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References

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Dissociation of Proteasomal Degradation of Biosynthesized Viral Proteins from Generation of MHC Class I-Associated Antigenic Peptides

Luis C. Antón,* Heidi L. Snyder,* Jack R. Bennink,2* Alexander Vinitsky,† Marian Orlowski,† Angel Porgador,‡ and Jonathan W. Yewdell2*

To study the role of proteasomes in Ag presentation, we analyzed the effects of proteasome inhibitors Cbz-Leu-Leu-Leucinal and lactacystin on the ability of mouse fibroblast cells to present recombinant vaccinia virus gene products to MHC class I-restricted T cells. The effects of the inhibitors depended on the determinant analyzed. For influenza virus nucleoprotein (NP), presentation of the immunodominant Kk-restricted determinant (NP50–57) was marginally inhibited, whereas presentation of the immunodominant Kα-restricted determinant (NP147–155) was enhanced, particularly by lactacystin. Biochemical purification of peptides confirmed that lactacystin enhanced the generation of Kα-NP147–155 complexes fourfold. Lactacystin also enhanced the recovery of one Kα-restricted vaccinia virus determinant from HPLC fractions, while inhibiting recovery of another. The inhibitors were used at sufficient concentrations to block presentation of biosynthesized full-length OVA and to completely stabilize a rapidly degraded chimeric ubiquitin-NP fusion protein. Strikingly, presentation of antigenic peptides from this protein was unaffected by proteasome inhibitors. We also observed that proteasome inhibitors induced expression of cytosolic and endoplasmic reticulum stress-responsive proteins. These data demonstrate first that the processes of protein degradation and generation of antigenic peptides from cytosolic proteins can be dissociated, and second that effects of proteasome inhibitors on Ag presentation may reflect secondary effects on cellular metabolism. The Journal of Immunology, 1998, 160: 4859–4868.

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3 Abbreviations used in this paper: TCD, T cell; CD8, CD8+ T lymphocytes; ER, endoplasmic reticulum; BFA, brefeldin A; N, nucleocapsid protein from vesicular stomatitis virus; NP, influenza virus nucleoprotein; SNP, nucleoprotein targeted to the secretory pathway; Ub, ubiquitin; VV, vaccinia virus; hsp, heat shock protein; grp, glucose-regulated protein; hsc, heat-shock cognate protein.

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LMP2 (18) or LMP7 (19). Three studies reported a correlation between the degradation rate of cytosolic proteins (presumably proteasome mediated), either biosynthesized by APCs (20, 21) or exogenously loaded into APCs (22), and the efficiency of antigenic peptide generation, while a fourth study reported no such correlation with biosynthesized Ags (23). Evidence has been presented showing a requirement for an intact Ub conjugation pathway in the presentation of some Ags (24), but we found no such requirement (25). More direct evidence came with the use of tripeptide aldehyde proteasome inhibitors that interfered with class I biogenesis and completely blocked the presentation of certain determinants (26–29). However, class I biogenesis is blocked only partially by proteasome inhibitors, and we have found that there is a wide variation in the effect of the inhibitors in the presentation of determinants from exogenous and endogenous influenza virus Ags, ranging from total inhibition to enhancement (10). Another suggestive, although indirect, line of evidence implicating the proteasome comes from cell-free experiments. In these studies, the efficiency of Ag presentation in vivo of different determinants, in various contexts, was positively correlated with in vitro degradation of substrates by purified proteasomes (30, 31). In some instances, these digests yielded the antigenic peptide as a final product. Most recently, it was reported that the 11S regulator is induced by cytokines (32), and that Ag presentation can be enhanced by expression of 11S regulators (33, 34).

Altogether the evidence is compelling that proteasomes are involved in the production of class I-binding peptides, but the extent of proteasomal involvement is uncertain. In the present study, we use proteasome inhibitors to examine the role of proteasomes in generating peptides from metabolically stable and unstable proteins produced by recombinant vaccinia viruses (rVVVs).

Materials and Methods

Materials

The peptide aldehyde inhibitors used, Cbz-Leu-Leu-Leucinal (zLL) and Cbz-Leu-Leucinal (zLLL), were synthesized as described (35). Lactacystin was obtained from Dr. E. J. Corey (Harvard University, Cambridge, MA). Brefeldin A (BFA) was from Sigma (St. Louis, MO). Abs to hsc70 (rat IgG1 mAb 1B5), heat shock protein 90 (hsp90) (rat IgM Ab 1R2D12p90), and grp94 (rat IgG2a Ab 9G10) were from StressGen (Victoria, British Columbia, Canada), and a mouse IgG1 Ab specific for hsp70 family of proteins (clone 3a3) was from Affinity Bioreagents (Nashanic Station, NJ). GammaBind Plus Sepharose was from Pharmacia (Piscataway, NJ), protein A-agarose from Pierce (Rockford, IL), and mouse anti-rat Ab Sepharose from Zymed (South San Francisco, CA).

Cells, virus stocks, and Abs

The thymidine kinase-deficient human osteosarcoma cell line 143B and the fibroblastoid cell line L929 (2H2), transfected with the gene encoding for either K\(^{\alpha}\) (L-K\(^{\alpha}\)) (36) or K\(^{\beta}\) (L-K\(^{\beta}\)) (a kind gift from Dr. J. Sheil, West Virginia University, Morgantown, WV), were maintained in DMEM supplemented with 7.5% (v/v) of FCS and 2 mM L-glutamine, in air/CO\(_2\) (91%/9%). The PB15 mastocytoma cell line was maintained in RPMI 1640, with the same supplements, and in air/CO\(_2\) (94%/6%). Long- and short-term T\(_{CD8+}\) cultures maintained in Iscove’s modified Dulbecco’s medium supplemented with 2-ME in air/CO\(_2\) (91%/9%). VV stocks were prepared from infected 143B cells and used as lysates after low speed centrifugation. Vaccinia virus recombinants (rVV) expressing the nucleoprotein from influenza virus A/Puerto Rico/8/34 (VV-NP\(_{PR8}\)) (37) or NT\(_{60}/68\) (VV-NP\(_{NT}\)) (20), as well as a secreted form of the former (VV-SNP\(_{NT}\)) (38), and a chimera form of the latter (VV-Ub-Ag-VP) (20) have been previously described. rVV expressing N protein from the Indian strain of vesicular stomatitis virus (VV-VSV-N) or a minigene containing the minimal K\(^{\alpha}\) restricted determinant of this protein (VV-N\(_{MA55-59}\)) has also been described (39, 40). rVV expressing minigenes containing the minimal determinants from NP restricted by K\(^{\alpha}\) (VV-NP\(_{MA56-254}\)) or K\(^{\beta}\) (VV-NP\(_{MA57-265}\)) were included, and have also been described (41). rVV expressing full-length OVA (VV-OVA) or a minigene expressing the minimal determinant (VV-OVA\(_{A357-266}\)) have been described (37). The mAb 25-D1.16, specific for the K\(^{\alpha}\)-OVA\(_{A357-266}\) complex (42), was used as a tissue culture supernatant.

Viral infection, metabolic labeling, and immunoprecipitation

Cells were incubated for 45 to 60 min with the different inhibitors (or DMSO as a control) at 37°C before infection with rVV at a multiplicity of infection of 10 plaque-forming units/cell. For the analysis of stress protein induction, uninfected cells were incubated with 5 \(\mu\)M ZLLL for 5 h before labeling. For radiolabeling, cells were incubated at 37°C for 30 min in methionine-free medium, and then radiolabeled in the same medium supplemented with \([\text{\textsuperscript{35}}\text{S}]\)methionine (Amersham, Arlington Heights, IL) for 5 min. In all instances, media contained the appropriate inhibitors. Cells were then lysed in extract buffer (50 mM Tris-HCl, pH 7.3, 100 mM NaCl, 1 mM EDTA, and 2% Triton X-100), with a mixture of protease inhibitors (Complete, from Boehringer Mannheim, Indianapolis, IN)). The nuclear fraction was discarded following centrifugation, and the extracts were analyzed by SDS-PAGE (43), under reducing conditions, either as lysates or after immunoprecipitation. For immunoprecipitation, Abs were coupled to either GammaBind Plus Sepharose (anti-hsc70, -hsp70, and -grp94), or anti-r Ab coupled to Sepharose (anti-hsp90). Samples were adjusted to contain the same volume and amount of radioactivity before immunoprecipitation. Samples were preclarified with the appropriate beads coupled with the appropriate serum autologous to the Ab used. After immunoprecipitation, the beads were washed as indicated (44), except for the anti-hsp90 Ab-coated beads, which were washed twice in MENG buffer (25 mM MOPS, 1 mM EDTA, 0.02% NaN\(_3\), pH 7.5, and 10% glycerol) (45). The beads were then boiled in sample buffer and analyzed by SDS-PAGE under reducing conditions. For the study of the Ub-Arg-NP stability, cells were infected for 2 h before labeling with \([\text{\textsuperscript{35}}\text{S}]\)methionine for 5 min. Aliquots were incubated for different times in medium supplemented with 10 mM cold methionine. Cells were lysed in boiling sample buffer for electrophoresis, containing 1% B-mercaptotananol, and Complete. The same volume/sample was loaded onto the gels. Gels were quantitated by analysis with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using the Image-Quant software provided. The counts obtained from the band corresponding to NP were normalized using those of a stable VV protein as a standard (this band is marked in Fig. IA with an asterisk). The image was arranged and labeled with Adobe Photoshop (Adobe Systems, Mountain View, CA), and printed with a digital printer (Fujix 3000 Pictography, Fuji Photofilm USA, Elmsford, NY).

Cytotracker

L-K\(^{\beta}\) cells infected in the presence or absence of proteasome inhibitors were infected with VV-NP, VV-OVA, or VV-OVA\(_{A357-266}\) for 5 h. Cells were then washed with ice-cold saline containing Na\(_2\)CO\(_3\) and stained with 25-D1.16 Ab (42), biotin-conjugated goat F(ab\(^{\prime}\))\(_2\) anti-mouse IgG, and FITC-conjugated streptavidan (Jackson ImmunoResearch, West Grove, PA). The amount of fluorescence associated with live cells was quantitated using a FACScan cytograph (Becton Dickinson, Mountain View, CA).

Mice and generation of specific T\(_{CD8+}\)

BALB/c (H\(^{\beta}\)) and C3H (H\(^{\beta}\)) mice, from Taconic Farms (Germantown, NY), were immunized with 5 \(\times\) 10\(^6\) plaque-forming units of VV-NP in 0.5 ml of PBS containing 0.2% BSA. At least 3 wk after immunization, spleens were harvested and restimulated in vitro for 1 wk with autologous splenocytes either infected with VV, or pulsed with synthetic peptides SYDYGRL\(_{K}\) or TYYRTRALV\(_{K}\), provided by the Biologic Resources Branch (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). For quantitation of antigenic peptides in acid extracts from infected cells, T\(_{CD8+}\) lines were generated as described (46).

Cytotoxicity assays

Cytotoxicity assays were performed as previously described (47). Cells were preincubated with the inhibitors for 30 min before infection with rVVVs. At different times after infections, the inhibitors were replaced by BFA at 5 \(\mu\)g/ml to block additional Ag presentation. Cells were kept in BFA-containing media for the remainder of the assay.

Peptide extraction and quantitation

This procedure has been described (46). Cells preincubated for 1 h with 10 \(\mu\)M lactacystin were infected with 6 h in 10 \(\mu\)M lactacystin. After acid extraction, low M\(_{\text{r}}\) material was fractionated by reverse-phase HPLC, as previously reported, collecting 0.25-ml fractions. The amount of peptide in these fractions was determined by their ability to sensitize target cells for...
recognition by specific T CD8+ cells, extrapolating the concentration from titration curves obtained with measured amounts of synthetic peptides.

Results

Effect of proteasome inhibitors on proteasome-dependent protein degradation

To gain a quantitative measure of the effectiveness of zLLL and lactacystin on proteasomal degradation, we examined their ability to inhibit degradation of a model substrate. To control for effects of zLLL on nonproteasomal proteases, we used zLL. zLL is a more potent calpain inhibitor than zLLL, similar in potency in inhibiting lysosomal proteases, and at least 250 times less active on proteasomes (10, 48). Townsend et al. (20) produced a rVV-expressing NP from NT60 influenza virus fused to the COOH terminus of Ub with an intervening Arg. Ub is cotranslationally removed from NP, leaving Arg as the NH2-terminal residue of NP. Arg-NP obeys the N-end rule defined by Varshavsky and colleagues (49) and is rapidly degraded (20). As seen in Figure 1 (A and B), Ub-Arg-NP is unstable, exhibiting a t1/2 of less than 20 min, whereas NP is stable throughout the chase period. zLL had no significant effect on protein degradation. In contrast, 10 μM zLLL or 100 μM lactacystin completely blocked degradation, while lactacystin at 10 μM had an intermediate effect on protein stability. Consistent with these results, both zLLL and lactacystin (but not zLL) induced the accumulation of polyubiquitinated cellular proteins in uninfected L-Kd cells, as determined by Western blotting of detergent lysates using anti-Ub Abs (results not shown). This is in agreement with previous reports on the accumulation of ubiquitinated proteins in the presence of proteasome inhibitors (50, 51).

Proteasome inhibitors block presentation of OVA

The processing of the Kβ-restricted determinant from OVA (OVA257–264) has been reported to be proteasome dependent, as determined by activation of a T cell hybridoma (26). To confirm that the proteasome inhibitors were capable of blocking Ag presentation in rVV-infected L929 cells, L-Kb cells incubated with proteasome inhibitors were infected with rVVs expressing OVA or the cytosolic minigene product, and the expression of cell surface OVA257–264-class I complexes was quantitated by cytofluorography using a mAb highly specific for the complex (42). As previously reported, this method easily detects quantitative differences in the presentation of the OVA257–264 peptide produced in different contexts: cells synthesizing cytosolic OVA257–264-class I complexes was quantitated by cytofluorography using a mAb highly specific for the complex (42). As previously reported, this method easily detects quantitative differences in the presentation of the OVA257–264 peptide produced in different contexts: cells synthesizing cytosolic OVA257–264-class I complexes was quantitated by cytofluorography using a mAb highly specific for the complex (42). As previously reported, this method easily detects quantitative differences in the presentation of the OVA257–264 peptide produced in different contexts: cells synthesizing cytosolic OVA257–264-class I complexes was quantitated by cytofluorography using a mAb highly specific for the complex (42). As previously reported, this method easily detects quantitative differences in the presentation of the OVA257–264 peptide produced in different contexts: cells synthesizing cytosolic OVA257–264-class I complexes was quantitated by cytofluorography using a mAb highly specific for the complex (42). As previously reported, this method easily detects quantitative differences in the presentation of the OVA257–264 peptide produced in different contexts: cells synthesizing cytosolic OVA257–264-class I complexes was quantitated by cytofluorography using a mAb highly specific for the complex (42). As previously reported, this method easily detects quantitative differences in the presentation of the OVA257–264 peptide produced in different contexts: cells synthesizing cytosolic OVA257–264-class I complexes was quantitated by cytofluorography using a mAb highly specific for the complex (42). As previously reported, this method easily detects quantitative differences in the presentation of the OVA257–264 peptide produced in different contexts: cells synthesizing cytosolic OVA257–264-class I complexes was quantitated by cytofluorography using a mAb highly specific for the complex (42). As previously reported, this method easily detects quantitative differences in the presentation of the OVA257–264 peptide produced in different contexts: cells synthesizing cytosolic OVA257–264-class I complexes was quantitated by cytofluorography using a mAb highly specific for the complex (42). As previously reported, this method easily detects quantitative differences in the presentation of the OVA257–264 peptide produced in different contexts: cells synthesizing cytosolic OVA257–264-class I complexes was quantitated by cytofluorography using a mAb highly specific for the complex (42). As previously reported, this method easily detects quantitative differences in the presentation of the OVA257–264 peptide produced in different contexts: cells synthesizing cytosolic OVA257–264-class I complexes was quantitated by cytofluorography using a mAb highly specific for the complex (42). As previously reported, this method easily detects quantitative differences in the presentation of the OVA257–264 peptide produced in different contexts: cells synthesizing cytosolic OVA257–264-class I complexes was quantitated by cytofluorography using a mAb highly specific for the complex (42). As previously reported, this method easily detects quantitative differences in the presentation of the OVA257–264 peptide produced in different contexts: cells synthesizing...
proteasomes in this process. This is confirmed by the inhibitory effect of lactacystin, which at 10 μM blocked complex formation to the same extent as zLLL. In contrast to the results with full-length OVA, the inhibitors had little effect on the generation of complexes from the cytosolic minigene product. The most straightforward explanation for these findings is that proteasome inhibitors block the formation of OVA257–264-Kb complexes from OVA by preventing proteasomes from contributing to the liberation of the peptides.

**Proteasome inhibitors do not inhibit the presentation of VV, and NP determinants to TCD8+.**

Cells present two immunodominant NP peptides to NP-specific TCD8: NP147-155 in association with Kd (53), and NP50–57 in association with Kb (54). We studied the effects of proteasome inhibitors on the presentation of these determinants in the following polypeptide contexts: full-length NP derived from PR8 or NT60 (termed NPPr8 and NPNT60, respectively), PR8 NP targeted to the ER by the IFN-β leader sequence (termed SNP), Ub-Arg-NP, and cytosolic minigene products. Despite its efficient translocation to the ER, generation of peptides from SNP is strictly TAP dependent (38), and therefore requires the action of cytosolic proteases.

We previously showed that 6 h after infection with rVVVs encoding the cytosolic minigene products, cells express approximately 50,000 Kd and Kb molecules with the respective peptides, as opposed to 30 copies/cell of NP147–155 and 1,800 copies of NP50–57 after infection with VV-NPPr8 (46). The extreme overexpression of the cytosolic minigene makes them insensitive to even major effects of proteasome inhibitors on viral gene expression, at least as detected by TCD8+–based assays (this limitation applies to a much lesser extent to cytofluorographic analysis using mAbs specific for class I peptide complexes, as described above). In the experiments shown below, therefore, rVVVs expressing cytosolic minigene products are included to demonstrate the maximal levels of lysis possible and to serve as controls for the specificity of the TCD8+ populations used.

rVV-infected L-Kd cells were used as target cells in a 51Cr release assay with TCD8+ specific for either NP50–57 (Fig. 3A) or NP147–155 (Fig. 3B). Cells were infected without inhibitor present or in the presence of 10 μM zLLL, 10 μM zLLL, or lactacystin at 10 or 100 μM. BFA was added to cells 250 min postinfection to block additional generation of cell surface class I peptide complexes (55, 56). This allowed us to remove the proteasome inhibitors from the TCD8+ assay, avoiding unidirectional effects of the inhibitors on TCD8+ function or 51Cr release from target cells. As we previously observed (38), targeting NP to the ER decreased the efficiency of generating NP147–155, while having little effect on the generation of NP50–57. The presentation of NP147–155 from NPNT60 was reduced relative to NPPr8. Since the sequence of the determinant is identical between the two, this represents an effect of flanking sequences or protein handling on peptide liberation.

In contrast to OVA, proteasome inhibitors did not effectively block the generation of NP determinants. zLLL did not significantly affect the generation of NP50–57 from any of the NP constructs. zLLL had no effect on presentation of NPPr8 to TCD8+ specific NP147–155, and presentation of SNP and NPNT60 was actually enhanced. This is unlikely to be due to the effect of zLLL on nonproteasomal proteases, since zLLL had little effect on NP147–155 presentation from SNP or NPNT60.

Lactacystin at 10 μM had little effect on presentation of NP50–57 and enhanced the presentation of NP147–155 from SNP and NPNT60, demonstrating the proteasome-specific nature of the latter effect. For NP50–57, 100 μM lactacystin partially blocked presentation from SNP, and did not affect presentation from NPPr8 or NPNT60. Lactacystin (100 μM) did not affect presentation of NP147–155 from either NPPr8 or NPNT60 and enhanced presentation from SNP.

In parallel experiments, we found that 100 μM lactacystin inhibits cell surface expression of rVV-encoded mouse CD54, human CD23, and human CD4, respectively, by 54, 52, and 38% after a 6-h infection, as determined using directly conjugated mAbs under saturating conditions. Thus, the inhibitory effects of 100 μM lactacystin on presentation of NP50–57 may in part be due to nonspecific effects on rVV gene expression or exocytosis of integral membrane proteins. The difference between 10 and 100 μM in enhancing NP147–155 presentation may reflect, to some extent, this nonspecific inhibition.

These findings were extended to another type of target cell (Fig. 4). P815 cells were coinfected with rVVVs expressing the target Ag of interest and a rVV expressing Kb. This allowed us to examine the presentation of another viral Ag, the nucleocapsid (N) of vesicular stomatitis virus, in addition to NPPr8. P815 cells treated with 25 μM zLLL demonstrated enhanced presentation of the NP147–155 peptide from NPPr8. In the same experiment, the presentation of the immunodominant N peptide (N52–59) to Kβ-restricted TCD8+ was severely compromised by zLLL. This shows that the inability of proteasome inhibitors to block generation of NP147–155 is not a peculiarity of L-Kd cells, and provides further evidence that proteasome inhibitors have disparate effects on different determinants.

**Increased production of NP147–155 in the presence of lactacystin: biochemical isolation of peptides**

These findings indicated that blocking proteasome activity enhances presentation of the NP147–155 determinant. This could be due to enhanced generation of NP147–155-Kb complexes or secondary effects due to alterations in other peptide class I complexes or other molecules involved in T cell recognition and lysis of target
cells. We therefore measured the amount of peptide class I complexes present in cells infected with either VV-NP PR8 or VV-SNP, in the presence or absence of 10 μM lactacystin. Acid-soluble peptides prepared from 5 × 10^8 cells were fractionated by HPLC, and the fractions containing antigenic peptides were titrated. Peptide concentrations were determined by comparative titration using a known quantity of synthetic peptide. We previously showed that virtually all peptides recovered by this method are derived from K_d molecules (46).

Peptide quantitation confirmed that the NP 147–155 is produced more efficiently from NP PR8 than from SNP: targeting NP to the ER reduced peptide production fourfold (Table I). Incubation of cells with 10 μM lactacystin enhanced peptide generation from both NP PR8 and SNP by approximately fourfold. This provides a direct demonstration of enhanced peptide generation following treatment with a proteasome inhibitor.

Analysis of the K_d-restricted, VV-derived peptides present in the HPLC fractions using polyclonal VV-specific T_CD8^+ populations (Fig. 5) revealed that lactacystin had little effect on the gen-

![Graph](image1)

**TABLE I.** NP 147-155 concentration in HPLC fractions from extracts of cells infected in the presence or absence of 10 μM lactacystin

<table>
<thead>
<tr>
<th></th>
<th>No Inhibitor</th>
<th>10 μM Lactacystin</th>
</tr>
</thead>
<tbody>
<tr>
<td>VV-NP</td>
<td>8.0 × 10^{-12} M^a</td>
<td>3.6 × 10^{-11} M</td>
</tr>
<tr>
<td></td>
<td>(29)_b</td>
<td>(130)</td>
</tr>
<tr>
<td>VV-SNP</td>
<td>1.9 × 10^{-12} M</td>
<td>6.8 × 10^{-12} M</td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td>(24)</td>
</tr>
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</table>

^a Peptide concentration in the HPLC fractions was estimated as described (46), by comparing their ability to sensitize uninfected targets for recognition by specific T_CD8^+ with that of serial dilutions of the corresponding synthetic peptide.

^b Numbers in parentheses show the calculated number of determinants per cell.

![Graph](image2)

**FIGURE 4.** Effect of proteasome inhibitors on the presentation of NP 147–155 and N 52–59 in P815 cells. Cells were coinfected with an rVV expressing K_b and with either VV-NP PR8, VV-VSV-N, or an rVV expressing the corresponding minimal epitopes. BFA was added 210 min after infection. Cells were labeled with ^51^Cr and tested for lysis by T_CD8^+ specific for NP 147–155 or N 52–59.

![Graph](image3)

**FIGURE 5.** Effect of lactacystin on generation of K_d-restricted VV peptides. HPLC profile of vaccinia-derived K_d-restricted peptides eluted from L-K_b cells infected either in the presence or absence of 10 μM lactacystin. Ten microliters of each fraction were used to sensitize P815 cells for recognition by K_d-restricted, VV-specific T_CD8^+ cells.
eration of K\textsuperscript{a}-restricted antigenic peptides eluting at fractions 91–92 and 98. Interestingly, lactacystin inhibited the generation of a VV peptide eluting at fraction 103, while enhancing the amount of peptide eluting at fraction 107. These findings provide biochemical evidence that supports the functional data indicating that proteasome inhibition has disparate effects on peptide generation covering a spectrum from inhibition (peptide in fraction 103) to enhancement (peptide in fraction 107). We cannot rule out at this point that the peptides in fractions 103 and 107 are related (e.g., the same core antigenic peptide, but with different flanking residues, or two overlapping epitopes, as has been described in a different system (57)), and that lactacystin enhances the production of one at the expense of the other.

**Disparate effect of proteasome inhibitors on protein stability and peptide generation**

The dissociation between cellular proteasome activity and peptide generation prompted us to investigate the role of proteasomes in generating peptides from Ub-Arg-NP. Despite the total inhibition of Ub-Arg-NP degradation by zLLL at 10 \( \mu \text{M} \), as shown in Figure 1, the generation of NP\textsubscript{50–57} and NP\textsubscript{147–155} at 5 \( \mu \text{M} \) of the inhibitor was unimpeded (Fig. 6). Increasing the concentration of zLLL to 15 \( \mu \text{M} \) still did not significantly block presentation. This demonstrates that antigenic peptides can be produced in a manner that is independent of the measurable proteasome-specific degradation of a full-length substrate.

**Treatment with proteasome inhibitors induces a cellular stress response**

In the course of characterizing the effects of proteasome inhibitors on VV-gene expression, we noted a major alteration in the expression of proteins in both VV-infected and uninfected cells. As seen in Figure 7A, treatment with zLLL strongly inhibited actin biosynthesis, and enhanced synthesis of several other proteins. As two of the most prominent bands migrate with molecular mass of \( \sim 70 \text{kDa} \), we conjectured that proteasome inhibitors induced a stress response. This was confirmed using Abs specific for different chaperones to collect specific gene products: hsc70 (heat-shock cognate protein (hsc), a cytosolic chaperone expressed at constitutively low levels and induced by stress), hsp70 (a family of cytosolic chaperones including proteins not expressed constitutively, as well as hsc70), hsp90 (another cytosolic chaperone), and grp94 (an ER resident chaperone, also known as gp96) were all induced by zLLL (Fig. 7B). Hsp90 was induced only marginally in these experiments, although its induction in other experiments was somewhat stronger. Similar results were obtained using lactacystin (not shown).

Heat-shock proteins act as molecular chaperones in vivo, binding to unfolded proteins, and some have been reported to bind antigenic peptides (58, 59). This raised the possibility that the enhanced presentation of NP\textsubscript{147–155} was a secondary effect of heat-shock protein induction. To test this possibility, L-K\textsuperscript{a} cells were heat shocked for 45 min at 42°C, in the presence or absence of 10 \( \mu \text{M} \) lactacystin, and then infected with VV-NP\textsubscript{PR8}. In independent experiments, we found a strong induction of heat-shock proteins in L-K\textsuperscript{a} cells treated at 42°C (data not shown). Heat shock resulted in an overall reduction of the specific \( ^{51}\text{Cr} \) release, but lactacystin was still able to enhance the recognition of the VV-NP\textsubscript{PR8}-infected cells by NP\textsubscript{147–155}-specific effectors (Fig. 7C). This suggests that the enhanced presentation of NP\textsubscript{147–155} is not a consequence of the heat-shock response induced by the proteasome inhibitors. The heat-shock response differs from that induced by blocking the proteasome activity, since grp94 and probably other stress proteins induced by proteasome inhibitors are not induced by heat shock (60). It remains plausible that these proteins are involved in the enhanced presentation of NP\textsubscript{147–155}.

**Discussion**

We recently reported that proteasome inhibitors only partially compromise the cell surface expression of VV-encoded mouse class I molecules, as determined using mAbs that recognize peptide-loaded class I molecules (10). In the same study, we found that proteasome inhibitors have a variable effect on the presentation of H2\textsuperscript{a}-restricted antigenic peptides by L929 cells infected with PR8 or loaded with exogenous viral proteins by fusion of noninfectious virus preparations with cells. The effects depended on the protein studied and the route of delivery of proteins to the cytosol. Of particular interest to the present study, proteasome inhibitors enhanced presentation of NP\textsubscript{60–57} from exogenous NP, and had little effect on presentation from biosynthesized NP.

In the present study, we extend these findings using rVV to express viral Ags. We found that for VV-produced cytosolic or ER-targeted NP, proteasome inhibitors have little effect on the generation of NP\textsubscript{60–57}, and enhance generation of NP\textsubscript{147–155}. A crucial question in analyzing these results is whether the inhibitors...
were used at sufficient concentrations to completely block proteasome activity. While this question cannot be definitively answered at present, our results argue toward the positive. We show that under the same conditions in which NP presentation is unaffected or enhanced, the generation of K\textsuperscript{d} -restricted determinants from OVA or N is severely compromised. Moreover, we also demonstrate a complete block of the degradation of Ub-Arg-NP. The latter finding is important as it shows that the inhibitors must be effective in 90% or more of the cells.

The resistance of NP\textsubscript{147–155} generation to proteasome inhibitors is consistent with the recent findings of Yellen-Shaw et al., who found that the proteasome inhibitor N\textsuperscript{-}acetyl-LLnL used at 50 μM enhances the generation of this determinant from rVV expressing different forms of NP (61). Under the same conditions, the generation of a determinant from another viral protein was inhibited (62). These findings are, however, apparently at odds with those of Cerundolo et al., who found that lactacystin inhibited presentation of VV-Ub-Arg-NP, VV-NP\textsubscript{327–498}, and a number of influenza virus-encoded proteins to T\textsuperscript{CD8}\textsuperscript{1} (63).

To examine presentation of VV-encoded NP constructs, Cerundolo et al. used T\textsuperscript{CD8}\textsuperscript{+} specific for a D\textsuperscript{b}-restricted peptide (residues 366–374). Unlike the K\textsuperscript{d}- and K\textsuperscript{k}-restricted NP determinants we have studied, the generation of this peptide requires expression of LMP2 and LMP7 proteasome subunits (64). It is not surprising, therefore, that generation of this peptide is proteasome dependent.

Cerundolo et al. treated cells with lactacystin at concentrations of 10 or 100 μM overnight before infection with influenza virus. Given the pleiotropic effects of proteasome inhibition on other aspects of cellular metabolism, it is not possible to eliminate the possibility that presentation of viral Ags was not due to indirect effects (e.g., altering delivery of substrates to other proteases due to modifications in chaperones, or modifying peptide handling in the ER). Moreover, as mentioned above, we found that preincubating L-K\textsuperscript{d} cells even for as short as 45 min with 100 μM lactacystin reduces the cell surface expression of a number of VV-encoded reporter proteins by approximately 50%. Additional experiments revealed that overnight treatment of cells with lactacystin at 10 or 100 μM greatly reduced influenza virus gene expression, as measured by cell surface expression of viral neuraminidase (expression reduced more than 10-fold at 10 μM and to background values at 100 μM). Increasing the concentration of the peptide aldehydes above 10 μM also results in reduction of rVV gene expression.

These findings reinforce the general principle that inhibitors should be used at the minimal concentration required to achieve inhibition of the target process. Use of inhibitors at higher concentrations greatly increases the chances of inhibiting unrelated, difficult to identify, targets. Even in the case of lactacystin, the observation that only proteasome β subunits are covalently labeled by following incubation of cells with [3H]lactacystin (65, 66) does not constitute proof of its specificity, particularly when the abundance of proteasomes (≈1% of cellular proteins) relative to other cytosolic proteases is considered. Indeed, it was reported recently that lactacystin inhibits the activity of a lysosomal protease (67). Despite these problems with nonspecific inhibition of cellular processes related to Ag presentation, it is remarkable that Cerundolo found that the presentation of an HLA-A1-restricted NP peptide persisted in cells treated overnight with 500 μM lactacystin. Thus, taken as a whole, the results of Cerundolo et al. are basically in accord with our findings that some determinants are presented in a proteasome-independent manner.

This conclusion is fully supported by the work of Hughes et al., who found that assembly of human class I molecules was only
partially compromised by the proteasome inhibitors Cbz-LLF and N-acetyl-LLnL (68). Analyzing radiolabeled peptides from purified class I molecules by HPLC, they noted that peptide yield was variably effected, and that the relative abundance of individual peptide species was greatly modified. This latter finding is the biochemical equivalent of our demonstration that lactacystin modifies the profile of VV-derived antigenic peptides recovered by HPLC.

An unexpected effect of the proteasome inhibitors was their enhanced generation of NP

\[ \text{NP}_{147-155} \]

This could reflect a number of molecular processes. First, since the proteasome is a major source of class I-binding peptides, peptides produced in a proteasome-independent manner will have less competition for all of the events entailed in locating and binding to class I molecules. This may be particularly important for NP

\[ \text{NP}_{147-155} \]

which does not display a particularly high affinity for K\( ^1 \) molecules relative to other peptides (38). Second, the proteasome may actively destroy NP

\[ \text{NP}_{147-155} \]

or its precursors in the cytosol. In a collaborative study with Tevethia and colleagues, we recently found an rVV-encoded cytosolic peptide whose presentation could only be detected by incubating cells with zLLL (69). Ossendorp et al. reported that substitutions in an antigenic viral peptide that result in intradeterminant cleavage by purified 20S proteasomes also abrogate presentation of the peptide from the full-length protein (31). Third, the inhibitors may modify proteasome activity in a manner favoring the creation of some determinants. This would be the case if one of the proteasome activities cleaves within the epitope, as has been suggested by Yellen-Shaw et al. (61). It is possible that in vivo the different proteasome activities are not equally sensitive to the inhibitors. This is particularly relevant with lactacystin, which has been shown in vitro to preferentially block the chymotryptic- and tryptic-like activities of 20S proteasomes (11). Fourth, as discussed below in more detail, enhanced generation may result from secondary effects of proteasome inhibition.

Probably the most important finding in the present study is the persistence of peptide generation from Ub-Arg-NP in the presence of proteasome inhibitors at sufficient concentration to completely block its degradation. The discrepancy between protein degradation rates and peptide generation was originally noted by Goth et al., who studied the generation of antigenic peptides from N-end rule substrates biosynthesized by APCs (23). Together, these findings indicate that proteasomal digestion of ubiquitinated proteins does not always result in the production of antigenic peptides. Indeed, the relevance of Ub targeting of proteins to proteasomes in antigenic processing is uncertain; in a prior study, we failed to detect diminished generation of antigenic peptides in cells with a compromised Ub-conjugation pathway (25). The observation that the generation of antigenic peptides is unrelated to fate of full-length conformed proteins is consistent with our recent proposal that antigenic peptides can be derived from defective ribosomal products: truncated or misfolded proteins that are shunted to peptidogenic proteases (70).

The major question raised by our findings is the nature of the protease(s) responsible for the generation of antigenic peptides in the presence of proteasome inhibitors. As mentioned above, we cannot eliminate the possibilities that either a subset of proteasomes or activities present in proteasomes are resistant to the inhibitors. Also plausible is that sensitive determinants require multiple rounds of proteasomal cleavage, while resistant determinants require fewer cycles. Arguing against this are the findings of Yang et al. that zLLL can block presentation of a peptide consisting only of 21 residues (27).

If not proteasomes, then what liberates antigenic peptides from NP and other proteins resistant to the effects of proteasome inhibi-


