A Role for a Novel Luminal Endoplasmic Reticulum Aminopeptidase in Final Trimming of 26 S Proteasome-generated Major Histocompatibility Complex Class I Antigenic Peptides*

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Arthur Komlosh‡, Frank Momburg§, Toni Weinschenk¶, Niels Emmerich‡, Hansjörg Schild‡, Eran Nadav‡, Isabella Shaked‡, and Yuval Reiss‡

From the ‡Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel, the §Division of Molecular Immunology, German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany, and the ¶Department of Immunology, Institute for Cell Biology, University of Tübingen, Auf der Morgenstelle 15, D-72076 Tübingen, Germany

Peptides presented to cytotoxic T lymphocytes by the class I major histocompatibility complex are 8–11 residues long. Although proteasomal activity generates the precise C termini of antigenic epitopes, the mechanism(s) involved in generation of the precise N termini is largely unknown. To investigate the mechanism of N-terminal peptide processing, we used a cell-free system in which two recombinant ornithine decarboxylase (ODC) constructs, one expressing the native H2-Kb-restricted ovalbumin (ova)-derived epitope SIINFEKL (ODC-ova) and the other expressing the extended epitope LESIINFEKL (ODC-LEova), were targeted to degradation by 26 S proteasomes following import into microsomes. We found that the cleavage specificity of the 26 S proteasome was influenced by the N-terminal flanking amino acids leading to significantly different yields of the final epitope SIINFEKL. Following incubation in the presence of purified 26 S proteasome, ODC-LEova generated largely ESINFEKL that was efficiently converted to the final epitope SIINFEKL following translocation into microsomes. The conversion of ESINFEKL to SIINFEKL was strictly dependent on the presence of H2-Kb and was completely inhibited by the metalloaminopeptidase inhibitor 1,10-phenanthroline. Importantly, the converting activity was resistant to a stringent salt/EDTA wash of the microsomes and was only apparent when transport of TAP, the transporter associated with antigen processing, was facilitated. These results strongly suggest a crucial role for a luminal endoplasmic reticulum-resident metalloaminopeptidase in the N-terminal trimming of major histocompatibility complex class I-associated peptides.

MHC1 class I molecules associate with peptides of 8–11 amino acids derived from the proteolytic degradation of intracellular protein antigens and present them to CD8+ T cells on the cell surface (1–3). Although the vast majority of class I ligands are translocated from the cytosol into the endoplasmic reticulum (ER) by the ATP-dependent transporter associated with antigen processing (TAP) (3), alternative pathways have been described including the liberation of minigene-encoded peptides from ER signal sequences (4–6), TAP-independent processing of signal sequences (7, 8), and processing of membrane-associated or soluble proteins in the secretary pathway (9–12). Peptides that are not retained in the ER by binding to class I or glycosylation are released back into the cytosol through the Sec61 channel (13–15).

Studies using membrane-permeable inhibitors of proteasomes have indicated that the proteasome is the major proteolytic activity responsible for the generation of antigenic peptides (16–21), although the incomplete inhibition of antigen processing by proteasome inhibitors has also demonstrated the involvement of nonproteasomal cytosolic proteases (22–28).

The proteasome is an abundant cytosolic multisubunit protease consisting of the proteolytic 20 S core particle that associates with PA700 regulatory complexes to form 26 S proteasomes or with PA28 complexes (29). The 26 S proteasome usually degrades ubiquitinated proteins (30). Ornithine decarboxylase (ODC) constitutes an exception because it is targeted to 26 proteasomes by antizyme in a ubiquitin-independent fashion (31–33). The 26 S proteasome seems to mediate the degradation of most antigenic proteins in living cells (34–36) and, therefore, deserves particular interest. The in vitro cleavage specificities of purified 20 S proteasomes and the relevance of proteasomal processing for the generation of immunodominant epitopes have been investigated in great detail (1, 37). Although 20 S proteasomes have been shown to precisely cleave a number of known class I ligands out of model polypeptide substrates, a minor part of cleavage products is longer than the canonical length of class I-binding peptides (38–41).

Limited information is, however, so far available about protein processing by 26 S proteasomes. A direct comparison of 26 and 20 S proteasomes revealed overlapping but substantially different cleavage patterns for the protein substrate β-casein (41). On average, the 26 S proteasomal cleavage products were found to be slightly shorter than peptides generated by 20 S proteasomes containing greater proportions of peptides that are too short for efficient TAP-mediated translocation as well as for class I binding (40, 41).

Proteasomes seem to be the dominant, if not the only, protease that generates the correct C terminus of class I ligands,
whereas the N-terminal trimming of proteasomal products is insensitive to proteasome inhibitors (20, 42, 43). Leucine aminopeptidase, puromycin-sensitive aminopeptidase, and bleomycin hydrolase have been implicated in the cytoplasmic trimming of proteasomal products (44, 45). In addition, tripeptidyl peptidase II has been suggested to play a role in the generation of class I ligands or precursors (46).

Different lines of evidence suggest that precursors of class I ligands can be trimmed in the ER lumen to their final lengths. Processing of peptide imported into microsomes was directly shown by biochemical methods (14, 47). The broad but not random substrate specificity of TAP precludes that 9-mer peptides containing a proline residue at position 2 or 3 are efficiently bound and transported by TAP (48–50), which can be rescued by extending such peptides with N- and/or C-terminal flanking residues (49). Nevertheless, several MHC class I alloforms in man and mouse prefer a proline residue at position 2 or 3 in the associated 9-mer peptide (51). Also for some peptides not containing proline, the addition of flanking residues to minimal epitopes strongly improved TAP affinities (50, 52, 53) implicating that precursors become trimmed in the ER.

The analysis of extended minimal epitopes or tandem arrays of epitopes that were directed into the ER of TAP-deficient T2 cells by a leader sequence have clearly indicated the predominance of an aminopeptidase activity (10, 47, 53–55), whereas the carboxypeptidase activity in the ER lumen seems to be very poor (5, 11, 53–55). N-terminal trimming of a precursor peptide was recently suggested to depend on the presence of the correct class I restriction element (47). However, the identity and biochemical properties of the aminopeptidase(s) in charge remains elusive.

To investigate the relative contribution of the proteasome and the cytosolic and ER peptidases to the generation of the definite class I epitope, we have developed an in vitro system in which recombinant ODCA containing ovalbumin (ova) peptides are targeted to degradation by 26 S proteasomes followed by import into purified microsomes. We find that the yield of a finally processed H2-Kb-binding epitope was strongly influenced by N-terminal flanking amino acids in the protein sequence. We show that a slightly extended oval peptide precursor was efficiently trimmed following TAP-mediated import into microsomes. The conversion into the definite epitope could be blocked by the aminopeptidase inhibitor 1,10-phenanthroline (PNT) and was strictly dependent on the presence of MHC class I molecules known to associate with the epitope.

EXPERIMENTAL PROCEDURES

Peptides

The peptide SIINFELK was synthesized by Anaspec (San Jose, CA). The peptide ESINFEK was synthesized by Dr. M. Fridkin at the Department of Organic Chemistry, The Weizmann Institute (Rehovot, Israel). The peptides HESINFEK, HLSINFEK, QSHEISINFEK, and QSHELSEINFEK were synthesized at the peptide synthesis core facility of the German Cancer Research Center (DKFZ) (Heidelberg, Germany). All peptides were purified to >95% homogeneity by HPLC, and their identity was confirmed by mass spectrometry. To accurately determine the concentration of peptides used in this study each peptide solution in double distilled water was analyzed by Edman degradation.

Chemicals

Bestatin and lactacystin were from Calbiochem. Complete-media EDTA-free protease inhibitors (referred to as protease inhibitors) were from Roche Molecular Biochemicals. 1,10-Phenanthroline, all standard reagents, and reagents for cell culture were from Sigma.

Cell Lines and Mice

The B3Z T cell hybridoma and the antigen-presenting cell line K5–L have been described by Karttunen et al. (56) and were granted by Dr. Chris Norbury (National Institutes of Health, Bethesda, MD). C57BL/6 (B6) and BALB/c mice were from local breeding in the animal facility of the Sackler Faculty of Medicine, Tel Aviv University (Tel Aviv, Israel). H2-DK–/– (B6-Kb) and H2-Kb–/– (B6-Dd) knockout mice (57) were a kind gift of Dr. Lea Eisenbach (Weizmann Institute, Rehovot, Israel). TAP1–/– mice (58) were courtesy of Dr. Natalio Garcia-Garbi (German Cancer Research Center, DKFZ, Heidelberg, Germany).

Preparation of 26 S Proteasome, Maltose-binding Protein–Antizyme Fusion Protein and Recombinant ODCA-ova Derivatives

The 26 S proteasome was purified from B6 livers, and the recombinant proteins, maltose-binding protein-antizyme fusion protein, and ODCA-ova derivatives were expressed in bacteria and purified by affinity chromatography as previously described (59). To generate the ODCA-ova derivatives, the peptide LEOSIINFEKL was synthesized by Dr. M. Fridkin at the Department of Organic Chemistry, The Weizmann Institute (Rehovot, Israel). The peptides HESIINFEKL, HLESIINFEKL, QSHESIINFEKL with BesXI-compatible ends was generated by annealing the two synthetic oligonucleotides 5’-ATCTGGAAGATATAACATCCTCGA-AAAAATGAGCC-3’ and 5’-CAGTTTTTCGAAGTTGATTTATTTTC- CAGATGGC-3’. The adapter was then inserted into the ODCA sequence as previously described for ODCA-ova (59). The production of 35S-labeled ODCA-ova and ODCA-Leova was in the methionine auxotroph strain B834(DE3) (Novagen Inc., Madison, WI), and purification of the 35S-labeled proteins was carried out as previously described (59).

Preparation of Subcellular Fractions

Lactacystin-treated Cytosol—The preparation of cytosol (100,000 g supernatant) from RMA cells was carried out as previously described (60). To inhibit proteasome activity, a 2-ml aliquot of the cytosol (20 mg/mL) protein extract was incubated for 35 μm lactacystin at 37 °C for 10 min at 37 °C. The lactacystin-treated cytosol was then dialyzed for 16 h at 4 °C against 500 ml of 20 mM Tris-HCl (pH 7.5), 0.5 mM DTT and stored in aliquots at −70 °C. Inhibition of proteasome activity in the cytosol was greater than 90% as determined by the inhibition of fluorogenic peptide Suc-LIYY-methyl coumarin hydrolizing activity (61).

Microsomes—Microsomes were prepared according to the protocol described by Shepherd et al. (58). Briefly, mice (8–10 weeks old) were injected intraperitoneally with 0.2 mg of polyinosine-cytosine/mouse 24 h prior to sacrifice. The livers and spleens were thoroughly washed with phosphate-buffered saline and then homogenized in a motor-driven Potter-Elvehjem Teflon tissue grinder. Homogenization was in 3 mL/mg buffer A (50 mM triethanolamine acetate (pH 7.5), 1 mM DTT, 5 mM magnesium acetate, 50 mM potassium acetate, 250 μM succinate, and a 1:25 (v/v) solution of protease inhibitors). The extract was then subjected to fractional centrifugation at 1,000 × g and then at 10,000 × g. The supernatant from the 10,000 × g spin was then placed on top of a sucrose cushion containing 1.3 M sucrose in buffer A at a ratio of 2:1 (sample:cushion) and then centrifuged at 140,000 × g for 2.5 h. The resulting pellet was then resuspended in buffer B (50 mM Hepes-KOH (pH 7.5), 1 mM DTT, 50 mM magnesium acetate, 50 mM potassium acetate, 250 μM succinate) to a concentration of 290 A280 nm units/mL. The microsome suspension was then stored in aliquots at −70 °C.

Stripping Off Microsome-associated Proteins

The microsome suspension was incubated on ice for 15 min in 10 volumes of buffer C (50 mM Hepes-KOH (pH 7.5), 1 mM DTT, 0.5 mM KCl, and 15 mM EDTA). The membrane suspension was then centrifuged at 10,000 × g in a microcentrifuge. The membrane pellet was then resuspended in 10 volumes of buffer D without EDTA and immediately centrifuged as described above. The final membrane pellet was resuspended in the processing reaction mixture. The protein content of the stripped microsomes was less than 40% of that of untreated membranes as determined by measurement of the absorbance at 280 nm.

Assay of TAP-mediated Peptide Transport

TAP-mediated peptide transport was determined by measuring the ATP-dependent transport of the radioiodinated peptide TNKTRIDGQY into isolated microsomes as previously described (63). Purified microsomes (1 unit) were incubated in a reaction mixture containing the following components in a final volume of 100 μl: 50 mM Hepes-KOH (pH 7.5), 1 mM DTT, 5 mM MgCl2, 10 mM creatine phosphate, 2.5 units of creatine phosphokinase, and 531,412,514–labeled peptide (37 ng, 106 cpm). In reactions without ATP, 2-deoxyglucoside (20 mM) and hexokinase (3 μg) were added instead of ATP and the ATP-regenerating system. Following incubation for 10 min at 37 °C the microsomes were pelleted by centrifugation at 10,000 × g in a microcentrifuge. The supernatant was removed, and the membrane pellet was resuspended in 1 ml of lysis buffer (20 mM Hepes-KOH (pH 7.5), 5 mM MgCl2, and 1% Nonidet P-40) and then sonicated for 30 s at 50% output power in a water bath...
sonicator (Ultrasonic Processor, Heat Systems Inc., Farmingdale, NY). The detergent extract was centrifuged for 10 min at 10,000 × g in a microcentrifuge. The resulting supernatant was then mixed with 25 μL of concanavalin A-Sepharose beads (Amersham Pharmacia Biotech), and the mixture was then mixed gently for 16 h at 4 °C. The beads were pelleted by centrifugation at 1,000 × g in a microcentrifuge and then washed twice with 1-mL portions of lysis buffer. The ATP-dependent transport of peptide was then determined by measuring the amount of radioactivity associated with the beads in the presence of ATP after subtraction of the concanavalin A-associated radioactivity obtained in a parallel reaction carried out in the absence of ATP.

Antigen Processing Assays

Antigen processing reaction mixtures contained the following components in a final volume of 75 μL: 50 mM Heps-KOH (pH 7.5), 5 mM MgCl₂, 0.5 mM DTT, 1 mM ATP, 10 mM creatine phosphate, 1.8 units of creatine phosphokinase, 10 μg of recombinant ODC, 15 μg of maltose-binding protein-antizyme fusion protein, and 10 units of 26 S proteasome (for the definition of proteasome units see Ref. 59). Where indicated, 1,10-phenanthroline (2 mM) or bestatin (250 μM) were added for a further incubation for 8 min at 37 °C. The reaction was preincubated for 4 min at 37 °C to allow for the maximal extraction of peptides from the proteasome. The activity was probably because of the presence of specialized proteases involved in the degradation of the proteasome under linear kinetic conditions we noticed that the amount of SIINFEKL produced from ODC-ova and ODC-LEova was 90 and 25%, respectively. Instead the predominant relative SIINFEKL was ESIINFEKL (ODC-LEova). We have previously shown that ODC-ova and ODC-LEova were processed by pu-

Peptide Processing Assays

Reactions were carried out in a final volume of 100 μL containing the following components: 50 mM Heps-KOH (pH 7.5), 5 mM MgCl₂, 0.5 mM DTT, 1 mM ATP, 10 mM creatine phosphate, 2.5 units of creatine phosphokinase, microsomes (1 unit), and 50 nmol of synthetic SIINFEKL or HLESIINFEKL. Where indicated, 1,10-phenanthroline (2 mM) or bestatin (250 μM) were added. In reactions without ATP, hexokinase (3 μg) (Roche Molecular Biochemicals) and 2-deoxyglucose (20 mM) were added instead of ATP and the ATP-regenerating system. The reaction was preincubated for 4 min at 37 °C and then SIINFEKL was added for a further incubation for 8 min at 37 °C. The reaction was stopped by the addition of 450 μl of lysis buffer and SIINFEKL was observed by western blotting with the 150C2 antibody. The finding that ESIINFEKL became the predominant relative SIINFEKL was ESIINFEKL (ODC-LEova). We have previously shown that ODC-ova and ODC-LEova were processed very efficiently to generate almost exclusively the optimal epitope SIINFEKL (59). When we compared the proteolytic processing of ODC-ova and ODC-LEova by pur-
epitope, we subjected the recombinant antigens to proteolytic processing by the 26S proteasome in the presence of a cytosolic fraction and isolated microsomes. Following incubation of the antigens in the cell-free system, the microsomes were pelleted, and the yield of SIINFEKL was measured by the ability to activate the K\textsuperscript{b}/SIINFEKL-specific T cell hybridoma B3Z.

When ODC-ova was incubated in the presence of 26S proteasome and B6.K\textsuperscript{b} microsomes, 54% of the SIINFEKL initially produced by the proteasome alone was recovered (6 of 11,000 cpm for ODC-ova and 6,000 cpm for ODC-LEova) were incubated in a volume of 25 μl in a standard reaction mixture. The percentage of degradation of the 35S-labeled ODC derivative was then determined as previously described (59) by measuring the amount of soluble radioactivity after addition of trichloroacetic acid and after subtraction of the soluble radioactivity obtained at time 0. The degradation results are the mean of duplicate incubations.

TABLE I

| Antigen     | Processed peptide | Relative amount % |
|-------------|-------------------|-------------------|
| ODC-ova     | SIINFEKL          | 90                |
|             | HSIINFEKL         | 5.5               |
|             | QSHSIINFEKL       | 4.5               |
| ODC-LEova   | SIINFEKL          | 25                |
|             | ESINFEKL          | 62                |
|             | HLESINFEKL        | 6                 |
|             | QSHLESINFEKL      | 7                 |

Processing of Synthetic ESINFEKL by Isolated Microsomes—To investigate the peptidase activity responsible for the N-terminal trimming, we studied the conversion of synthetic SIINFEKL to SIINFEKL by isolated microsomes. As observed in the antigen processing experiments, incubation of synthetic ESINFEKL with isolated B6.K\textsuperscript{b} microsomes resulted in a remarkable enhancement of SIINFEKL recovery (Fig. 3). In agreement with the results of the ODC-LEova processing experiment, TAP\textsuperscript{−/−} BALB/c as well as B6.D\textsuperscript{b} microsomes could not generate SIINFEKL (Fig. 3 and data not shown).

To further characterize the specific trimming activity associated with the microsomes, we tested the sensitivity of the putative peptidase to the metalloaminopeptidase inhibitor bestatin (64) as well as to the ion chelator PNT that was previously shown to inhibit various metallopeptidases (65). We found that when both inhibitors were present, the ESINFEKL to SIINFEKL conversion was completely blocked (Fig. 4). PNT alone was sufficient to sustain the block. Surprisingly however, in the presence of bestatin alone, there was a significant augmentation in the yield of SIINFEKL. These results suggest that at least two types of peptidases influence the fate of ESINFEKL: a bestatin-sensitive peptidase that had a deleterious effect and a PNT-sensitive “trimmase” that converted ESINFEKL to SIINFEKL. The PNT-mediated inhibition was not due to inhibition of peptide transport into the ER because
In this work we have reconstituted in vitro the entire antigen processing pathway, from proteolysis of the antigen by the 26 S proteasome to the binding of the final epitope to the specific MHC class I molecule in the ER. This cell-free system that is comprised of the 26 S proteasome, a protein antigen, cytosol, and isolated microsomes enables the analysis of the relative contribution of the proteasome and cytosolic and microsomal peptidases to the generation of the definite epitope. The major impediment for the analysis of the mechanism of the 26 S proteasome in antigen processing lies in the difficulty to produce large enough ubiquitinated protein antigen for in vitro localization of the trimming aminopeptidase would have been resistance to proteolysis, this approach could not have been applied because treatment of microsomes with trypsin, for example, would have also digested TAP and prevented peptide transport. Thus, to establish the localization of the ESINFEKL to SIINFEKL-converting enzyme, we attempted to remove the external peptidases. To this end the microsomes were washed with 15 mM EDTA and 500 mM KCl. This washing procedure removed over 60% of the proteins associated with the microsomes (data not shown) and should have removed almost entirely any peptidase activity associated with the outer face of the microsomes. As shown in Fig. 5A, when synthetic SIINFEKL was incubated with the washed microsomes in the absence of ATP (i.e. when TAP transport was blocked) there was only a small reduction in the amount of SIINFEKL relative to the input level obtained in the control incubation with buffer alone (Fig. 5A, first two columns on the left). Low and intermediate PNT concentrations (50–200 μM) were sufficient to restore the input level of SIINFEKL indicating that the remaining peptidase activity on the cytosolic face was completely inhibited (Fig. 5, A and C). However, when ESINFEKL was incubated with the washed microsomes in the presence of ATP, the conversion of ESINFEKL to SIINFEKL was significantly inhibited only at higher PNT concentrations (Fig. 5, B and C). These results demonstrate that under conditions in which the activity of the peptidase on the external surface of the microsomes was completely inhibited, there was an additional PNT-sensitive activity that became apparent only in the presence of ATP (see also Fig. 4). We conclude that an ER-luminal metallopeptidase contributes to the conversion of ESINFEKL to SIINFEKL.

**FIG. 2.** Processing of ODC-ova and ODC-LEova by the 26 S proteasome in the presence of cytosol and microsomes. ODC-ova (A) and ODC-LEova (B and C) were incubated in a standard antigen processing reaction mixture with the indicated additions as described under “Experimental Procedures.” Following incubation at 37 °C for 10 min, microsomes were isolated and extracted. The peptides were then purified by reverse phase HPLC, and the amount of processed SIINFEKL was quantified based on the ability to activate the B3Z T cell hybridoma as described under “Experimental Procedures.” Results of A–C were calculated as the mean value of duplicate incubations. D, recognition of synthetic ODC-ova peptides by B3Z. Various amounts of synthetic peptides were incubated with K′L cells and then tested for recognition by B3Z. Squares, SIINFEKL; triangles, ESINFEKL; circles, HLESINFEKL.

**FIG. 3.** Processing of synthetic ESINFEKL by isolated microsomes. Synthetic ESINFEKL was incubated with 1 A_{280} unit of the indicated microsomes either in the presence or absence of ATP. Microsomes were then extracted, and the peptides were isolated as described under “Experimental Procedures” and in the legend to Fig. 2. The amount of SIINFEKL was then determined by the B3Z activation assay as described under “Experimental Procedures.” Results were calculated as the mean value of duplicate incubations.

**FIG. 4.** Effect of peptidase inhibitors on processing of synthetic ESINFEKL by isolated microsomes. Synthetic ESINFEKL was incubated with 1 A_{280} unit of B6.K′ microsomes with the indicated additions. Microsomes were then extracted, and the amount of SIINFEKL was determined by the B3Z activation assay. Results were calculated as the mean value obtained in two independent experiments.

Although the processing of ESINFEKL was dependent on TAP transport and was class I-specific it was still possible that peptidases operating at the cytoplasmic leaflet of the microsomes initially converted ESINFEKL to SIINFEKL and that only subsequent transport into the ER lumen and binding to the correct class I molecule prevented further degradation of the peptide epitope. Although the ultimate proof for luminal
processing experiments to allow mass spectrometric analysis of the cleavage products. We have overcome this crucial problem by targeting the protein antigen to the 26 S proteasome through the ubiquitin-independent mechanism used by ODC (31, 59). By varying the amino acid residues immediately N-terminal to SIINFEKL we were able to generate an ODC-ova derivative, ODC-LEova, that was degraded at a similar rate by the 26 S proteasome; however, the proteolytic processing resulted in a significant reduction in the abundance of SIINFEKL (see Fig. 1 and Table I). The fact that ESIINFEKL, the dominant extended SIINFEKL, was a poor activator of B3Z T cells allowed us to follow for the first time postproteasomal processing activity in a physiologically relevant fully reconstituted cell-free system.

Consistent with our previous results (59), we found by mass spectrometric quantification that the 26 S proteasome efficiently liberates the SIINFEKL peptide from the ODC sequence context ( . . . WQLMKQGSH–SIINFEKL–SHGFPEVEE . . . ), whereas the N-terminal extended peptides HSIINFEKL and QSHSINFEKL were only minor products. When introducing the extended epitope LESIINFEKL into the same ODC sequence context, the predominant cleavage now occurred between the N-terminal Leu and Glu residues, which is in full agreement with the cleavage specificities of both the 20 S and the 26 S human proteasome. ROCK and co-workers (43, 44) have shown the bestatin-sensitive peptidase acted prior to TAP transport into the ER by TAP. This follows from our observation that the addition of cytosol led to further degradation of SIINFEKL when the activity of the external peptidase was inhibited (see Fig. 2A). Nevertheless, the significant proportion of SIINFEKL that was retained in the microsomes suggested that the TAP-mediated translocation of peptide occurred at a higher rate.

Three cytosolic peptidases have thus far been implicated in the trimming of N-terminally extended peptide precursors to their final size. Rock and co-workers (43, 44) have shown that a bestatin-sensitive leucine aminopeptidase can cleave SIINFEKL precursors to generate the final 8-mer. In a recent study, puromycin-sensitive aminopeptidase and bleomycin hydrolase have been implicated in the N-terminal trimming of a vesicular stomatitis virus nucleoprotein-derived epitope precursor (45). These observations indicate that individual peptidases contribute to limited N-terminal trimming of epitope precursors. Nevertheless, the cumulative effect of cytosolic peptidases on SIINFEKL and its precursors seems to be deleterious. This can be concluded from our finding that the addition of cytosol to the antigen processing reaction caused a major reduction in the amount of SIINFEKL retained in the ER (see Fig. 2). In other experiments not presented in this study we found that cytosol completely destroyed 26 S proteasome-processed peptides in the absence of microsomes and that this degradation could not be inhibited by bestatin. The stimulatory effect of bestatin on the yield of SIINFEKL in the absence of cytosol (see Fig. 4) likely resulted from inhibition of a particular membrane-associated peptidase(s). This also suggested that the bestatin-sensitive peptidase acted prior to TAP transport and thus limited the availability of peptide substrates.

López and co-workers (28) have recently provided evidence for a PNT-sensitive cytosolic aminopeptidase activity. When the metallopeptidase inhibitor PNT was administered to cells infected with recombinant vaccinia virus expressing a human immunodeficiency virus Env epitope either in the context of the natural human immunodeficiency virus envelope protein or of a recombinant hepatitis B virus core protein, the authors (28) noticed efficient blockade of antigen presentation. However, the processing of another recombinant hepatitis B virus construct or a long epitope precursor targeted to the ER in a TAP-independent manner was not affected by PNT suggesting that a PNT-sensitive peptidase was operative prior to TAP transport. We have also identified a PNT-sensitive peptidase associated with the external leaflet of the microsomal membranes. However, in addition we show that a distinct PNT-sensitive peptidase is operative in the lumen of the ER and that this luminal peptidase in involved in the final processing of 26 S proteasome-processed peptides subsequent to introduction into the ER by TAP. This follows from our observation that the final epitope SIINFEKL was generated by EDTA/salt-stripped microsomes from ESIINFEKL when the activity of the external peptidase was inhibited and when peptide translocation was facilitated (see Fig. 5).

The presence of H2-Kb was strictly required to convert ESIINFEKL to SIINFEKL, whereas only background levels could be recovered from H2-Db- or H2-Kb/Dd-expressing microsomes (cf. Figs. 2B, 2C, and 3). This would invoke a model.
accompanying which N-terminal trimming is initiated by binding of the precursor peptide to the appropriate class I receptor followed by recruitment of the aminopeptidase to the class I-peptide complex as originally proposed by Rammensee and co-workers (51). In this model, the class I molecules would prevent N-terminal trimming beyond the optimal peptide length.

Earlier work has provided evidence that selected peptides could only be extracted from tissues expressing class I molecules able to associate with these peptides (66). Furthermore, it has been shown that antigenic peptides that are not retained in the ER undergo retrotranslocation to the cytosol for degradation (13–15, 58). The herein presented findings that SINFEKL processed from OVA-ova was undetectable in reactions containing H2-Kb, but not H2-Kk-expressing B6 microsomes (Fig. 2A) or H2-Kk-expressing BALB/c microsomes are fully consistent with the idea that class I molecules retain and protect TAP-translocated peptides.

Recently, Shastrl and colleagues (47) demonstrated the efficient conversion of an N-terminally extended SINFEKL derivative in H2-Kk but not H2-Kk-expressing cells. We have shown that in a cell-free system the presence of H2-Kk is essential for the recovery of SINFEKL following processing of ESINFEKL by a microsomal metalloaminopeptidase. However, we cannot conclusively determine whether binding of the precursor peptide to the correct restriction element is strictly required for the initiation of the processing or whether class I molecules merely capture partially digested peptides released from slowly acting lumenal aminopeptidases in a random process and protect them from further degradation. Additional work is required to distinguish between these two potential mechanisms. Furthermore, the general role of this aminopeptidase will have to be dissected using additional protein antigens.

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REFERENCES
1. Rock, K. L., and Goldberg, A. L. (1999) Annu. Rev. Immunol. 17, 739–779
2. Pamer, E., and Cresswell, P. (1998) Annu. Rev. Immunol. 16, 323–358
3. Momburg, F., and Ha¨ mmerling, G. J. (1998) Adv. Immunol. 68, 191–256
4. Anderson, K., Cresswell, P., Gaetani, M., Herms, J., Williamson, A., and Zweerink, H. (1991) J. Exp. Med. 174, 489–492
5. Eisenlohr, L. C., Bacik, I., Bennik, J. R., Bernstein, K., and Yewdell, J. W. (1992) Cell 71, 963–972
6. Bacik, I., Cox, J. H., Anderson, R., Yewdell, J. W., and Bennik, J. R. (1994) J. Immunol. 152, 381–387
7. We, M. L., and Cresswell, P. (1992) Nature 356, 43–44
8. Henderson, R. A., Cox, A. L., Sakaguchi, K., Appella, E., Shabanowitz, J., Hunt, D. F., and Engelhard, V. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10275–10279
9. Hammond, S. A., Bollinger, R. C., Tobery, T. W., Siliciano, R. F. (1993) Nature 364, 158–161
10. Elliott, T., Willis, A., Cerundolo, V., and Townsend, A. (1995) J. Exp. Med. 181, 1481–1491
11. Snyder, H. L., Bacik, I., Yewdell, J. W., Behrens, T. W., and Bennik, J. R. (1998) Eur. J. Immunol. 28, 1339–1346
12. Gil-Torregrosa, B. C., Castaño, A. R., and Del Val, M. (1998) J. Exp. Med. 188, 1315–1320
13. Schumacher, T. N., Kanzler, D. V., Heemels, M. T., Ashton-Rickardt, P. G., Shepherd, J. C., Früh, K., Yang, Y., Peterson, P. A., Tonge, S., and Hoffmann, G. (1995) J. Exp. Med. 19, 1101–1116
14. Roelse, J., Gromme B., and Momburg, F. (1999) Eur. J. Immunol. 29, 117–122
15. Rock, L. K., Gram, C., Rothstein, L., Clark, K., Stein, R., Dick, T., Hwang, D., and Goldberg, A. L. (1994) Cell 78, 671–771
16. Harding, C. V., Franze, J., Song, X., Farah, J. M., Chatterjee, S., Iqbal, M., and Siman, R. (1999) J. Immunol. 155, 1767–1775
17. Bai, A., and Forman, J. (1997) J. Immunol. 159, 2139–2146
18. Cerundolo, V., Benham, A., Braid, V., Mukherjee, S., Gould, K., Mastro, B., Neefjes, J., and Townsend, A. (1997) Eur. J. Immunol. 27, 336–341
19. Crain, A., Akopian, T., Goldberg, A., and Rock, L. K. (1997) Proc. Natl. Acad.
