Re-evaluating the Generation of a "Proteasome-Independent" MHC Class I-Restricted CD8 T Cell Epitope

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Re-evaluating the Generation of a “Proteasome-Independent” MHC Class I-Restricted CD8 T Cell Epitope

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The proteasome is primarily responsible for the generation of MHC class I-restricted CTL epitopes. However, some epitopes, such as NP<sub>147-155</sub> of the influenza nucleoprotein (NP), are presented efficiently in the presence of proteasome inhibitors. The pathways used to generate such apparently “proteasome-independent” epitopes remain poorly defined. We have examined the generation of NP<sub>147-155</sub> and a second proteasome-dependent NP epitope, NP<sub>50-57</sub>, using cells adapted to growth in the presence of proteasome inhibitors and also through protease overexpression. We observed that: 1) Ag processing and presentation proceeds in proteasome-inhibitor adapted cells but may become more dependent, at least in part, on nonproteasomal protease(s), 2) tripeptidyl peptidase II does not substitute for the proteasome in the generation of NP<sub>147-155</sub>, 3) overexpression of leucine aminopeptidase, thymet oligopeptidase, puromycin-sensitive aminopeptidase, and bleomycin hydrolase, has little impact on the processing and presentation of NP<sub>50-57</sub> or NP<sub>147-155</sub>, and 4) proteasome-inhibitor treatment altered the specificity of substrate cleavage by the proteasome using cell-free digests favoring NP<sub>147-155</sub> epitope preservation. Based on these results, we propose a central role for the proteasome in epitope generation even in the presence of proteasome inhibitors, although such inhibitors will likely alter cleavage patterns and may increase the dependence of the processing pathway on postproteasomal enzymes. The Journal of Immunology, 2006, 176: 2249–2261.

Several important experimental observations support a central role for the proteasome in the cytosolic phase of MHC I-restricted Ag processing. First, proteasome inhibitors that block at least some functions of the proteasome inhibit the presentation of numerous epitopes and can hinder the proper maturation of many MHC I alleles, presumably due to the consequent paucity of peptides in the ER (2). Second, the expression of several proteasome components, including catalytically active β subunits (LMP2, LMP7, and LMP10) and a potential activator complex (PA28), can be upregulated in response to IFN-γ and are incorporated into the proteasome. The resulting “immunoproteasomes” favor the generation of peptides suited for presentation by MHC I molecules (2). Third, it has been possible in some cases to demonstrate precise epitope generation in cell-free digests using purified proteasome and synthetic epitope precursors (2). Finally, the posthydrophobic cleavage activity of the proteasome (the chymotryptic-like activity) is considered to be critical for generating the hydrophobic C-termini of peptides that is preferred by murine TAP and many human and murine class I alleles (1). This correlation between proteasome cleavage and TAP and MHC preference suggests a coevolution of the degradation and presentation components of the class I processing pathway (1). Notably, posthydrophobic cleavage is the rate-limiting activity for protein degradation and is the activity most potently blocked by proteasome inhibitors (4). Following initial proteasomal cleavage, additional trimming events may be required to generate precise MHC class I-binding peptides (5, 6). This trimming may occur in the cytosol (7–9) or ER (6, 10–13) and may occur more efficiently at the N terminus of epitope precursors than at the C terminus, further supporting the notion that the proteasome plays a central role in generating the C terminus of epitopes during initial processing.

Despite the abundant evidence implicating the proteasome in Ag processing, its obligatory role in some cases is unclear. We have demonstrated previously that N-Ac-Leu-Leu-norleucinal (LNNL) and lactacystin (Lac), two potent inhibitors of the proteasome, fail
to block, and actually enhance, presentation of the H-2Kd-restricted epitope from influenza A PR/8/34 nucleoprotein (NP), NP147–155 (14, 15). Furthermore, mutation of an immediately flanking alanine residue to a proline (influenza NP with aa 146 mutated from alanine to proline (A146P)) results in a substrate that can be processed to yield the antigenic peptide only in the presence of these inhibitors (14). Subsequent reports described a similar presentation phenotype for several other epitopes (16–22). In addition, cell-free proteasome digests do not always result in the generation of epitopes produced in, and presented by, intact cells (14, 23, 24), suggesting that other proteases may be involved in the generation of some epitopes. NP147–155 is destroyed rather than generated by the proteasome during such digests (14, 23), a result that is greatly accentuated by the A146P mutation (14). Therefore, NP147–155 production may involve the action of proteases operating in parallel with the proteasome that are effective only in the absence of fully functional proteasome (which could serve to destroy the epitope). Alternatively, production may involve a combination of both proteasomal and nonproteasomal activities.

A proteolytic activity that can compensate for reduced proteasome function has been identified (25, 26). The activity of a high m.w. complex, larger than, and distinct from, the proteasome is increased in cells adapted to grow in the presence of irreversible inhibitors of the proteasome (25, 26). In Lac-adapted EL-4 cells, this enzyme has been identified as tripeptidyl peptidase II (TPP II) (26). This large cytosolic protease was originally described as a high m.w. oligomeric, extralysosomal aminopeptidase purified from rat liver homogenates (27, 28). TPP II is an efficient tripeptide aminopeptidase and has been demonstrated to generate peptides ranging from 7 to 27 aa from a 41 aa substrate through both exo- and endoproteolytic activity, implying that it may function to generate peptides for MHC I-restricted presentation (26). In support of this possibility, EL-4 cells adapted to grow in the presence of the irreversible inhibitor of the proteasome carboxbenzoxyl-Leu-Leu-Leu-vinyl sulfone (Z-L3-VS) continue to express stable, irreversible proteasome inhibitors. Our results do not support the existence of a “proteasome-independent” pathway for the production of epitopes such as NP147–155. Rather, our data favor a model in which NP147–155 generation depends on proteasome activities that persist in the presence of proteasome inhibitors along with the action of additional proteases.

Materials and Methods

Cells, viruses, and inhibitors

L929 cells transfected with the Kd gene (L-Kd) and B8 fibroblasts were maintained in DMEM with 5% FCS, L-Kd and B8 murine fibroblasts were adapted to grow in the presence of increasing concentrations of the irreversible proteasome inhibitor Z-L3-VS as described previously (25). Adapted cells were maintained in 10 µM Z-L3-VS, and adaptation was confirmed by the ability to grow in the presence of 50 µM Z-L3-VS and altered cleavage of fluorogenic substrates (see below). PB15 cells adapted to grow in serum-free medium were maintained in OPTI-MEM (Invitrogen Life Technologies) without any serum supplement. Vaccinia virus (vac) encoding NP, NP50–57, NP147–155, NP (A146P), OVA, and all hairpin (HP) NP constructs have been described previously (14, 37). vac expressing TPP II was generated by excising the cDNA encoding murine TPP II from pCDNA3 (provided by Dr. B. Tomkinson, University of Uppsala, Uppsala, Sweden, through S. Renn and Dr. P. Taghert, Washington University School of Medicine, St. Louis, MO) with EcoRI and NotI, ligating into EcoRI/NotI cut pBluescript (Stratagene), excising from pBluescript with Sall/NotI, and cloning into the Sall/NotI sites of the plasmid pSCL1. Recombinant vac was generated as described previously (38). The irreversible proteasome inhibitor Z-L3-VS (39) was a gift from Drs. M. Bogoy (University of San Francisco, San Francisco, CA) and H. Ploegh (Harvard University, Boston MA). Lac was purchased from E. J. Correy (Harvard University, Boston MA). LNL (calpain inhibitor I) was purchased from Calbiochem. Butabindide was purchased from Tocris Bioscience, and AAF-cmk and other chemicals were purchased from Sigma-Aldrich. All inhibitors were used as a 1000X stock in DMSO unless otherwise noted. All restriction endonucleases were purchased from New England Biolabs.

Enzyme assays

Purified bovine 20S proteasome, used in initial experiments, was a gift from Dr. G. DeMartino (University of Texas Southwestern Medical Center, Dallas, TX), and purified drosophila TPP II was a gift from S. Renn and Dr. P. Taghert. 20S proteasome used for all reported experiments was later acquired from Biochim. The fluorogenic peptide substrates N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (AAF-AMC) and Ala-Ala-Phe-chloromethyl ketone (AAF-cmk) sensitive postproteasomal activities were used as a 1000X stock in DMSO unless otherwise noted. Purified enzymes (60 ng of proteasome or 150 ng of TPP II) or cell extracts were incubated in protease assay buffer (50 mM Tris (pH 7.4), 0.06 mM EGTA, 2 mM MgCl2, 0.5 mM 2 ME, and 100 µM BME) containing the indicated substrates and inhibitors for 1–1.5 h. The reaction was stopped by the addition of 2.5 M HCl (4 µl of 100 µM HCl). Luminescence was measured in a Luminescence Spectrometer or a Wallac Victor2 plate reading fluorometer at an excitation of 360 nm and emission of 460 nm. A bandwidth of 40 nm allowed detection of cleavage of N-cbz-Leu-Leu-Glu-β-naphthylamide despite the optimal excitation/emission of 335 nm/410 nm.
CTL assays

CTL assays were performed essentially as described previously (14). Briefly, epitope-specific CTL were generated by priming mice with mini- gene vaccines encoded either the NP50–57 or NP147–155 epitopes. After at least 2 wk, spleens were removed, homogenized, and restimulated with influenza A PR/8/34 for 6–7 days. Epitope presentation was assessed by incubating target cells with the indicated vac recombinants for 4 h, labeling with 100 μCi 51Cr/106 cells for 1 h, washing with cold PBS, and coculturing the infected targets with dilutions of epitope-specific CTL populations for an additional 4 h. One-half of the supernatant was harvested, and 51Cr release was measured by a gamma counter. In assays using inhibitors, breflidil A (BFA) (Sigma-Aldrich) was added just before cocultivation with CTL to a final concentration of 5 μg/ml to alleviate the need to include protease inhibitors when T cells were present. For some assays, cells were pretreated overnight with the protease inhibitor AAF-cmk, washed twice in buffered salt solution/BSA (BSS/BSA), and CTL assays were performed as above in the absence of inhibitors. In experiments involving butabindide, P815 cells adapted to grow in serum-free OPTI-MEM were treated overnight with the inhibitor at 100 μM. Cells were supplemented with fresh butabindide every step till the addition of in vitro-stimulated splenocytes.

Gel filtration

A total of 4 × 107 L-Kd cells was treated for 12 h with 50 μM AAF-cmk, washed twice with BSS/BSA, and infected with 3 pfu/cell of either control vac or TPP II expressing vac in the absence of inhibitors. After 9.5 h, cells were lysed in 200 μl of 50 mM Tris plus 1% Triton X-100 (pH 7.4) on ice for 10 min. The lysate was centrifuged at 12,000 × g for 10 min, and the supernatant was loaded directly onto a Superose 6 column (Pharmacia). The column was eluted at a flow rate of 0.2 ml/min, and 2-min (0.4 ml) fractions were collected. Fifty micrograms of the column was eluted at a flow rate of 0.2 ml/min, and 2-min (0.4 ml) fractions were collected into chilled tubes containing 100 μl each 5% polyacrylamide storage buffer (500 mM KPO4, 5 mM DTT, and 75% glycerol). Each fraction was assayed for cleavage of fluorescent substrates as above.

Peptide digestion with purified proteasome

Short-term digestion. Five micrograms of purified 20S proteasome (Boston Biocraft) with or without 20 μM Lac was incubated in reaction buffer containing 50 mM HEPES (pH 7.6), 0.5 mM EDTA, 2.0 mM MgCl2, 1.0 mM DTT, and 0.03% SDS for 30 min at 37°C. Fifty micrograms of the NLNDA TYR QRALVTRGLMD peptide was added, and the samples were again incubated for 2 h at 37°C.

Long-term digestion. The procedure was similar to the short-term digestion, except 40 μg of the NLNDA TYR QRALVTRGLMD peptide was incubated with 5 μg of purified 20S proteasome with or without 20 μM Lac in 50 mM HEPES (pH 7.6), 0.5 mM EDTA, and 0.03% SDS for 24 h at 37°C. In both cases, proteasomes were then removed by filtering the reaction sample through a Centricon YM-10 unit (Millipore) at 4°C. SDS was removed from the sample as described previously (40). The digests were separated by reverse phase HPLC on a Vydac C18 column. Eluent A contained 0.1% trifluoroacetic acid, eluent B, and 0.1% trifluoroacetic acid in 99.9% acetonitrile. Elution was performed at a 1.0 ml/min flow rate in the linear gradient of eluent B (0–60%) over 40 min. Peak fractions were collected, vacuum-dried, and analyzed by surface-enhanced laser desorption ionization mass spectrometry.

Results

Proteasome inhibitor sensitivity of NP epitopes

We have observed previously that two MHC I-restricted epitopes of influenza A PR/8/34 NP exhibit opposite sensitivities to protease inhibitors that affect the proteasome (14, 15). Processing and presentation of NP50–57 to CTL is diminished in the presence of the proteasome inhibitor Lac (Fig. 1A), especially when Ag expression is limited (15). In contrast, generation of NP147–155 from the proteasome inhibitor Lac (Fig. 1B), especially when Ag expression is limited (15). In contrast, generation of NP50–57 and NP147–155 from full-length NP expressed by vac was assessed in a standard 51Cr release assay in the absence (●) or presence (○) of Lac (10 μM). Similar results were observed using LLnL (50 μM) and ZL1YS (50 μM; data not shown). B, Presentation of NP50–57 and NP147–155 from vac minigenic expression constructs was unaffected by Lac (10 μM). Similar results were observed using LLnL (50 μM) and ZL1YS (50 μM; data not shown). C, Presentation of NP147–155 from the A146P mutated full-length NP was assessed by 51Cr release assay in the absence (●) or presence (○) of the indicated proteasome inhibitors at the concentrations indicated in A and B. Control vac indicates a recombinant vac expressing an irrelevant Ag.

FIGURE 1. The effect of inhibitors of the proteasome on presentation of NP50–57 and NP147–155. A, Presentation of NP50–57 and NP147–155 in L-Kd2 cells from full-length NP expressed by vac was assessed in a standard 51Cr release assay in the absence (●) or presence (○) of Lac (10 μM). Similar results were observed using LLnL (50 μM) and ZL1YS (50 μM; data not shown). B, Presentation of NP50–57 and NP147–155 from vac minigenic expression constructs was unaffected by Lac (10 μM). Similar results were observed using LLnL (50 μM) and ZL1YS (50 μM; data not shown). C, Presentation of NP147–155 from the A146P mutated full-length NP was assessed by 51Cr release assay in the absence (●) or presence (○) of the indicated proteasome inhibitors at the concentrations indicated in A and B. Control vac indicates a recombinant vac expressing an irrelevant Ag.

Development of adapted cells

As a first step in investigating the possibility that proteasome-independent Ag processing pathways exist for the production of NP147–155, cell lines were adapted to grow in the presence of proteasome inhibitors. Similar proteasome-inhibitor adapted cells have been previously demonstrated to exhibit a dramatic reduction in proteasome activity and a compensatory increase in TPP II activity (25, 26). Despite several observations that suggest peptides are available for MHC I folding in adapted cells and that up-regulated TPP II can prevent the accumulation of ubiquitinylated proteins, the processing and presentation of specific epitopes in proteasome-inhibitor adapted cells has not been fully examined. In addition, although TPP II can trim protein fragments under cell-free conditions to generate antigenic peptides (26, 32), the precise Ag processing role of this protease in intact cells remains unclear. L-Kd2 cells were adapted to grow in the presence of the irreversible inhibitor Z-Lys- VS essentially as described by Gläs et al. (25). A low concentration of Z-Lys- VS (1–10 μM) was toxic to most cells, but by 2–3 wk of culture, a small population of resistant cells emerged. Adapted L-Kd2 cells were unaffected by higher concentrations of Z-Lys- VS (50 μM) while normal cells died within 2–3 days. In contrast, AAF-cmk, a potent inhibitor of TPP II, killed adapted L-Kd2 cells in 24–48 h, as observed for adapted EL-4 cells model Ag to investigate the role of different proteases in the processing of the apparently proteasome-independent NP147–155 epitope. Interestingly, a similar alanine to proline mutation in the flanking region of an HIV gag epitope also impairs processing (41). The unusual, but not unique (16–22), presentation phenotype of NP147–155 suggests that there may be proteasome-independent Ag processing pathways that favor the generation of epitopes like NP147–155.
of AAF-AMC by purified proteasome (H11021 1% the activity of TPP II in AAF-AMC hydrolysis) was detected. Numbers indicate percent inhibition. ND, not done.

The large cytosolic protease TPP II can substitute for some functions of the proteasome when proteasome activity is impaired (25, 26, 29). Because the processing and presentation of NP<sub>147–155</sub> was more efficient in the presence of proteasome inhibitors, we next examined the potential involvement of TPP II in epitope production. As a first step, we investigated protease inhibitors that block

**Table I. Inhibition of protease activity (% inhibition)**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Purified Protease</th>
<th>TTP II</th>
<th>Cell Lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Substrate:</td>
<td>AAF-AMC</td>
<td>LLYY-AMC</td>
</tr>
<tr>
<td>Lactacystin (10μM)</td>
<td>LLVY-AMC</td>
<td>96 ± 2.4</td>
<td>ND</td>
</tr>
<tr>
<td>Lactacystin (50μM)</td>
<td>LLVY-AMC</td>
<td>98 ± 0.5</td>
<td>ND</td>
</tr>
<tr>
<td>ZL&lt;sub&gt;2&lt;/sub&gt;VS (50μM)</td>
<td>LLVY-AMC</td>
<td>94 ± 1.4</td>
<td>34 ± 1.7</td>
</tr>
<tr>
<td>AAF-cmk (50μM)</td>
<td>LLVY-AMC</td>
<td>5 ± 0.5</td>
<td>−13 ± 1.2</td>
</tr>
</tbody>
</table>

*Activity was determined as described in Materials and Methods. Note that purified TPP II did not hydrolyze LLYY-AMC, LLE-bNA, or GGR-bNA, and little or no cleavage of AAF-AMC by purified proteasome (<1% the activity of TPP II in AAF-AMC hydrolysis) was detected. Numbers indicate percent inhibition. ND, not done.*
the activity of either TPP II or the proteasome. The chloromethyl ketone, AAF-cmk, is a potent inhibitor of TPP II (26). As expected, this compound inhibited purified TPP II, abrogating 100% of the detectable enzyme activity (Table I). Lac at higher concentrations (50 μM) also inhibited TPP II activity (~40%) (26), but at 10 μM (the concentration used in this study), no change in TPP II activity was detected (Table I). We observed no change in the chymotryptic-like or caspase-like activity of purified proteasome in the presence of AAF-cmk while Lac or Z-L3-VS completely blocked the chymotryptic-like activity (Table I; data not shown). Z-L3-VS also inhibited ~35% of the caspase-like activity of purified proteasome (Table I). This inhibition profile was also observed in cell lysates (Table I) where Z-L3-VS inhibited ~35% of the caspase-like activity of purified proteasome (Table I). This inhibition profile was also observed in cell lysates (Table I) where Z-L3-VS inhibited only activities consistent with the proteasome while AAF-cmk decreased the TPP II-like activity ~70% (Table I). The efficient inhibition of purified TPP II by AAF-cmk suggests that the residual (~30%) AAF-AMC hydrolysis observed in cell lysates may be due to other protease activities. No significant cleavage of a substrate for the trypsin-like activity of the proteasome (cbz-GGR-bNA) was detected using two different sources of purified proteasome. Cleavage of this substrate was observed in cell lysates, but this activity was not blocked by either Z-L3-VS or AAF-cmk. Importantly, the specificity of inhibition observed using purified proteases and cell lysates was also maintained in intact cells as treatment with AAF-cmk did not significantly affect proteasome activity while nearly completely eliminating AAF-AMC hydrolyzing activity (data not shown and see below). These data demonstrate that AAF-cmk potently inhibits TPP II without affecting the proteasome, while Lac and Z-L3-VS preferentially block the chymotryptic-like activity of the proteasome.

Impact of AAF-cmk on epitope presentation

To examine the impact of a TPP II-like activity on the processing and presentation of NP50–57 and NP147–155, AAF-cmk was included during CTL assays using both normal and proteasome-inhibitor adapted cells. In nonadapted cells, AAF-cmk inhibits processing and presentation of NP147–155 but not NP50–57 from vac-expressed NP (Fig. 4A). This is significant because AAF-cmk represents the first protease inhibitor we have tested that decreases processing and presentation of NP147–155. For both epitopes, presentation from minigene constructs was unaffected by AAF-cmk. Minigene expression can lead to high epitope levels that mask...
more subtle effects on Ag presentation. However, lack of AAF-cmk impact on NP50–57 presentation by untreated cells argues against nonspecific inhibitory effects of this compound. Importantly, in Z-LV-VS-adapted cells, AAF-cmk inhibited presentation of NP147–155. We also observed a moderate but consistent (four of four experiments) inhibition of NP50–57 presentation (Fig. 4B), in contrast to results with nonadapted cells. Presentation of each epitope from a minigene construct was also not substantially affected by AAF-cmk in the adapted cells (Fig. 4, A and B). These results suggest that an AAF-cmk-sensitive proteolytic activity can contribute to the processing of NP147–155 in normal cells and NP147–155 and other epitopes in proteasome inhibitor adapted cells. TPP II is an obvious candidate for this processing activity given its role in compensating for chronic proteasome inhibition (25, 26, 29) and studies reporting a trimming (31, 32) or proteasome-independent (30) processing role for this enzyme. However, AAF-cmk may affect other proteases and despite the potent inhibition of TPP II activity by AAF-cmk, epitope presentation was blocked by only ~10–50% in adapted cells. Therefore, we developed alternate approaches to examine the role of TPP II in Ag processing more directly.

TPP II RNAi

RNAi duplexes were designed to limit TPP II expression and then tested for their ability to inhibit TPP II-like AAF-AMC hydrolysis in cell extracts. This approach, in contrast to the inhibitors used above, represents a highly specific method to reduce TPP II activity. As shown in Fig. 5, A and B, two RNAi duplexes (TPP II no. 1 and TPP II no. 2) targeting different regions of the TPP II mRNA resulted in ~50% reduction in AAF-AMC cleavage compared with a control RNAi duplex. At least some of the residual AAF-AMC cleavage activity likely derives from other proteases capable of cleaving this substrate. No reduction, and perhaps a slight increase, in proteasome-like activity was detected following TPP II RNAi transfection (Fig. 5B). Despite this inhibition of TPP II activity using RNAi, NP147–155 processing and presentation was not significantly changed (Fig. 5C). These data call into question the role of TPP II in the processing of NP147–155.

Impact of butabindide, a TPP II-specific inhibitor

We next evaluated the impact of butabindide, a specific TPP II inhibitor. Because butabindide has been shown to be inactivated by serum factors (31), we used P815 cells that are adapted to grow in serum-free medium. As shown in Fig. 6A, treatment of P815 cells with butabindide did not have any impact on the presentation of NP147–155. To determine whether butabindide-treated cells indeed have reduced TPP II activity, we performed TPP II (AAF-AMC hydrolyzing) as well as proteasome (bNA hydrolyzing) activity assays using extracts obtained from a portion of butabindide-treated cells. Results show that this activity is reduced remarkably, while proteasome activity is in fact slightly enhanced in butabindide-treated cells (Fig. 6, B and C). Although siRNA exclusively targets TPP II, butabindide may affect other proteases, a potential explanation for the persisting AAF-AMC-cleaving activity with siRNA treatment. Butabindide has, in fact, been demonstrated to inhibit the lysosomally located TPP I (47). In any event, it is clearly very effective at inhibiting TPP II (and any other AAF-AMC-hydrolyzing activity) but has no impact on NP147–155 presentation.

Overexpression of TPP II

As a final approach to examine specifically whether TPP II plays a key role in the pathway that is responsible for the generation of NP147–155, a recombinant vac was generated expressing functional TPP II. If TPP II activity can enhance NP147–155 generation in a proteasome-independent manner, then higher levels of this enzyme

![Figure 5](http://www.jimmunol.org/)

**Figure 5.** TPP II RNAi. A and B, RNAi constructs were designed targeting two regions of TPP II mRNA. Forty-eight hours after transfection of L-K<sup>+</sup> cells with a control RNAi, or each TPP II RNAi individually or together, AAF-AMC (A) and LLE-bNA (B) activity was assessed in cell lysates as described in Materials and Methods. Similar results to LLE-bRNA were observed for LLVY-AMC. C, L-K<sup>+</sup> cells transfected with the TPP II RNAi constructs were tested in a 51Cr release assay for presentation of NP147–155 from vac-expressed NP<sub>13–498</sub>. No change in killing of cells infected with a control vac lacking NP or minigene-expressing vac was observed in cells expressing TPP II RNAi constructs compared with nontransfected or control RNAi transfected cells (data not shown). Similar experiments using control, reporter, constructs routinely achieve ~50–60% of the cells transfected (data not shown).
These conditions for overexpression of functional, oligomerized TPP II were used to assess directly the influence of this enzyme on NP$_{50-57}$ and NP$_{147-155}$ generation. Fig. 8 demonstrates that, even with a $\sim$10-fold increase in TPP II-like activity, no significant impact on the processing and presentation of either epitope from full-length protein was detected. The HP system of limiting Ag expression was again used to discern differences that may be obscured by Ag overexpression. Even under conditions of limited substrate expression, no significant increase in NP$_{50-57}$ or NP$_{147-155}$ presentation discerned in adapted cells (see Fig. 3) using the HP system. Overexpression of TPP II also did not restore the presentation of NP$_{147-155}$ from A146P NP (Fig. 8C), a key property of any putative autonomous NP$_{147-155}$ Ag processing pathway. Rather, presentation from A146P was only recovered by inclusion of proteasome inhibitor regardless of TPP II overexpression (Fig. 8C). These results demonstrate that TPP II is unlikely to play a significant role-limiting role in the generation of either proteasome-dependent epitopes such as NP$_{50-57}$ or apparently proteasome-independent epitopes such as NP$_{147-155}$.

**Overexpression of other proteases**

One possible explanation for the results described above is that a protease other than the proteasome or TPP II is involved in the generation of NP$_{147-155}$. Indeed, several other cytosolic proteases have been implicated in Ag processing. These include at least two enzymes, PSA and BH, that have been reported to be inhibited by AAF-cmk (32, 36). In addition, TO and the IFN-γ-inducible LA have been proposed to act as either productive or destructive post-proteasomal trimming enzymes (33–35, 49). To test whether any of these proteases contributes to productive NP$_{147-155}$ generation, recombinant vacs expressing each of these four proteases were constructed. Overexpression by this approach resulted in a 5- to 10-fold increase in protease activity (data not shown). To discern even subtle changes in the efficiency of NP$_{147-155}$ production in the presence of higher activity of each of these proteases, two methods of limiting Ag presentation were used (Fig. 9). First, a HP recombinant (HP18) was used as described above to limit Ag expression (Fig. 9A). Second, BFA was used to limit presentation of NP$_{147-155}$ and NP$_{50-57}$. BFA limits egress from the Golgi to the cell surface and prevents the continued increase in cell surface peptide MHC complexes during CTL assays (14). Fig. 9 demonstrates that, even under limiting conditions, no substantial change in epitope presentation was observed, positively or negatively, as a result of overexpression of any of the five proteases.

**Altered proteasome activity**

Having determined that TPP II and other cytosolic proteases are unlikely to play a role in the generation of the proteasome-independent NP$_{147-155}$ epitope, we turned to a second possible explanation for this unusual presentation phenotype—that proteasome inhibitors do not completely inactivate the proteasome, but rather alter cleavage specificity in a manner that leads to greater NP$_{147-155}$ production. To investigate this, purified proteasome was incubated for 2 h with a 19 aa substrate containing the NP$_{147-155}$ epitope flanked by five additional NP residues on either side (NLNDATYQRTRLAVRTGMD, epitope in bold) in the absence or presence of Lac. The digest was then separated by HPLC, and the peptide species were identified by mass spectrometry as described in Materials and Methods. Analyses revealed that Lac causes substantial reduction in activity at the previously described major cleavage site within the epitope (Fig. 10A). This is indicated by the nearly complete loss of the readily detectable N$_{142}$-R$_{152}$ product.
and commensurate preservation of the input peptide. More prolonged incubation (24 h) caused the generation of many species under both conditions (Fig. 10B), indicating that the proteasome is not incapacitated by Lac. Of note, the fragmentation patterns were clearly distinct, and this was also apparent on the HPLC tracings (data not shown). The large number of

(NLNDATYQRTR) and commensurate preservation of the input peptide. More prolonged incubation (24 h) caused the generation of many species under both conditions (Fig. 10B), indicating that

FIGURE 8. Overexpression of TPP II does not enhance epitope presentation. A and B, Conditions that allowed a 10-fold overexpression of TPP II activity using TPP II vac were used to assess presentation of NP50–57 (A) and NP147–155 (B). Normal L-Kd cells were pretreated overnight with 50 μM AAF-cmk, washed twice, and coinfected with 5 PFU/cell of the indicated Ag expressing vacs and 5 PFU/cell control vac (OVA vac) or TPP II vac. A series of HP-NP vacs were again used to limit Ag expression. No increase in presentation of either epitope is observed. In neither case is the presentation of epitopes from minigene constructs affected. C, Overexpressed TPP II does not restore presentation of NP147–155 from the A146P NP and does not alter presentation of NP147–155 from A146P NP in the presence of proteasome inhibitor (50 μM LLNL). The four bars indicate the E:T ratios 77:1, 26:1, 9:1, and 3:1.
fragments and their frequent coelution precluded determination of relative amounts. Nevertheless, it is clear that Lac allows for the generation of many more species that contain the epitope (indicated by a thicker line in Fig. 10B). Indeed, in the no Lac sample, the complete epitope is present in only the input peptide while it is completely contained within several products extended at the N and/or C termini in the plus Lac digest (indicated by arrows in Fig. 10B). Three other aspects of the long-term digests are worth noting. First, addition of the inhibitor produces more species that are N-terminally extended, particularly at D145 and A146. Second, although addition of the inhibitor does not completely prevent cleavage at R, the activity is presumably much lower given the presence of many more species in which the R152-T153 bond is preserved. Finally, the minimal epitope itself is generated in the presence of Lac, but this peptide is within a relatively minor HPLC peak (data not shown). The strong bias toward extended versions of the epitope may explain the greater dependence on AAF-cmk-sensitive activity for presentation of NP50–57 and NP366–374 in adapted cells (Fig. 4). Taken together, these results indicate that Lac strongly reduces action at the major cleavage site within the epitope while modulating activity at secondary cleavage sites.

Discussion

There is little doubt that the proteasome is the main cytosolic protease involved in the turnover of proteins and the generation of many MHC I-bound peptides. However, a clear understanding of how unusual epitopes, including NP147–155, are generated in the presence of proteasome inhibitors does not currently exist. Because many epitopes exhibit this presentation phenotype (14, 16–22), it is important to investigate the mechanism by which they are generated. A priori, two models are possible. In the first, epitopes such as NP147–155 that are resistant to, or enhanced by, proteasome

![Figure 9](http://www.jimmunol.org/)

**FIGURE 9.** Overexpression of four additional proteases does not augment epitope presentation even under limiting conditions. The coinfection approach was used to overexpress PSA, BH, TO, and LA (and TPP II again) and test the processing and presentation of NP50–57 and NP147–155. A, A standard 51Cr release assay using normal L-K+ cells was performed to test NP147–155 presentation from an HP-limited NP expression construct in the absence of additional protease expression or when each of the indicated enzymes was overexpressed. B and C, BFA-limited NP50–57 (B) or NP147–155 (C) processing and presentation was not enhanced by any of the overexpressed proteases. BFA (5 μg/ml final) was added at 150 min postinfection or not at all (NP bars), and presentation was assessed in a standard 51Cr release assay. Control indicates infection with vac expressing an irrelevant Ag. The three bars for each condition indicate the E:T ratios 33:1, 11:1, and 4:1.

![Figure 10](http://www.jimmunol.org/)

**FIGURE 10.** Cell-free proteasome digest of 19-mer peptide containing the NP147–155 epitope flanked by 5 aa from NP on either side reveals qualitative differences in substrate cleavage in the presence of Lac. A, The 19-mer peptide was digested with purified 20S proteasome in the absence (top panel) or presence of 20 μM Lac for 2 h. The digest was then separated by HPLC (tracings shown), and the indicated peptide species were determined by mass spectrometry. B, Prolonged (24 h) digestion of the 19-mer peptide with proteasome. A similar analysis was conducted, and the various products identified under both conditions are shown. Those products containing the NP147–155 are indicated with an arrow at the right. The major proteasome cleavage site between R152 and A153 is indicated by a dashed line.
inhibitors are generated from protein substrates by a proteasome-independent processing pathway. TPP II has been suggested to have the potential to operate in this capacity (26, 30). However, there is currently no consensus on the precise role of TPP II in Ag processing since other reports support more of an accessory role for TPP II in the production of CTL epitopes (31, 32). The second possible model is that the proteasome is an integral component in the presentation of epitopes such as NP^{147-155}, even in the presence of proteasome inhibitors. This second alternative must receive strong consideration since, by most accounts, standard proteasome inhibitors do not completely inactivate all functions of the proteasome (19, 29, 50, 51) and, in fact, may alter the specificity of residual activities. Considering the evidence discussed further below, we expected our results to support the first model that TPP II was a rate-limiting protease in the proteasome-independent production of NP^{147-155} in intact cells. However, our data fail to support this notion and rather suggest a model in which the proteasome, in combination with downstream protease(s), is instrumental in the generation of NP^{147-155}. In the case of NP^{147-155}, altered proteasome activity in the presence of proteasome inhibitors is favorable for epitope production in that the major intraepitopic cleavage activity is reduced substantially by proteasome inhibitor. The sensitivity of NP^{147-155} presentation to the nonproteasomal protease inhibitor AAF-cmk, especially when the proteasome functions suboptimally, implicates the involvement of additional proteases downstream of the proteasome.

Our focus on TPP II was driven by the suggestion from several studies that TPP II can compensate for some proteasome functions. In cells chronically treated with the proteasome inhibitors Z-L^{2}VS or Lac, TPP II activity is markedly up-regulated (25, 26), and inhibition of TPP II in these adapted cells, an innocuous treatment in normal cells, results in cell death (see above and Ref. 25). TPP II is capable of preventing accumulation of polyubiquitinated proteins in the presence of acute proteasome inhibition, suggesting that the presence of TPP II can prevent the major block in protein degradation that is caused by reduced proteasome function (29). Several observations have also suggested that TPP II is capable of contributing to class I-restricted Ag processing. In Z-L^{2}VS-adapted cells where proteasome is impaired but TPP II activity is substantially up-regulated, MHC I molecules continue to be properly assembled, indicating the availability of MHC I-binding peptides (25, 26, 29). Also, cell-free digests have demonstrated that TPP II can degrade substrates by both endo- and exoproteolytic cleavage and can generate peptide fragments that are the correct size for TAP transport and MHC I binding (7–27 aa; Ref. 26). With respect to the generation of specific CTL epitopes, three observations could be interpreted as the participation of TPP II. First, Levy et al. provided evidence based on cell-free digests that TPP II (and PSA) could trim proteasomal degradation products to generate a precise CTL epitope, thus placing TPP II in a downstream role (32). Second, a recent study by Reits et al. (31) suggests that TPP II has a major role in handling proteasomal degradation products >15 aa long and that the action of TPP II can be either endoproteolytic or N-terminal exoproteolytic. This model also places TPP II downstream of the proteasome but perhaps upstream of aminopeptidases involved in N-terminal trimming (e.g., ER-AAP (52)). In a third study, RNAi was used to inhibit TPP II expression (30). Presentation of the HIV Nef^{73-82} epitope was reduced in cells in which TPP II expression was blocked, and this was interpreted as evidence of a proteasome-independent/TPP II-dependent processing event. However, it is also possible that the required activity of TPP II for Nef^{73-82} production occurs following initial proteasomal cleavage since the proteasome would still have been active under conditions of RNAi treatment.

In the present study, we demonstrate that AAF-cmk, a potent inhibitor of TPP II, but not the proteasome, significantly impairs the generation of NP^{147-155} in normal cells and reduces processing and presentation of NP^{147-155} and a second epitope (NP^{50-57}) to a lesser extent in Z-L^{2}-VS-adapted cells. While these results are suggestive of the involvement of TPP II, AAF-cmk has a rather broad specificity, and these results could indicate the involvement of other proteases. For a more direct examination of the role of TPP II as a proteasome-independent processing pathway, we used three approaches. First, we blocked TPP II expression using RNAi. In contrast to the study examining the presentation of the HIV Nef^{73-82} CTL epitope (30), inhibiting TPP II expression using RNAi had no effect on NP^{147-155} processing. Because the level of TPP II inhibition was not reported in the earlier study, it is hard to determine whether the discrepancy between ours and the previous study represents a difference in the level of TPP II inhibition, a difference in human vs mouse cells, or a differential dependency on TPP II activity for the Nef and NP epitopes. As a second approach to address the role of TPP II in NP^{147-155} Ag processing, we overexpressed this enzyme and examined presentation of the two NP epitopes. Overexpressing components of the proteasome complex has been shown to enhance epitope presentation in other systems (53–55), indicating that changes in Ag processing efficiency can be discerned using this approach if the protease in question has a central role in epitope generation. However, substantial differences in levels of TPP II expression failed to alter epitope presentation. Inhibition of endogenous TPP II, a treatment that reduces NP^{147-155} presentation, followed by 10-fold overexpression of TPP II activity failed to enhance NP^{147-155} generation. Even when the synthesis and presentation of NP was limited, an approach that can reveal Ag processing patterns obscured by protein overexpression (15), no change in NP^{50-57} or NP^{147-155} was detected in the presence of increased levels of functional TPP II. Finally, butabindide, a more specific inhibitor of TPP II than AAF-cmk (31), had no detectable impact on NP^{147-155} presentation, despite eliminating nearly all detectable TPP II activity. Taken together, these experiments suggest that any role TPP II might play in the generation of NP^{147-155} is minimal and not critical.

Based on these results, we turned to the seemingly paradoxical possibility that the proteasome, even in the presence of proteasome inhibitors, might be required for NP^{147-155} generation. Digestion of a synthetic 19-mer segment of NP containing NP^{147-155} with purified proteasome has demonstrated the major cleavage site to be within the epitope (TYQRTR_	ext{ALV}) (14), and this same cleavage is enhanced in the presence of the A146P mutation (14). Including proteasome inhibitors during the cell-free digests leads to an altered pattern of substrate cleavage that results in greatly reduced activity at the major cleavage site. In addition, prolonged digestion shows that cleavage at the precise N and C termini of NP^{147-155} by the proteasome is not efficient, suggesting that postproteasomal proteases are required to generate the precise 9 aa peptide recognized by CTL. In contrast to the likely scenario in intact cells, substrates may reenter the proteasome in cell-free digests and be subject to multiple cycles of cleavage. Thus, the proportion of N-terminally extended species generated in intact cells may be underestimated by cell-free digestion. Notably, in the presence of a proteasome inhibitor, a number of epitope precursors were generated. Although AAF-cmk has no effect on the generation of NP^{50-57} under normal conditions, presentation appears to be partially reduced by this inhibitor under conditions of chronic proteasome impairment. The reduction is not as dramatic as for NP^{147-155}. Altogether these results suggest that the altered Ag processing in chronically treated cells is due not only to modulated proteasome
but also a shift in the proteolytic profile of the cell to a state that depends more heavily on postproteasomal enzymes. The lack of survival of adapted cells in the presence of AAF-cmk observed by Glas et al. (25) may be due to the compensatory increase in TPP II, and perhaps other proteases, that act downstream of the proteasome. In such a scenario, postproteasomal proteases might play a critical role in cell metabolism as well as Ag processing since the proteasome, while still operable in proteasome-inhibitor adapted cells (29, 50), is probably less efficient than under normal conditions. Further work will be necessary to clarify these issues.

Although, in principle, modulated proteasome could account entirely for the processing of NP147–155, the participation of a nonproteasomal activity in epitope production is a reasonable concept given the demonstrated contribution of other proteases to MHC I Ag processing (25, 26, 30, 32–36, 49, 52, 56). The majority of these proteases has been suggested to act downstream of the proteasome, in a trimming capacity. The demonstration of proteases such as leucine aminopeptidase, PSA, and BH as cytosolic postproteasomal trimming enzymes fits well with published data showing that, in many cases, proteasomal degradation generates N-terminally extended epitope precursors (57, 58). Of note, in addition to TPP II, AAF-cmk also inhibits the activity of PSA and BH (36). However, using a protease overexpression approach, increased levels of leucine aminopeptidase, PSA, BH, and TO do not appear to have a major impact on NP epitope production. In no case did we observe a significant change (either positive or negative) in the processing and presentation of either NP147–155 or NP50–57, even under limiting conditions. Thus, none of the five nonproteasomal proteases that has been associated with Ag processing appears to have a significant impact on the presentation of NP50–57 and NP147–155. It is possible that there is already sufficient activity of the relevant postproteasomal trimming enzyme available to act on a limited amount of NP147–155 containing substrate generated by another rate-limiting step (e.g., proteasomal processing). In this situation, increased expression might not increase epitope production. We do note, however, that TO overexpression (achieved with a transfection system that is likely not as potent as the recombinant vac system we used) was found to inhibit substantially the presentation of the Ova257–264 epitope (49), suggesting that overexpression is a valid strategy for evaluating the role of proteases in Ag processing. We speculate that our failure to observe similar destructive activity indicates that such effects are epitope specific. It is also possible that the action of postproteasomal processing enzymes may be redundant, in which case it would be difficult to discern the specific requirement of an individual protease. A third, intriguing possibility is that additional, as yet unidentified cytosolic protease(s) may be involved in the processing of NP147–155 in a model that is accommodated by the broad specificity of AAF-cmk. Given our initial goal of finding potential substitutes for the proteasome, our analysis was confined to cytosolic proteases; howevert, ERAP1/ERAAP may also be an important postproteasomal peptide modifier, although we are not aware of reports demonstrating inhibition of ERAP1/ERAAP by AAF-cmk. Future experiments will be necessary to distinguish between these possibilities and to determine whether there is a novel AAF-cmk-sensitive protease in this pathway. It should be noted that our data do not eliminate the possibility that, in the generation of NP147–155 and NP50–57 (especially in adapted cells), another AAF-cmk-sensitive protease substitutes for the proteasome, but, for reasons elaborated above, we disfavor this notion.

The data presented in the current study support a model for the generation of epitopes such as NP147–155 that are produced more efficiently in the presence of proteasome inhibitors in which initial, inefficient, proteasomal processing is followed by the action of postproteasomal enzymes that liberate precise epitopes from epitope-containing precursors. The effect of proteasome inhibitors in this model is to alter, but not completely ablate, proteasome activity preserving epitopes that would otherwise be destroyed. However, many important questions about this pathway remain unanswered. First, both acute and chronic proteasome inhibition leave the tryptic-like cleavage activity as the major residual proteasomal activity based on fluorogenic substrates. The destructive activity of NP147–155 appears tryptic-like, but this Arg152-Ala153 cleavage is reduced rather than accentuated in the presence of Lac. Because allosteric effects of inhibitors and substrates may influence the specificity of proteasomal cleavage (4), it will be important to determine the specificity of residual proteasome activity on protein substrates in the presence of both acute and chronic proteasome inhibition. The three proteasome inhibitors used in this study, LLnL, Lac, and ZL3VS, all accentuate NP147–155 presentation. This suggests that, if an altered proteasome is responsible for NP147–155 generation in the presence of inhibitors, then LLnL, Lac, and ZL3VS induce similar functional changes in the proteasome. Indeed, including LLnL or Lac during CTL assays using the ZL3VS-adapted cells did not substantially change presentation of NP147–155 (data not shown). Given the similar effects on proteasome function and increase in presentation of NP147–155 in normal cells, this result is perhaps not surprising. It will be important to develop additional proteasome inhibitors that can target different activities of the proteasome, including those that may be modulated by short-term or chronic treatment with LLnL, Lac, or ZL3VS. Second, the majority of the nonproteasomal proteases implicated in MHC I Ag processing have N-terminal trimming activity, but C-terminally extended precursors of NP147–155 are also likely to be generated based on cell-free digests. Although some data suggests the presence of C-terminal trimming activities are not as abundant or as active as N-terminal processing enzymes, the possible involvement of cytosolic C-terminal trimming proteases is currently being assessed. Lastly, what are the potential implications of these results for protective immunity? Many epitopes have now been identified that are produced or presented more efficiently when proteasome activity is impaired or altered (16–22). During chronic infections or cancer, it may be beneficial to alter the spectrum or level of epitopes produced in vivo to boost T cell recognition or to increase the potential targets for T cell recognition. The HIV protease inhibitor ritonavir has been reported to impair proteasome activity (59). It may be interesting to determine whether this compound or other proteasome inhibitors that can be used in vivo alter CTL activity toward epitopes such as NP147–155 in animal models. A more complete understanding of this degradation pathway may lead to approaches that selectively modify epitope presentation in vivo.

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