

Need for Tripeptidyl-peptidase II in Major Histocompatibility Complex Class I Viral Antigen Processing when Proteasomes are Detrimental*

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CD8⁺ T lymphocytes recognize infected cells that display virus-derived antigenic peptides complexed with major histocompatibility complex class I molecules. Peptides are mainly byproducts of cellular protein turnover by cytosolic proteasomes. Cytosolic tripeptidyl-peptidase II (TPPII) also participates in protein degradation. Several peptidic epitopes unexpectedly do not require proteasomes, but it is unclear which proteases generate them. We studied antigen processing of influenza virus nucleoprotein epitope NP_{147–155}, an archetype epitope that is even destroyed by a proteasome-mediated mechanism. TPPII, with the assistance of endoplasmic reticulum trimming metallo-aminopeptidases, probably ERAAP (endoplasmic reticulum aminopeptidase associated with antigen processing), was crucial for nucleoprotein epitope generation both in the presence of functional proteasomes and when blocked by lactacystin, as shown with specific chemical inhibitors and gene silencing. Different protein contexts and subcellular targeting all allowed epitope processing by TPPII as well as trimming. The results show the plasticity of the cell's assortment of proteases for providing ligands for recognition by anti-viral CD8⁺ T cells. Our observations identify for the first time a set of proteases competent for antigen processing of an epitope that is susceptible to destruction by proteasomes.

Protein degradation is essential for cell metabolism. Erroneous or expired proteins are first broken down into peptides and

then into amino acids (aa)⁶ (1). As a byproduct of this process, some peptides can be rescued from final degradation by translocation to the endoplasmic reticulum (ER) by the transporters associated with antigen processing and binding to major histocompatibility complex (MHC) class I molecules (2, 3). These complexes are presented at the cell surface and are continuously screened by circulating CD8⁺ T lymphocytes, which detect pathogen-derived peptides and clear infection from infected hosts (4).

The proteasome is the most abundant, multifunctional and multispecific degradative protease in the cytosol and is not dispensable for cell viability. Cell lines that survive in the presence of proteasome inhibitors have a residual proteasome activity (5) and overexpress at least one degradative enzyme (6). This protease, tripeptidyl-peptidase II (TPPII) (7, 8), partially compensates for some of the functions of the handicapped proteasome. TPPII is a serine protease that has an exopeptidase activity that removes stretches of preferentially 3 aa from the amino terminus of peptides (9) as well as a less efficient endopeptidase activity (7, 10).

The proteasome is involved in the generation of many epitopes recognized by CD8⁺ T lymphocytes (11, 12). However, it also destroys epitope-containing peptides. Because very few peptides suffice for immune surveillance by CD8⁺ T cells, the productive/destructive balance is in most instances, but not always (13–15), positive for the generation of MHC class I ligands. TPPII cooperates with proteasomes in the generation of a viral epitope (10, 16), is essential for the proteasome-independent generation of a second one (17), and plays a certain role in downstream cleavage of proteasomal products relevant for antigen presentation (10, 18).

MHC class I ligands, thus, derive from byproducts of proteolytic activities, which may not have the precise final size for optimal binding affinity. Trimming by exopeptidases, therefore, plays a significant role, notably by the ER aminopeptidases

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⁶ The abbreviations used are: aa, amino acids; ER, endoplasmic reticulum; MHC, major histocompatibility complex; TPPII, tripeptidyl-peptidase II; NP, nucleoprotein; CTL, cytotoxic-T-lymphocyte; ENV, envelope; ERAAP, endoplasmic reticulum aminopeptidase; ERAAP, endoplasmic reticulum aminopeptidase associated with antigen processing; LC, lactacystin; rVV, recombinant vaccinia viruses; m.o.i., multiplicity of infection; AAF-cmk, Ala-Ala-Phe-chloromethyl ketone; LeuSH, leucinethiol; ICS, intracellular cytokine staining; AAF-amc, Ala-Ala-Phe-4-methyl-7-coumarilamide; IFN, interferon; Ct, carboxyl termini; siRNA, small interfering RNA; WR, Western Reserve.

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ERAAP/ERAP1 and ERAP2 (19–22). However, there is limited access to carboxypeptidase activity in the classical antigen processing pathway (2, 23, 24), which means that all correct carboxyl termini (C_c) have to be generated by endopeptidases such as proteasomes (2). Notably, TPPII has been shown to generate the correct C_c of one epitope *in vitro* (17) and to act on cytosolic precursor peptides *in vivo* (10).

Influenza virus nucleoprotein (NP) contains several cytotoxic-T-lymphocyte (CTL) epitopes. Processing of some of them requires proteasomes, whereas others are largely resistant to proteasome inhibitors (13, 14, 25). Presentation of the NP_{147–155} epitope is even enhanced in the presence of a number of proteasome inhibitors. This is true in the natural context of NP from two strains of influenza virus, A/Puerto Rico/8/34 (NP_{PR8}) and A/NT/60/68 (NP_{NT60}), as well as in a few mutated constructs of these proteins. Among several possible explanations (14) including the recent suggestion that an altered enzymatic activity of inhibitor-treated proteasomes might generate *in vitro* products compatible with peptide presentation (27), the current hypothesis derived from *in vitro* digestions (13, 29) is that the naturally presented peptide or a precursor is destroyed by proteasomes.

This leaves the question open as to which is the protease that generates this and related epitopes that do not benefit from proteasome activity. Our results show the vital role of TPPII together with trimming aminopeptidases in generating this NP_{147–155} epitope from several diverse protein contexts both in the presence and in the absence of lactacystin (LC)-sensitive proteasome action.

EXPERIMENTAL PROCEDURES

Recombinant Vaccinia Viruses (rVVs)—The rVVs employed in this study have been described previously; that is, those encoding full-length influenza virus NP from the PR8 and NT60 strains (14, 30) as well as the rVVs encoding a secretory variant of NP_{PR8} that differs from it in that it contains an interferon- β signal peptide (31), rVVs encoding minigenes expressing the cytosolic NP_{147–155} epitope (rVV-NP_{M147–155}) or the epitope preceded by a signal sequence (rVV-SNP_{A147–155}) (30), and rVV-ENV (vPE16), which expresses ENV envelope glycoprotein gp160 from the strain IIIIB of human immunodeficiency virus-1 (32). All these rVVs had a Western Reserve (WR) wild-type vaccinia background. In addition, a series of rVVs based on the wild-type Copenhagen strain and encoding the influenza virus NP antigenic nonamer NP_{147–155} in a chimeric protein context was also employed. Chimeric proteins had insertions at position 179 at the carboxyl terminus of the hepatitis B virus core or precore proteins (cytosolic HBc or secretory HBe, respectively) of the NP_{147–155} sequence either non-flanked or flanked on either side of the epitope by 4 aa that represent the natural flanking sequences from NP_{PR8} and NP_{NT60} (Fig. 1). The chimeric proteins cC-NP147, cC-NP147P, and cC-NP147N were based on HBc and were, thus, expressed in the cytosol, as they lacked a signal sequence. Chimeras sC-NP147, sC-NP147P, and sC-NP147N entered the secretory pathway. The negative control encodes the carrier protein HBe.

Cell Lines—L cells, murine kidney fibroblasts transfected with K^d (30) or D^d (33), were used. P13.1 cells are derived from

P815 mastocytoma cells (H-2^d) by transfection with the lacZ gene encoding β -galactosidase (34). Human kidney 293 cells were transfected with K^d (35). Human T2/K^d are human lymphoblastoid T2 cells deficient in transporters associated with antigen processing and transfected with K^d (36).

Viral Infections—L cells were infected as described (37). Briefly, a 1-h virus adsorption period at the indicated multiplicity of infection (m.o.i.) at a cell density of 10^5 cells/cm² was followed by washes and by the indicated incubation periods. To study the effect of inhibitors, cells were treated with a 5-fold concentration for 15 min before virus adsorption, and the inhibitor was kept throughout adsorption and infection at the standard concentrations. As the negative control, cells were similarly infected with WR at a m.o.i. of 30 in the case of full-length NPs or with rVV-HBe at a m.o.i. of 30 or 90 for the chimeric constructs. As positive control, cells were infected with these viruses and pulsed during the 1-h adsorption period with 10^{-9} M NP_{147–155} peptide.

Inhibitors—LC inhibits proteasomes (38) and was used at 10 μ M and purchased from E. J. Corey (Harvard University). Ala-Ala-Phe-chloromethyl ketone (AAF-cmk) is a TPPII substrate analog (7) and was used at 110 μ M. Leucinethiol (LeuSH) inhibits metallo-aminopeptidases including ERAAP (19) and was used at 30 μ M in RPMI medium containing freshly added 5×10^{-5} M 2-mercaptoethanol. All three were from Bachem. Butabindide (Tocris) is a substrate analog that acts as a competitive inhibitor of TPPII (39), was used from 50–400 μ M, and was added again at every step and every 90–120 min (10). One butabindide lot showed higher potency.

Polyclonal Monospecific CTL Lines; Intracellular Cytokine Staining (ICS)—Polyclonal ENV-IIIIB-monospecific CTL were generated by infection of BALB/c mice with rVV-ENV followed by weekly restimulation of splenocytes with 10^{-6} M G9I synthetic peptide (sequence GPGRAFVTI) and interleukin 2 as described elsewhere (40). Similarly, polyclonal NP_{147–155}-specific CTL lines were generated from splenocytes of BALB/c mice after two intraperitoneal injections of 10^7 PFU of rVV-NP_{PR8} 3 weeks apart and using 10^{-8} M NP_{147–155} peptide (sequence TYQRTRALV) for weekly restimulation. ICS assays were performed as described (41, 42). CTL lines were stimulated overnight in the presence of brefeldin A (Sigma) with infected cells or control infected cells previously pulsed for 1 h with 10^{-9} M NP_{147–155} peptide. An effector to target ratio of 0.2:1 was used, with the exception of minigene-expressing targets, where it was 10:1 to decrease sensitivity of detection. After stimulation cells were incubated with fluorescein isothiocyanate-conjugated anti-CD8 α monoclonal antibody (Proimmune), fixed, and incubated with phycoerythrin-conjugated monoclonal antibody to IFN- γ (BD Pharmingen) during permeabilization. Events were acquired using a FACScalibur flow cytometer (BD Biosciences), and data were analyzed using CellQuest software (BD Biosciences). Background activation obtained with cells infected with negative control virus (usually 0 to 5%) was subtracted. Percent specific inhibition was calculated as % specific inhibition = $(A - Ai / A - N) \times 100$, where A is the % CD8⁺ IFN- γ ⁺/total CD8⁺ cells with target cells infected with rVVs, Ai is the value with rVVs plus inhibitor, and

N is the value with the negative control. Mean and S.D. was calculated typically from at least two independent experiments.

Western Blot—NP proteins and chimeric HBe proteins were detected in pellets of L/K^d cells infected for 2 h as for the ICS assays with rVVs. They were separated by SDS-PAGE, electrotransferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences), and developed with rabbit anti-HBe serum (37) by standard ECL procedures (Amersham Biosciences). Full-length NPs were detected using a rabbit antibody raised to the carboxyl terminal peptide C⁴⁸⁸FFGDNAEEYDN⁴⁹⁸ coupled to keyhole limpet hemocyanin (Imject[®] maleimide-activated mCKLH from Pierce). Antibodies were purified by ammonium sulfate precipitation and anion exchange chromatography on DE52 (Whatman). ENV was detected in rVV-ENV-infected L/D^d cells as described (43).

Reverse Transcription and PCR—RNA was extracted from transfected cells with RNeasy (Qiagen), and mRNA was reverse-transcribed using oligo-dT primers with Sensiscript (Qiagen). It was then amplified with Taq DNA polymerase (Applied Biosystems) using primers specific for human TPPII (forward, CAC TGC AGT TAT AGC AGC AAA AGT; reverse, TAT TTC AAG GAG GAC TGA ACA TCA) to yield a 579-bp band or for murine TPPII (reverse, CCA ACG AGC AAT ACA AAA TTC A) to give a 519-bp band and separated by agarose electrophoresis. Expression levels of the housekeeping gene encoding β -actin were tested as control (human: forward, GCA TGG AGT CCT GTG GCA TCC; reverse, GGT GTA ACG CAA CTA AGT CAT AG; mouse: forward, GTG GGC CGC TCT AGG CAC CAA; reverse, CTC TTT GAT GTC ACG CAC GAT TTC).

Small Interfering RNA (siRNA)—The antisense strand of siRNA duplexes targeting human TPPII had the published sequence (17) (Qiagen). For silencing murine TPPII, the following siRNA duplexes with two 3' end overhang dT nucleotides were used separately or pooled: 5'-UCU UCU ACG AGU UUG GUU GdTdT-3' (#1), 5'-AGA UAC CAC GGU UAU UUC CdTdT-3' (#2), 5'-GAA UCU GUA AUC GAA UUG UdTdT-3' (#3), 5'-UAG UUG ACA AGA UCA CAC UdTdT-3' (#4) (Dharmacon). As negative controls, siRNA duplex for human ERAP1 (19) (Qiagen) and for human TPPII was used in murine cells, and siRNA for mouse ERAAP was used for human cells (19) using each time the same amount as of the experimental siRNA. For transfection, 293K^d, L/D^d, or L/K^d cells ($3\text{--}5 \times 10^6$ /sample) were resuspended in 100 μ l of nucleofection reagent V, V, or T (Amaxa), respectively. From siRNA to human TPPII and from the pool of four siRNAs to mouse TPPII 3 μ g of siRNA was added, whereas 6 μ g of siRNA was added from each individual siRNA to murine TPPII. The three cell lines were transfected using Nucleofector II (Amaxa) programs A23, T25, or U33, respectively, between 3 and 6 days before performing the enzymatic assay or infecting with rVVs and performing the ICS assay. Occasionally, cells were transfected again at day 6 and tested 3 days later in ICS, with similar results. Efficiency of transfection was more than 95% of cells, as assessed with fluorescein isothiocyanate-labeled or Alexa 647-labeled control siRNA (Qiagen). Percent specific inhibition was calculated with respect to control cells transfected with control siRNA as indicated.

Enzyme Assays—L/K^d, L/D^d, and 293K^d cells ($2\text{--}5 \times 10^4$ /sample) were washed and then lysed at 4 °C in 500 μ l of 50 mM Tris, pH 7.4, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM ATP, 1% Triton-X100 (5). Fluorogenic TPPII substrate Ala-Ala-Phe-4-methyl-7-coumarilamide (AAF-amc) (7, 44) was added to 100 μ M (5), and the reaction was incubated for 30–60 min at 37 °C in a fluorimeter CaryEclipse (Varian). Measurements were taken every minute for 1 s with excitation at 370 nm and emission at 430 nm and are presented as arbitrary units. Blank reactions without lysates were subtracted, and percent inhibition was calculated with respect to control cells either untreated with inhibitors or transfected with control siRNA as indicated.

RESULTS

NP_{147–155} Epitope Processing from Full-length NP Is Susceptible to AAF-cmk in the Presence of Functional Proteasomes—We studied cytosolic TPPII as a candidate antigen processing enzyme for influenza virus NP. This enzyme (6, 7) as well as marginally others (45) is susceptible to treatment of living cells with the covalent irreversible inhibitor AAF-cmk, but proteasomes are not (7).⁷ When NP from influenza virus strain PR8 (scheme in Fig. 1) is expressed in L/K^d cells by a rVVs, the NP_{147–155} epitope is presented to K^d-restricted CTL. Treatment of NP_{PR8}-expressing cells with AAF-cmk completely blocked NP_{147–155} presentation in an ICS assay (Fig. 2A). As a control, endogenous presentation by D^d of the proteasome-dependent human immunodeficiency virus envelope (ENV) epitope R10I (40) was not affected. The same was true for exogenous presentation of limiting amounts of the NP_{147–155} synthetic peptide. NP_{PR8} protein expression in the presence of this inhibitor was controlled by Western blot (Fig. 2E, lanes 2). These three controls were routinely included in each experiment throughout this report. Thus, an AAF-cmk-susceptible proteolytic activity is indispensable for NP epitope processing from full-length NP in the presence of a functional proteasome.

NP_{147–155} Epitope Processing Requires the AAF-cmk-susceptible Proteolytic Activity Also When Proteasomes Are Blocked with LC—The NP epitope is presented from several of the NP variants with somewhat different efficiencies (13, 14). NP_{NT60} has a 6% sequence difference, notably at aa 146, immediately upstream of the epitope. An engineered secretory variant of NP_{PR8}, SNP_{PR8}, contains a signal peptide for translocation into the ER (Fig. 1). Presentation of NP_{147–155} from NP_{NT60} or SNP_{PR8} to the same CTL cannot be achieved under the same infection conditions used for NP_{PR8} (Fig. 2B, open bars). Therefore, antigen presentation was potentiated by treating cells with the proteasome inhibitor LC, which blocks the presumed proteasome destructive activity (Fig. 2B, light gray bars). Under these conditions, the TPPII inhibitor AAF-cmk reduced antigen presentation from all three full-length proteins by ~65% (Fig. 2B, black bars), whereas exogenous presentation of limiting amounts of the synthetic peptide was unaffected by the mix of both inhibitors (Fig. 2C). The most stringent control for the specificity of the inhibitory effect was the lack of effect of the combined inhibitory treatment on limited endogenous presen-

⁷ S. Guil, M. Rodríguez-Castro, F. Aguilar, E. M. Villasevil, L. C. Antón, and M. Del Val, unpublished results.

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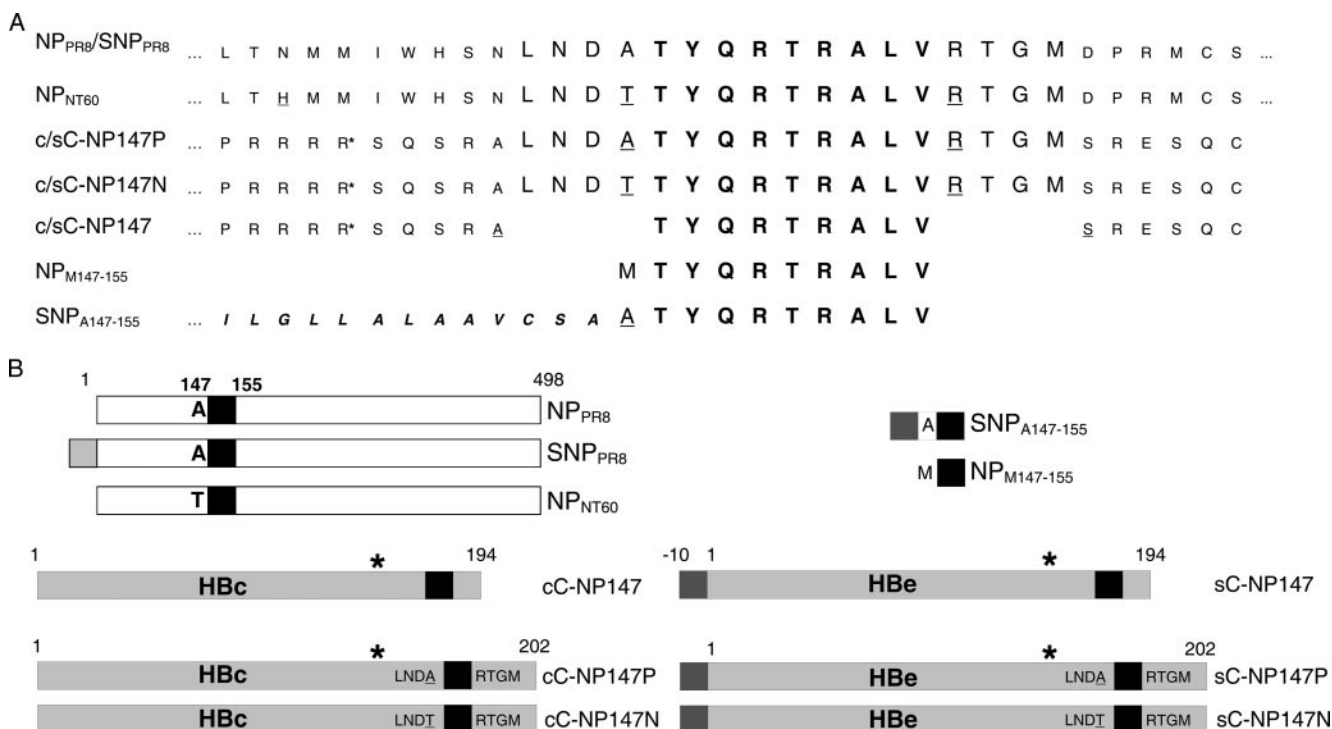


FIGURE 1. Relevant sequence (A) and schematic representation (B) of native and chimeric proteins and miniproteins employed in this study. The epitope is shown in *boldface*. Relevant flanking residues are *underlined*, and signal sequence is *italicized*. The *black box* always represents the NP₁₄₇₋₁₅₅ epitope. Leader peptides are denoted by *boxes* at the amino termini of several constructs. The chimeric proteins are denoted *c* or *s* for cytosolic or secretory, *C* for C_i insertion of the NP₁₄₇₋₁₅₅ epitope, and none, *P*, or *N* for native flanking sequences not included or 4 aa on each side derived from NP_{PR8} or NP_{NT60}, respectively. Maturation of the chimeric proteins involves cleavage by furin at the *asterisks*.

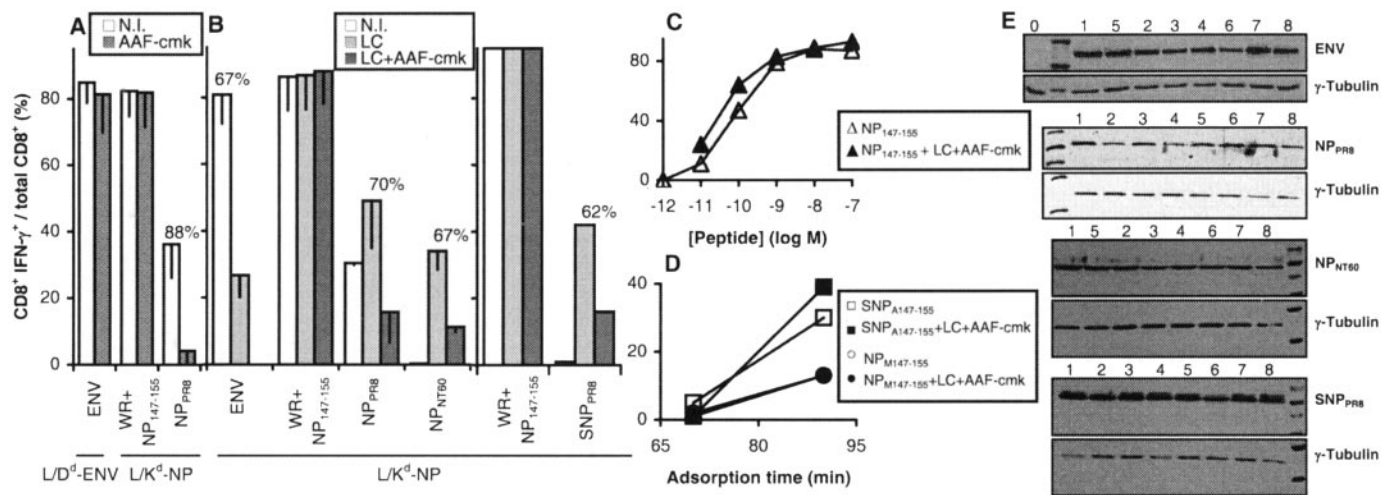


FIGURE 2. NP₁₄₇₋₁₅₅ epitope processing from full-length NP is susceptible to AAF-cmk in the presence and in the absence of a fully functional proteasome. A, L/K^d cells were infected with rVV-NP_{PR8} at a m.o.i. of 30 for 2 h in the absence (*white bars, N.I.*) or presence of AAF-cmk (*dark gray bars*) and assayed by ICS for NP₁₄₇₋₁₅₅ presentation. As a control, peptide-loaded WR-infected cells were used. As a further control, L/D^d cells were infected with rVV-ENV for 2 h at a m.o.i. of 10 and used for activation of the ENV-specific CD8⁺ T lymphocyte line. In *panels A and B*, numbers on the *tops of pairs of bars* indicate percent specific inhibition by AAF-cmk. *Error bars, S.D.* B, L/K^d cells were infected with rVVs encoding the indicated proteins at a m.o.i. of 30 for 2 h in the presence of LC (*light gray bars*) or LC and AAF-cmk (*black bars*) and assayed by ICS. As a control, peptide-loaded WR-infected cells were used. The *two graphs* in *panel B* represent different experiments. C, L/K^d cells were incubated for 1 h with different NP₁₄₇₋₁₅₅ peptide concentrations in the presence (\blacktriangle) or absence (\triangle) of LC and AAF-cmk and then assayed by ICS. D, L/K^d cells were infected with minigenes SNP_{A147-155} (\square , \blacksquare) or NP_{M147-155} (\circ , \bullet) at a m.o.i. of five for the indicated times in the absence (*open symbols*) or presence of LC and AAF-cmk (*closed symbols*) and assayed by ICS at an effector to target ratio of 10:1. E, L/K^d cells were infected at a m.o.i. of 30 for 2 h with the indicated rVVs in the absence of inhibitors (*lane 1*), in the presence of AAF-cmk (*lane 2*), butabindide (*lane 3*), LeuSH (*lane 4*), LC (*lane 5*), LC and AAF-cmk (*lane 6*), LC and butabindide (*lane 7*), or LC and LeuSH (*lane 8*). L/D^d cells were similarly infected with rVV-ENV. As a control, cells were infected with WR (*lane 0*). Proteins were detected with a purified rabbit antibody to the conserved NP C_i peptide 488–498 or with a monoclonal antibody to ENV. Expression was controlled with an antibody to γ -tubulin (Sigma). A lane with molecular weight markers is included.

tation from a rVV that encodes the minimal epitope NP_{M147-155} and expresses it in the cytosol as well as on an ER-targeted minigene construct (Fig. 2D) (Fig. 1). Of note, it will be shown

later that these limited levels of minigene presentation can actually be blocked by appropriate inhibitors (see Fig. 6A). Presentation was deemed endogenous because it was susceptible to

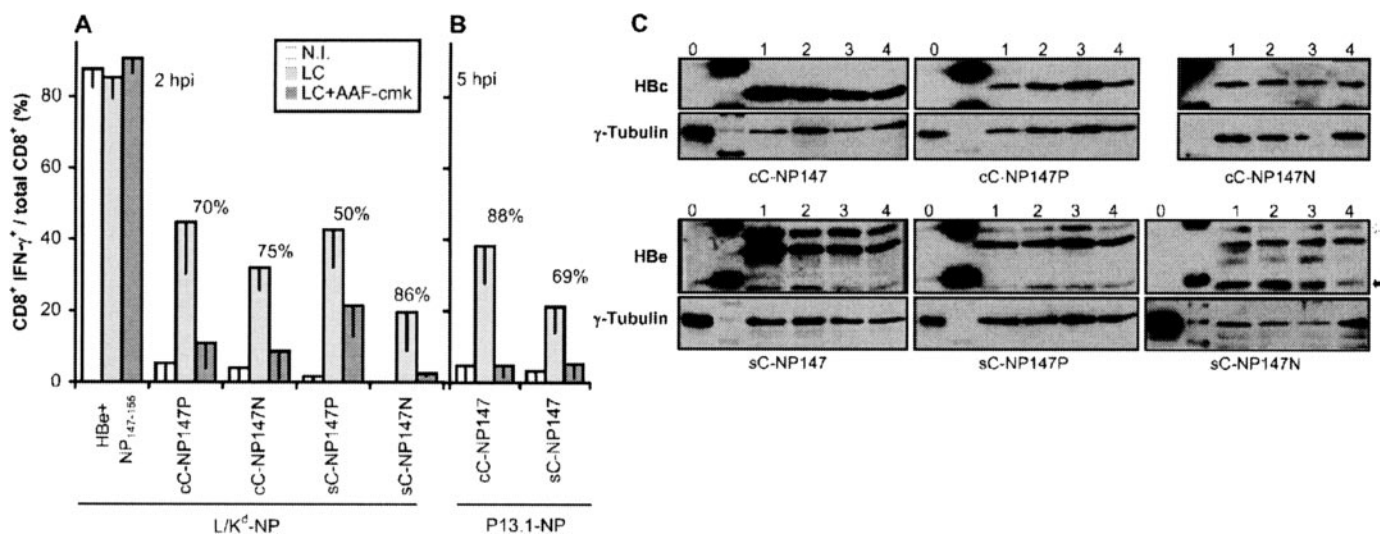


FIGURE 3. NP₁₄₇₋₁₅₅ epitope processing from different protein contexts is mediated by the AAF-cmk-susceptible proteolytic activity in the absence of functional proteasomes. A, L/K^d cells were infected at a m.o.i. of 30 for 2 h (2 hpi) with rVVs encoding the indicated proteins that contain the NP epitope in an HBc/HBe context and flanked by the native NP_{PR8} or NP_{NT60} sequences (white bars, N.I.), in the presence of LC (light gray bars), or LC and AAF-cmk (black bars) and assayed by ICS for NP₁₄₇₋₁₅₅ presentation. As a control, peptide-loaded cells that were infected at a m.o.i. of 30 for 2 h with control rVV-HBc were used. B, P13.1 cells were infected at a m.o.i. of 30–90 for 5 h (5 hpi) with rVVs encoding the indicated proteins that contain the naked NP epitope in an HBc/HBe context in the presence of LC (light gray bars) or LC and AAF-cmk (black bars) and assayed by ICS. Numbers on the tops of pairs of bars indicate percent specific inhibition by AAF-cmk. As control, 4% inhibition by AAF-cmk of synthetic peptide presented by LC-treated cells was observed. C, L/K^d cells were infected at a m.o.i. of 30 for 2 h with the indicated rVVs or with a control rVV (lane 0) in the absence of inhibitors (lane 1), in the presence of LC alone (lane 2), LC and AAF-cmk (lane 3), or LC and LeuSH (lane 4). Non-flanked constructs were infected for 7 h. Proteins were detected with a HBC/HBe-specific rabbit antiserum (37). A lane with molecular weight markers is included. The white arrow denotes the glycosylated bands in the secretory constructs, and the black arrow denotes mature, lower molecular weight bands after removal of C₁ by furin.

brefeldin A treatment (data not shown). Thus, treatment with AAF-cmk in the presence of LC was specifically blocking antigen presentation from all three full-length NPs.

The AAF-cmk-susceptible Proteolytic Activity Is Required for NP₁₄₇₋₁₅₅ Epitope Processing from Different Protein Contexts—We next tested antigen processing requirements when the NP₁₄₇₋₁₅₅ epitope was expressed in unrelated protein contexts, those of the cytosolic or secretory variants of the hepatitis B virus core protein (Fig. 1). In these chimeras, the NP epitope is also destroyed by a proteasome-mediated event, both when only the epitope sequence is inserted and also when it carries the natural flanking sequences from either NP_{PR8} or NP_{NT60}.⁸ Therefore, these chimeras also need potentiation by LC for efficient presentation to specific CD8⁺ T cells (Fig. 3A). When the epitope was expressed by the chimeras with the NP flanking sequences in either carrier protein, antigen presentation potentiated by LC was equally susceptible to treatment with the TPPII inhibitor, with an average inhibition of ~70% (Fig. 3A, black bars). Again, protein expression was controlled (Fig. 3C).

LC-potentiated presentation from the constructs that lacked the native flanking sequences was very inefficient in L/K^d cells and required strong (m.o.i. 90) and long (7 h) infection conditions. We, therefore, used more permissive cells, which permitted m.o.i. of 30 and 5 h, and found that antigen presentation from non-flanked constructs was also fully AAF-cmk-susceptible in the presence of LC (Fig. 3B). This indicates that, acting independently of the LC-susceptible activities of the proteasome, the AAF-cmk-susceptible protease was proficient for liberating the NP epitope even from a fully unrelated protein context, that of HBc/HBe.

Gene Silencing of TPPII Impairs Presentation of the NP₁₄₇₋₁₅₅ Epitope—Because AAF-cmk is not a well characterized inhibitor, we sought to examine the involvement of candidate TPPII by other means. The approach used before (10, 17, 18) with human TPPII-specific siRNA was used. A pool of four newly designed siRNAs to target murine TPPII was transfected into mouse L/K^d cells and shown by reverse transcription-PCR to have a similar inhibitory effect on murine TPPII gene expression (Fig. 4A). Transfection of human- or mouse-specific siRNAs into the relevant cells had also an average inhibitory effect of 68 ± 13% or 65 ± 15%, respectively, on hydrolysis of the fluorogenic substrate AAF-amc (see Fig. 4B for a representative experiment). TPPII is responsible for this hydrolyzing activity (7). It was then tested whether transfection of TPPII-specific siRNAs prevented NP antigen presentation in an ICS assay. Longer infection times were used than before to allow an inhibition to be observed when the proteasome function was intact. As shown in Fig. 4C for human 293 cells transfected with K^d and in Fig. 4E (p) for murine cells, presentation of NP_{NT60} was impaired in both species, whereas that of NP_{PR8} was only marginally affected.

Because the inhibitory effect of siRNA was dose-dependent and using higher concentrations of the murine-specific siRNA pool was not technically feasible, the four individual murine TPPII-specific siRNAs were next used separately at the highest concentration. All were similarly effective in blocking the transfected cell AAF-amc hydrolyzing activity (Fig. 4D). In addition, antigen presentation from NP_{NT60} was inhibited by 60–70%, as was the case before for the pooled siRNAs (Fig. 4E). Genetic inhibition did not have any effect on ENV presentation, in accordance with its resistance to inhibition by AAF-cmk. Again, NP_{PR8} was more resistant to inhibition, with values of

⁸ S. Guil, P. de León, L. C. Antón, and M. Del Val, manuscript in preparation.

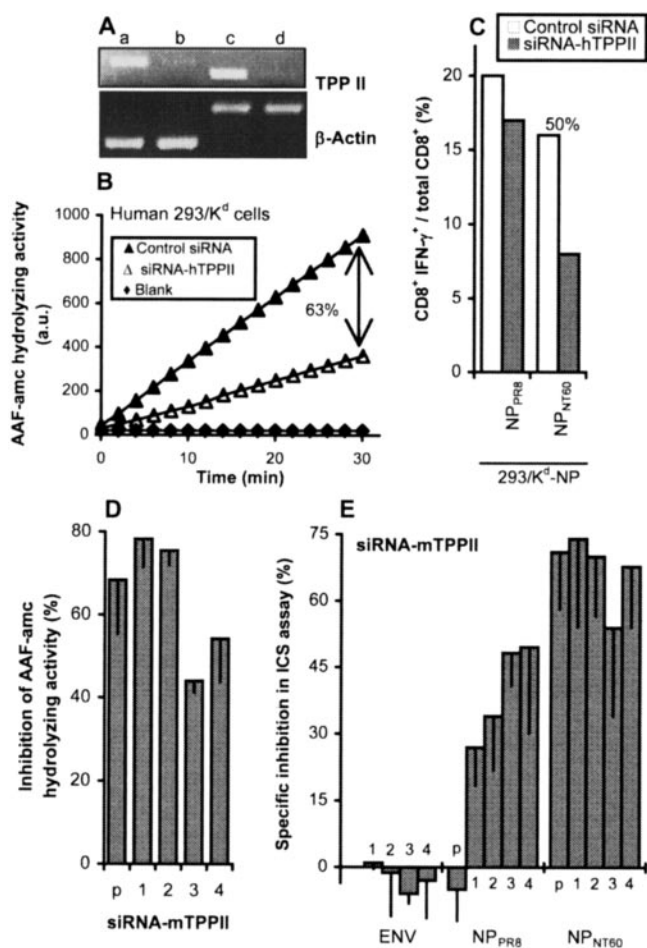


FIGURE 4. Gene silencing of tripeptidyl-peptidase II impairs presentation of the NP₁₄₇₋₁₅₅ epitope. *A*, human 293K^d cells were transfected with control siRNA (siRNA to murine ERAAP) (*a*) or with human TPPII siRNA (*b*) and murine L/K^d cells were transfected with control siRNA (human ERAP1 siRNA) (*c*) or with a pool of four siRNAs to murine TPPII (*d*) and 3 days later analyzed by reverse transcription-PCR for expression of human TPPII gene (*a* and *b*) or murine TPPII gene (*c* and *d*) (upper panel). β -Actin gene expression served as control (lower panel). *B*, human 293K^d cells that had been transfected 3 days before with control siRNA (siRNA to murine ERAAP) (\blacktriangle) or with human TPPII siRNA (siRNA-hTPPII, \triangle) were lysed, and TPPII activity was assayed with the fluorogenic substrate AAF-amc. Percent inhibition is indicated with respect to control cells transfected with control siRNA. Control substrate without cell lysate is shown (\blacklozenge). Plateau inhibition was achieved 2 days after transfection and remained stable at least until day 6. *a.u.*, arbitrary units. *C*, human 293K^d cells that had been transfected 3 days before with control siRNA (siRNA to murine ERAAP) (white bars) or with siRNA-hTPPII (dark gray bars) and assayed in panels *A* and *B*, were infected with NP_{PR8} for 2 h or with NP_{NT60} for 3 h (longer than in Fig. 2) at a m.o.i. of 30 and assayed by ICS for NP₁₄₇₋₁₅₅ presentation. Numbers on the tops of pairs of bars indicate percent specific inhibition. *D*, murine L/K^d cells that had been transfected before with each of four different siRNA-mTPPII as indicated or with the pool (*p*) were lysed, and TPPII activity was assayed with the fluorogenic substrate AAF-amc. Percent specific inhibition was calculated with respect to cells transfected with control siRNA (siRNA-hTPPII). *E*, murine L/D^d or L/K^d cells as indicated that had been transfected before with a pool of four different siRNA-mTPPII (*p*) or with each individual siRNA-mTPPII as indicated were infected with rVV-ENV (L/D^d cells) or infected with NP_{PR8} for 2 h or with NP_{NT60} for 3 h (longer than in Fig. 2) at a m.o.i. of 30 and assayed by ICS. Percent specific inhibition was calculated with respect to cells transfected with control siRNA (siRNA-hTPPII). Average % in ICS was 45 for ENV, 36 for NP_{PR8}, and 20 for NP_{NT60}.

30–45% inhibition of antigen presentation. It is worth recalling that, in the absence of LC, presentation of NP_{PR8} is significantly stronger than that of NP_{NT60} and, thus, presumably more difficult to block. Collectively, given the high specificity of the

genetic silencing approach, the results strongly implicate TPPII in the NP antigen processing pathway in living cells.

TPPII Is Crucial in the Generation of the NP₁₄₇₋₁₅₅ Epitope—We turned then to butabindide, an indol-based analog of a natural substrate of TPPII, that acts as the best and most specific competitive inhibitor known for TPPII (39). Butabindide does not affect proteasome-mediated degradation nor all other steps relevant for endogenous peptide presentation (10, 18). In support of the use of the fluorogenic substrate AAF-amc as a measure of TPPII activity, butabindide blocked most of the cellular AAF-amc-hydrolyzing activity (on average $74 \pm 12\%$), which was in turn completely blocked by AAF-cmk (Fig. 5A).

In our system butabindide affected neither exogenous peptide nor endogenous ENV presentation (Fig. 5B). It blocked though presentation to specific CTL of the NP₁₄₇₋₁₅₅ epitope from NP_{PR8} in a dose-dependent fashion, even in the presence of functional proteasomes (Fig. 5C). Because fetal bovine serum contributes to inactivation of butabindide in solution (10), inhibition was more prominent in the absence of serum (Fig. 5C). Inhibition of NP_{NT60} by butabindide was also observed (Fig. 5D) and found to be dose-dependent, reaching maximal values under conditions of limited antigen expression (Fig. 5E). Presentation of NP_{PR8} and NP_{NT60} was prevented on average by 68 or 61%, respectively, even in the presence of a functional proteasome. The same selective inhibition of antigen presentation from both NP_{PR8} and NP_{NT60} was true in human 293K^d cells (Fig. 5F). The data demonstrate that TPPII is indeed the AAF-cmk-susceptible proteolytic enzyme that is crucial for the generation of this NP epitope that is presumably destroyed by proteasomes.

Trimming by ER Aminopeptidases of the TPPII-generated Product—Although TPPII has a wide substrate specificity, it was nevertheless questionable that it would be able to precisely excise the epitope from several distinct protein contexts. One possibility is that excision is not so accurate and that amino-terminal variants of the minimal NP epitope are tolerated by the K^d molecule and the T-cell receptor, as shown for the ENV natural peptides presented by D^d that we have used as control throughout this report (47). The most proximal amino-terminal aa in the NP_{PR8} sequence is Ala. This is also the only aa that remains in the ER-targeted miniprotein SNP_{A147-155} after removal of the leader peptide by the signal peptidase. To test if presentation of the Ala-extended NP epitope is feasible, we treated cells expressing limited amounts of the miniprotein with LeuSH. LeuSH is a potent inhibitor of microsomal metallo-aminopeptidases that blocks ERAAP (19, 20), the only epitope trimming aminopeptidase described so far in the ER of mouse cells. As depicted in Fig. 6A, presentation from the ER-targeted minigene was fully blocked by LeuSH. The same inhibitory effect of LeuSH on presentation from the cytosolic miniprotein (Fig. 6A), which has an initiating methionine, could be explained by the fact that methionyl-aminopeptidases are also metallopeptidases (48). Thus, precise trimming to the final size NP₁₄₇₋₁₅₅ nonamer seems to be vital, and as little as one amino-terminal Ala or Met severely interferes with antigen presentation. The ER-targeted miniprotein was also presented in cells deficient in the transporters associated with antigen processing, and this was also impaired by LeuSH (Fig. 6B). Therefore,

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trimming involves at least an ER metallo-aminopeptidase, probably ERAAP.

When the same experiment was performed with either NP_{PR8} or NP_{NT60}, a substantial implication of a LeuSH-susceptible aminopeptidase could also be demonstrated in the presence of functional proteasome (Fig. 6C) as well as under conditions where proteasomes are inhibited by LC to allow antigen presentation, as presentation was reduced by ~70% in the presence of the ERAAP inhibitor (Fig. 6D). Combined treatment with LC and LeuSH did not have any negative effect on exogenous peptide presentation over an entire dose-response curve (Fig. 6E). Treatment of infected cells with LeuSH did not affect TPPII activity (Fig. 6H).

Presentation from the HbC/HbE constructs was also found to be susceptible to LeuSH in all cases (Fig. 6, F–G) while somewhat affecting protein expression of the secretory constructs (Fig. 3C, lanes 4). Comparing identical assay conditions, sensitivity to LeuSH in the presence of LC was reproducibly lower when the amino-terminal flanking aa was Ala (as in the two full-length PR8s and in the two PR8-flanked HbC/HbE constructs) (inhibitions of 48–67%, *n* = 4), than when it was Thr (full-length NP_{NT60} and the two NP_{NT60}-flanked constructs) (84–92%, *n* = 3) (Fig. 6, D and F). In conclusion, as is generally the case for proteasomes and a number of epitopes (19, 49), TPPII needs cooperation from ER aminopeptidases, probably ERAAP, to efficiently generate the final NP_{147–155} epitope.

DISCUSSION

By studying antigen processing and presentation to CD8⁺ T lymphocytes of one remarkable influenza virus NP epitope that is destroyed by a proteasome-mediated event, we show that the cytosolic protease TPPII, with the aid of trimming aminopeptidases, probably ERAAP, is a key proteolytic activity required for its generation, acting independently of the LC-susceptible proteasome activity. Presentation of NP_{PR8} and NP_{NT60} was prevented by 88% by AAF-cmk, by up to 50 or 74% by TPPII-specific siRNA, respectively, and by 68 or 61% by the TPPII-specific inhibitor butabindide, respectively, even in the presence of a functional proteasome.

Processing of chimeric constructs expressing the NP_{147–155} epitope flanked on each side by four native NP aa in an unrelated protein context was almost completely dependent on the AAF-cmk-susceptible protease, whereas the activity of the LC-

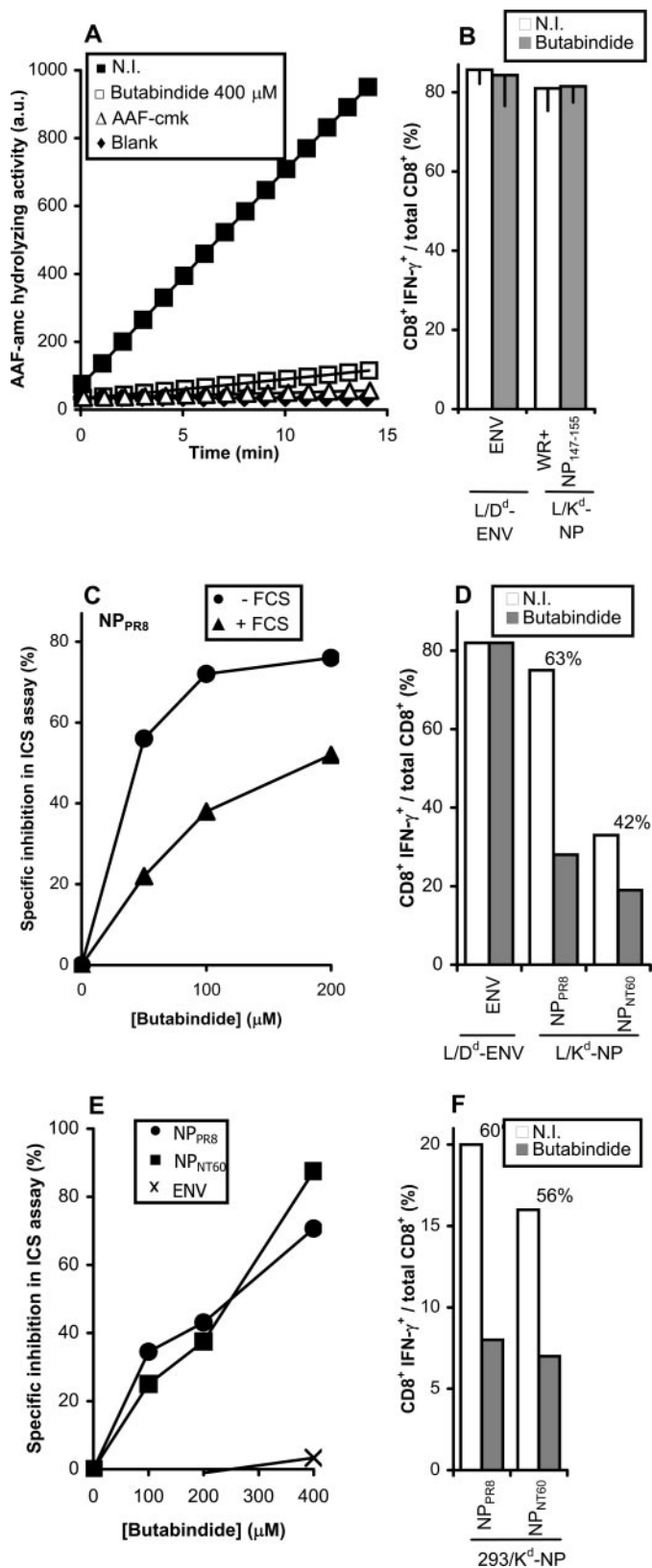


FIGURE 5. Tripeptidyl-peptidase II is crucial in the generation of the NP_{147–155} epitope. A, L/K^d cells were untreated (N.I.) or treated for 1 h as indicated and lysed, and TPPII activity was analyzed with the fluorogenic substrate AAF-amc. Control substrate without cell lysate is shown (♦) a.u., arbitrary units. B, L/D^d cells were infected with rVV-ENV in the absence (white bars) or presence of 200 μM butabindide (dark gray bars) and assayed by ICS for ENV presentation. Similarly, butabindide-treated peptide-loaded WR-infected

L/K^d cells were assayed by ICS for NP_{147–155} presentation. C, L/K^d cells were infected with rVV-NP_{PR8} at a m.o.i. of 30 for 2 h in the presence of increasing concentrations of butabindide in the absence (●) or presence (▲) of fetal calf serum (FCS), and assayed by ICS. Percent specific inhibition was calculated with respect to untreated cells. D, L/K^d cells were infected with NP_{PR8} for 2 h or with NP_{NT60} for 3 h at a m.o.i. of 30 in the absence (white bars) or presence of 400 μM butabindide (dark gray bars) and in the absence of serum and assayed by ICS. As control, L/D^d cells were infected with rVV-ENV and tested with ENV-specific CTL. E, L/K^d cells were infected with NP_{PR8} for 2 h (●) or with NP_{NT60} for 3 h (■) at a m.o.i. of 30 in the absence of serum and in the presence of increasing butabindide concentrations and assayed by ICS. Control L/D^d cells infected with ENV (×) were treated identically and assayed with ENV-specific CTL. Percent specific inhibition was calculated with respect to untreated cells. F, human 293K^d cells were infected with NP_{PR8} for 2 h or with NP_{NT60} for 3 h at a m.o.i. of 30 in the absence (white bars) or presence of 100 μM butabindide (dark gray bars) and in the absence of serum and assayed by ICS. In panels D and F, numbers on the top of pairs of bars indicate percent specific inhibition. Data in C–F are representative experiments.

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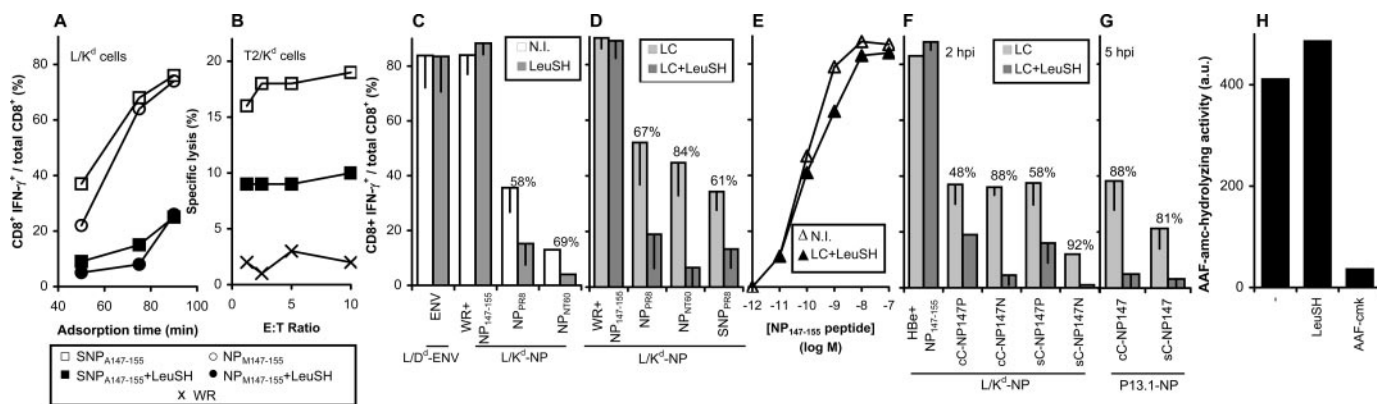


FIGURE 6. Trimming by aminopeptidases of the TPPII-generated product. A, L/K^d cells were infected with minigenes SNP_{A147-155} (□, ■) or NP_{M147-155} (○, ●) at a m.o.i. of 5 for the indicated times in the absence (*open symbols*) or presence of LeuSH (*closed symbols*) and assayed by ICS for NP₁₄₇₋₁₅₅ presentation at an effector to target (E:T) ratio of 10:1. B, TAP-deficient T2/K^d cells were infected with minigene SNP_{A147-155} (□, ■) or WR (×) at a m.o.i. of 3 for 1 h in the absence (*open symbols*) or presence of LeuSH (*closed symbols*) and assayed by cytotoxicity for NP₁₄₇₋₁₅₅ presentation at different E:T ratios. C, L/K^d cells were infected at a m.o.i. of 30 with rVV-NP_{PR8} for 2 h or with NP_{NT60} for 3 h in the absence (*white bars, N.I.*) or presence of LeuSH (*dark gray bars*) and assayed by ICS. As a control, peptide-loaded WR-infected cells were used. Similarly, L/D^d cells were infected with rVV-ENV and used for activation of ENV-specific CD8⁺ T lymphocytes. D, L/K^d cells were infected with rVVs encoding the indicated proteins at a m.o.i. of 30 for 2 h in the presence of LC (*light gray bars*) or LC and LeuSH (*black bars*) and assayed by ICS. As a control, peptide-loaded WR-infected cells were used. E, L/K^d cells were incubated for 1 h with different NP₁₄₇₋₁₅₅ peptide concentrations in the presence (▲) or absence (△, N.I.) of LC and LeuSH and then assayed by ICS. F, L/K^d cells were infected at a m.o.i. of 30 for 2 h with rVVs encoding the indicated proteins that contain the NP epitope in an HBC/HBe context and flanked by the native NP_{PR8} or NP_{NT60} sequences in the presence of LC (*light gray bars*) or LC and LeuSH (*black bars*) and assayed by ICS. As a control, peptide-loaded cells that were infected at a m.o.i. of 30 for 2 h (2 hpi) with control rVV-HBe were used. G, P13.1 cells were infected at a m.o.i. of 30 to 90 for 5 h (5 hpi) with rVVs encoding the indicated proteins that contain the naked NP epitope in an HBC/HBe context in the presence of LC (*light gray bars*) or LC and LeuSH (*black bars*) and assayed by ICS. In panels C, D, F, and G, numbers on the tops of pairs of bars indicate percent specific inhibition by LeuSH. H, L/K^d cells were infected at a m.o.i. of 10 with rVV-NP_{PR8} for 5 h in the absence (-) or presence of LeuSH or AAF-cmk, as indicated, and lysed, and TPPII activity was analyzed with the fluorogenic substrate AAF-amc. Similar results were obtained when the inhibitors were included only in the lysates of untreated infected cells. *a.u.*, arbitrary units.

susceptible proteasome was detrimental. Non-flanked constructs were presented much less efficiently. Therefore, the results suggest that these 4 aa on each side of the epitope provide sites for efficient TPPII cleavage. It is tempting to speculate that one of these sites could be after position 155, producing the correct C_t of the epitope (Fig. 7). The obligatory role of proteasomes in antigen processing seems to be generating the correct C_t of epitopes. In these experiments proteasomes are inhibited by LC, which very effectively inhibits the chymotryptic activity that would generate the correct C_t of this epitope by cleaving after Val-155. The main processing activity for NP₁₄₇₋₁₅₅, TPPII, which is also an endopeptidase, could perform this task as suggested (10). Indirect evidence from *in vitro* digestions of peptide substrates with purified and LC-inhibited 20 S proteasomes suggests that an altered proteasome activity (27) might also contribute to processing in living cells, but only secondarily, as this is strongly inhibited by AAF-cmk both in this report and in that of Wherry *et al.* (27).

This publication (27) reported results that seem to conflict with those presented here. Whereas in both reports LC enhanced presentation and AAF-cmk blocked it, we reached apparently different conclusions based mainly on results obtained with the TPPII-specific inhibitor butabindide and with gene silencing of the protease. Some differences in the experimental approach used in both reports may account for some of the discrepancies. First, Wherry *et al.* (27) infected for longer periods and added the unstable butabindide less frequently than we did (as recommended (10)), which may leave a window of enzyme activity enough to allow for efficient presentation. Regarding gene silencing, Wherry *et al.* (27) reported that only 50–60% of cells were transfected, and thus, still many cells may present antigen, whereas we reached values of 95%

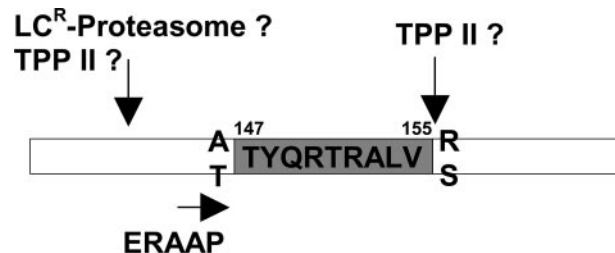


FIGURE 7. A model for antigen processing of influenza virus NP protein. The model is compatible with the results reported in this article, although other possibilities exist. TPPII endopeptidase activity would produce the C_t of the final NP₁₄₇₋₁₅₅ peptide, although the site of cleavage does not concur with known TPPII specificity. TPPII may also produce a previous necessary amino-terminal endoproteolytic cleavage. ERAAP would trim the amino terminus to the final size. Participation of additional proteases, perhaps including LC-resistant activities of proteasomes, is not excluded.

leading to inhibition of antigen presentation. These differences may explain why we positively identify TPPII involvement. Accordingly, our model of the sequential pathway of NP₁₄₇₋₁₅₅ antigen processing includes TPPII as a key activity among others (Fig. 7), whereas Wherry *et al.* put weight only on the contribution of the altered proteasome, which we do not exclude.

We have defined as well the requirement for trimming aminopeptidases in this pathway. Even proteasomes need help from trimming enzymes. A paradigm is the model epitope OVA₂₅₇₋₂₆₄ presented by K^b. It can be generated precisely and efficiently by proteasomes *in vitro* (50), and yet proteasomes need help from ERAAP in living cells for efficient ovalbumin presentation (18, 49). Likewise, intermediate products generated by TPPII can profit from aminopeptidases to generate final size MHC class I ligands. ERAAP, previously implicated in antigen processing

(19), is the most likely candidate to cooperate with TPPII, because it is LeuSH-sensitive and because it resides in the ER, where it would trim all proteins studied here, specifically the secretory miniprotein.

The elevated efficiency of presentation of NP_{PR8} over NP_{NT60} could explain the higher difficulty to inhibit NP_{PR8} , SNP_{PR8} , and the NP_{PR8} -like chimeras with the ERAAP inhibitor. Because NP_{PR8} and NP_{NT60} differ at the -1 residue, which is an obligatory target of ERAAP, one hypothesis is that strain-specific differences in processing efficiency could be due, at least in part, to ERAAP cleavage specificity. Additionally, the higher difficulty to inhibit NP_{PR8} with murine and human TPPII-specific siRNA may suggest that TPPII cleavages also contribute to the strain-specific differences in processing efficiency. For TPPII to act fully independently of the proteasome, it would have to produce the necessary amino-terminal distal cut in Fig. 7. This would imply that its endoproteolytic activity would have to reach far within a protein, a notion maybe difficult to reconcile with its need for a free amino terminus in the substrate (44). TPPII can cleave 30-aa-long peptides (18), but further research is needed to assess if TPPII can directly act on full-length proteins, on defective ribosomal products, DRiPs (46), or only as a downstream protease on preprocessed products.

It should be emphasized that our data do not exclude a role for proteasomes in $NP_{147-155}$ epitope generation. Even in the presence of LC, residual caspase-like proteasome activity (8) or altered proteasome activity (16, 27) might be required for antigen processing, maybe in a sequential pathway involving proteasomes and TPPII (10) (Fig. 7). If the LC-resistant activities of the proteasome were indeed involved and if they were inefficient in the absence of the remaining LC-susceptible activities, they might generate longer products than usual. Because TPPII is the rate-limiting enzyme *in vivo* for hydrolyzing long peptides (10, 18), this may explain why TPPII is such a relevant enzyme for $NP_{147-155}$. On the other hand, it is clear that in all cases the main action of proteasomes or at least of their inhibitor-sensitive activities is destructive rather than constructive, as presentation of all full-length and chimeric proteins was enhanced by treatment with the proteasome inhibitor.

The sequential complementary action of TPPII and the trimming aminopeptidase in $NP_{147-155}$ antigen processing would also predict TPPII involvement in generation of yet more MHC class I ligands. It is unclear whether this alternative pathway generates mainly low abundance, yet immunodominant epitopes such as $NP_{147-155}$ (14). Further work will be necessary to establish whether processing of NP by TPPII represents an exceptional case among the more than 10 epitopes susceptible or independent of proteasomes or else indicates an important alternative mechanism for generation of MHC class I ligands.

TPPII was previously suggested to generate an epitope presented by HLA-A3 and -A11 (17). Surface expression of these allotypes and that of -B35 is particularly resistant to proteasome inhibitors (28), and they share a peptide motif with a basic C_t (26). However, neither the $NP_{147-155}$ epitope nor K^d share these features. Thus, the suitability of TPPII for antigen processing appears to be more general than previously thought.

Our results show the plasticity of the cell's assortment of proteases for providing ligands for MHC class I molecules and recognition by $CD8^+$ T cells. It is interesting to note that we show that TPPII, which compensates for some of the functions of the proteasome in cell metabolism, also compensates for some of the proteasome deficits in antigen processing for T-cell immunosurveillance. Although undoubtedly the proteasome will be instrumental in producing many epitopes, as it is the single most abundant protease in the cell, it is also becoming increasingly clear that the cumulative action of all other cellular proteases can significantly contribute to generating and trimming peptides for presentation by MHC class I molecules and detection and elimination of infected cells.

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**Need for Tripeptidyl-peptidase II in Major Histocompatibility Complex Class I
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