with the cDNA-encoding HLA-B51 and either the RU1 cDNA or two minigenes coding for, respectively, peptide RU1_{34–42}, displaying the exact N terminus, but carrying a C-terminal extension, and RU1_{34–42}, the minimal HLA-B51-restricted epitope (Fig. 1). The vectors used for the expression of the minigenes were designed based on the ubiquitin/protein/reference technique described previously (39). As expected, these cells were not recognized by specific CTL, and not due to the transfection of the cells with plasmid constructs containing the RU1 full-length cDNA or minigenes coding for the CTL-defined epitope RU1_{34–42} with a C-terminal extension (pEGFP/Ub-RU1_{34–42}) or the minimal epitope RU1_{34–42} (pEGFP/Ub-RU1_{34–42}). The peptides encoded by the latter two constructs are produced as a result of the proteolytic cleavage after the last residue of Ub, a system that allows to bypass the need for an N-terminal methionine. Some of the cells were also transfected with a plasmid encoding the herpes simplex protein ICP47, which blocks the TAP-mediated transport of antigenic peptides. The presentation of RU1_{34–42} by HLA-B51 in absence (open bars) or presence (filled bars) of ICP47 was monitored by measuring the amount of TNF-α released by CTL 381/84 incubated for 24 h with the transfected cells. HEK293 cells expressing EGFP-Ub-RU1_{34–42} and EGFP-Ub_{G75,76-RU1_{34–42}}, respectively, were metabolically labeled with 35S and lysed, and the lysate was immunoprecipitated, using an Ab against a peptide sequence located between the C terminus of EGFP and the N terminus of Ub. The precipitated material was separated by SDS-12% PAGE and subjected to autoradiography. As control, EGFP-Ub was immunoprecipitated from radiolabeled cells expressing the 118-aa-long protein Melan-A and the star indicates unidentified species. See text for details.

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Lactacystin and AAF-CMK block the recognition of tumor cells by specific CTL. BB64-RCC cells were treated with the proteasome inhibitor lactacystin and the protease inhibitor AAF-CMK or left untreated. CTL clone 381/84 was added to the cells, and TNF release was measured (open bars). As control, saturating amounts of the antigenic peptide RU1_{34–42} were added exogenously (filled bars).

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** Peptide RU1_{34–42} is transported by the TAP complex. A, HEK293-EBNA cells were transfected with a plasmid coding for HLA-B51 and constructs containing the RU1 full-length cDNA or minigenes coding for the CTL-defined epitope RU1_{34–42} with a C-terminal extension (pEGFP/Ub-RU1_{34–42}) or the minimal epitope RU1_{34–42} (pEGFP/Ub-RU1_{34–42}). The peptides encoded by the latter two constructs are produced as a result of the proteolytic cleavage after the last residue of Ub, a system that allows to bypass the need for an N-terminal methionine. Some of the cells were also transfected with a plasmid encoding the herpes simplex protein ICP47, which blocks the TAP-mediated transport of antigenic peptides. The presentation of RU1_{34–42} by HLA-B51 in absence (open bars) or presence (filled bars) of ICP47 was monitored by measuring the amount of TNF-α released by CTL 381/84 incubated for 24 h with the transfected cells. B, HEK293 cells expressing EGFP-Ub-RU1_{34–42} and EGFP-Ub_{G75,76-RU1_{34–42}}, respectively, were metabolically labeled with 35S and lysed, and the lysate was immunoprecipitated, using an Ab against a peptide sequence located between the C terminus of EGFP and the N terminus of Ub. The precipitated material was separated by SDS-12% PAGE and subjected to autoradiography. As control, EGFP-Ub was immunoprecipitated from radiolabeled cells expressing the 118-aa-long protein Melan-A instead of the RU1 peptides. The arrow indicates the position of EGFP-Ub, and the star indicates unidentified species. See text for details.
could lead to the production of N-terminally extended antigenic peptides. Therefore, we compared, by SDS-PAGE, the mobility of two EGFP-Ub minigene constructs. The first one codes for EGFP-Ub–RU134–47, which is cleaved by Ub-specific peptidase to produce EGFP-Ub and RU134–47. The second plasmid encodes EGFP-UbG75–76–RU134–42, which lacks the two C-terminal Gly residues of Ub, thereby producing an uncleavable form of Ub. Indeed, the C-terminal Gly have been shown to be essential for the Ub peptidase-mediated cleavage of Ub (40). Both constructs contained a sequence derived from the influenza A HA between the C terminus of EGFP and the N terminus of Ub, which can be recognized by a specific Ab (22). Cells were transfected with both plasmids, metabolically labeled with [35S]Met/Cys, and lysed in the presence of alkylating agent to prevent postlysis deubiquitylation, and the cleared lysate was immunoprecipitated using the anti-HA mAb linked to protein G-Sepharose. The length of the RU1 minigenes was selected so as to maximize the migration differences after separation on SDS-PAGE. As shown in Fig. 8B, the SDS mobility of EGFP-Ub–RU134–47 carrying the wild-type Ub moiety was indistinguishable from EGFP-Ub derived from a plasmid encoding a melanoma-associated protein fused to EGFP-Ub. In contrast, EGFP-UbG75–76–RU134–42 migrated with a slightly slower mobility. This result indicated that the cleavage at the Ub minigene junction had occurred in the wild-type construct and produced a polypeptide that differs from the mutant construct by 7 aa. A very faint band with slower mobility could be observed for the nonmutated construct, but its precise identity could not be determined because a band of identical mobility was also visible in the construct carrying the mutated Ub and could be sometimes detected in the EGFP-Ub control plasmid. Even though the vast majority of EGFP-Ub–RU134–42 molecules was cleaved, the possibility that a small fraction of EGFP-Ub–RU134–42 may not be cleaved by Ub-specific proteases and produces an amino-terminally extended antigenic peptide precursor cannot be completely ruled out. Nevertheless, the data presented in this work support the notion that the final processing of RU134–42 can take place in the cytosol.

Discussion
In this work, we investigated the N-terminal trimming of the two RU134–42 precursor peptides, RU129–42 and RU131–42, liberated by the proteasome in vitro. We show that two cytosolic peptidases can trim the N-terminal extensions of these peptide precursors. The peptidases, TPP II and PSA, acted sequentially on peptide RU129–42 and redundantly on peptide RU131–42. In all cases, this process led to the transient accumulation of a species displaying the final N terminus of the antigenic peptide. Moreover, we showed that proteasome and TPP II/PSA inhibitors blocked the presentation of RU134–42 by HLA-B51+ tumor cells. Taken together, these data suggest that the production of the epitope RU134–42/HLA-B51 may be a cytosolic process involving at least three distinct peptidases, the proteasome, TPP II, and PSA.

The N-terminal trimming peptidases identified to date share the properties of having broad specificities, of being redundant, and of trimming antigenic peptide precursors without detectable accumulation of a species with the correct N terminus. We were therefore surprised that the generation and the transient accumulation of the final N terminus of RU134–42 from RU129–42 required the sequential action of two distinct peptidases. The two identified peptidases, TPP II and PSA, have already been implicated, in different experimental conditions, in the MHC class I Ag-processing pathway. The first one, TPP II, was identified using cells that had been adapted to a proteasome inhibitor (32). This treatment induced the overexpression of TPP II, and indirect evidences, such as cell surface expression of MHC class I and HPLC profile of peptides eluted from MHC class I molecules, suggested that TPP II may compensate for the lack of proteasome activity (32, 41). However, the exact contribution of TPP II to this process remains to be elucidated, as the activities of the proteasome may not be completely blocked in those cells (42). Another study demonstrated that TPP II did not only remove tripeptides from the N terminus of peptides, but also had some endoproteolytic activities, which could potentially produce antigenic peptides (30). Of note is that in this study we did not observe any other endoproteolytic activity of human TPP II than the one removing tripeptides from free N termini (data not shown). However, in none of the two cases mentioned above was TPP II directly identified in the trimming of a specific antigenic peptide precursor. Contrary to the role of TPP II in the production of antigenic peptides, the contribution of PSA to the processing of a CTL-defined epitope was recently revealed by an experimental approach similar to the one described in this work (13). In that approach, PSA was shown to degrade N-terminally extended precursors of the antigenic peptide VSV nuclear protein52–59 and to generate, among many other fragments, a peptide with the final N terminus. Using the precursor peptide RU129–47, we showed that the production of the final N terminus of the antigenic peptide was an ordered process, which required the sequential activities of the peptidases TPP II and PSA (Figs. 3, 4, and 6). This was also the case in a cytosolic extract, which contained other active peptidases (Figs. 6 and 7J). A molecular explanation for this resides probably in the specificity of the individual peptidases. It appears that neither TPP II nor PSA can cleave a peptide bond before or after Pro (P). In addition, PSA does not cleave efficiently peptide bonds involving a Gly (G) residue (34). Because the sequence of the precursor peptide is TGSTAVP... in which VP corresponds to the first 2 aa of the CTL-defined epitope (Fig. 1), this may explain why TPP II has to remove the first 3 aa (it cannot cleave further because this would involve the residue P) before PSA, which does not cleave the full-length precursor because of the amino acid G, can trim the last 2 aa. PSA will then stop at the exact N terminus of the antigenic peptide because of the residue P. Such a process may be specific for antigenic peptides carrying the residue P at position 2, as is the case for certain ligands of the HLA-B7, HLA-B8, HLA-B15, HLA-B51, and other class I molecules. However, the fact that the same peptidase (PSA) has been identified using two different peptide precursors may not be coincidental, and leads us to postulate that a limited number of peptidases will be responsible for the final editing of MHC class I ligands. At present, we do not know whether this sequence of action is identical in all cell types nor in situations in which the expression of other peptidases is induced, as is the case for leucyl aminopeptidase induced by IFN-γ (15).

The production of the final antigenic peptide from the N-terminally extended precursor RU131–42 could be achieved by TPP II and PSA in a redundant fashion. This result is in agreement with the findings reported on the generation of the N-terminal end of VSV nuclear protein52–59, namely that the final N terminus of this antigenic peptide could be obtained by two redundant processes, mediated either by PSA or, in that case, by bleomycin hydrolase (13). The likely explanation for the different processing of RU131–42 and RU129–47 resides in the length and the sequence of the N-terminal amino acid extension. Therefore, the choice of peptidases(s) responsible for the postproteasomal trimming of antigenic peptide precursors may be dictated by the nature of the N-terminal extension produced by the proteasome. We have analyzed the processing of several antigenic peptide precursors in vitro and have noticed that the proteasome can, in some instances, directly generate the exact antigenic peptide (our unpublished data). However,
this species is frequently accompanied by the presence of peptides that carry additional N-terminal amino acids. It will be interesting to study whether the final antigenic peptide produced within a cell will be more efficiently loaded onto MHC class I molecules than the precursors requiring further N-terminal processing.

N-terminal trimming in the ER has also been described (5). However, because no specific peptide has been identified at the molecular level, it is difficult to speculate on the role of this ER trimming. The fact that most peptides isolated from the HLA-A2 molecules of the TAP-deficient cell line T2 are considerably longer in size than those isolated from normal cells (43) indicates that if aminopeptidases are active in the ER, they may be very specific or play a minor role in the processing of antigenic peptides derived from signal sequences released by the signal peptidase in the ER. In this study, we directly searched for membrane-embedded or luminal peptides that would mediate the final trimming of our precursor (Fig. 7). Although we could detect a proteolytic activity other than the one mediated by TPP II in our membrane preparation, it was only acting on the precursor RU151–42, and was also found in the cytosol. Moreover, this activity was not inhibited by AAF-CMK even though this drug efficiently blocked the CTL recognition of APCs, suggesting that this peptidase is not partic-


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