Proteasomes Can Either Generate or Destroy MHC Class I Epitopes: Evidence for Nonproteasomal Epitope Generation in the Cytosol

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Proteasomes Can Either Generate or Destroy MHC Class I Epitopes: Evidence for Nonproteosomal Epitope Generation in the Cytosol

Chance John Luckey,* Gina M. King,† Jarrod A. Marto,† Sunita Venketeswaran,* Bernhard F. Maier,* Victoria L. Crotzer,* Teresa A. Colella,* Jeffrey Shabanowitz,† Donald F. Hunt,‡ and Victor H. Engelhard†‡

Proteasomes have been implicated in the production of the majority of peptides that associate with MHC class I molecules. We used two different proteasome inhibitors, the peptide aldehyde N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) and the highly specific inhibitor lactacystin, to examine the role of proteasomes in generating peptide epitopes associated with HLA-A*0201. Neither LLnL nor lactacystin was able to completely block the expression of the HLA-A*0201. Furthermore, the effects of LLnL and lactacystin on the expression of different categories of specific epitopes, TAP independent vs TAP dependent and derived from either cytosolic or membrane proteins, were assessed. As predicted, presentation of two TAP-dependent epitopes was blocked by LLnL and lactacystin, while a TAP-independent epitope that is processed in the endoplasmic reticulum was unaffected by either inhibitor. Surprisingly, both LLnL and lactacystin increased rather than inhibited the expression of a cytosolically transcribed and TAP-dependent peptide from the influenza A virus M1 protein. Mass spectrometric analyses of in vitro proteasome digests of a synthetic 24 mer containing this epitope revealed no digestion products of any length that included the intact epitope. Instead, the major species resulted from cleavage sites within the epitope. Although cleavage at these sites was inhibitable by LLnL and lactacystin, epitope-containing species were still not produced. We conclude that proteasomes may in some cases actually destroy epitopes that would otherwise be destined for presentation by class I molecules. These results suggest that some epitopes are generated by nonproteosomal proteases in the cytosol.


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(27–29). However, experiments with cells as well as knockout mice lacking LMP2 and/or LMP7 suggest the LMP subunits affect a minority of class I binding peptides (30–35). A second line of evidence for proteasome involvement in Ag processing came from the demonstration that the 26S proteasome is involved in the degradation of polyubiquitin-tagged proteins and that ubiquitination was necessary for the generation of some class I epitopes. Initial work with temperature-sensitive mutants in ubiquitin conjugation demonstrated that presentation of cytosolically loaded OVA depended on ubiquitination (36). However, a subsequent study was unable to confirm this observation (37). Other studies have shown that the rate of the degradation by the ubiquitin-proteasome pathway of cytosolically loaded OVA, β-galactosidase, and bacterially expressed Pep60 protein correlated with the rates of class I Ag presentation (38–40). However, the necessity for ubiquitin-mediated degradation in generating the majority of class I-associated peptides, many of which are derived from endogenously expressed proteins, remains unclear. Still others have used in vitro proteasome digestion systems to generate epitopes from either whole proteins or synthetic polyproteic substrates that contain known epitopes internally (41–46). More recently, the proteasome-associated regulator PA28 has been shown to enhance the production of class I binding peptides when overexpressed within cells (47) as well as when added to in vitro proteasome digests (48–50). The studies with the LMPs, ubiquitin, and PA28 clearly demonstrate at least a limited role for proteasomes in the generation of class I-associated peptides.

Further evidence for proteasome involvement in class I epitope generation comes from the use of various proteasome inhibitors. Initial studies using the peptide aldehydes and later the more specific proteasome inhibitor lactacystin (Lac) demonstrated a significant decrease in the surface expression of the murine class I molecule H2-KK (50–52) as well as the human HLA-A*0201 molecule (53). The peptide aldehydes as well as Lac have further demonstrated proteasome involvement in the production of several individual class I-associated peptides: exogenously loaded, virally expressed, or in one case constitutively expressed (51, 52, 54–57). These results also imply that proteasomes are involved in the generation of some class I-associated peptides.

In contrast, recent studies have suggested the possibility that not all class I epitopes require proteasome function for their generation. The TAP-independent expression of HLA-A*0201 appears to be largely insensitive to the effects of proteasome inhibition (53, 58). Likewise, the expression of two virally expressed murine class I alleles is either not blocked or only partially blocked by proteasome inhibitors (59). Furthermore, individual epitopes derived from virally expressed influenza proteins also appear insensitive to proteasome inhibition (59, 60). The generalizability of the role of proteasomes to all class I alleles and epitopes therefore remains uncertain.

In the inhibitor studies to date, proteasome function was assessed in systems in which epitopes were derived from proteins that were either cytosolically loaded by electroporation (51, 54) or expressed after bacterial (55) or viral infection (56, 57, 59, 60). However, in the majority of cells at the target level, class I-associated peptides are derived from endogenously expressed proteins rather than from cytosolically loaded proteins. Although viral or bacterial infection is the normal route leading to epitope expression, proteasome inhibitors could potentially have effects on viral or bacterial protein expression unrelated to class I epitope generation. We therefore established a system to investigate the effects of the proteasome inhibitors Lac and LLnL on the generation of HLA-A*0201 epitopes that were constitutively expressed within cells. Constitutive expression avoids experimental concerns about possible inhibitor effects on viral or bacterial infection and/or gene expression. One of the HLA-A*0201-associated epitopes tested in our system, M158-96, has previously been shown to be blocked by proteasome inhibitors in a viral infection system (57). The M158-96 epitope therefore provides a means to compare the constitutive and viral expression systems. In addition, many of the epitopes in this study are expressed in different subcellular locations before their association with class I, allowing the assessment of proteasome function in several class I processing pathways. Finally, we use an in vitro proteasome digestion system to further explain the results observed in cells.

**Materials and Methods**

**Inhibitors**

Lac was a gift from Dr. S. Omura (The Kitasato Institute, Tokyo, Japan) and is a Streptomyces metabolite that irreversibly inhibits proteasomes via covalent binding to the active sites of the catalytically active β subunits (52, 61). LLnL, also known as calpain inhibitor I, was purchased from Calbiochem (La Jolla, CA) and reversibly inhibits proteasomes as well as several other classes of proteases (51). Brefeldin A (BFA) was purchased from Sigma (St. Louis, MO) and inhibits egress of all proteins through the secretory pathway at the level of the cis-Golgi, including newly generated class I-peptide complexes (62–64).

**Tumor lines**

All tumor lines were of human origin and were maintained in cell medium (RPMI 1640 medium supplemented with 5% FCS containing SerXtend (Irvine Scientific, Santa Ana, CA) and 2 mM glutamine) in a humidified 5% CO2 atmosphere at 37°C. The melanoma line DM93 was the gift of Drs. Hilliard F. Seigler and Timothy L. Darrow (Duke University, Durham, NC). The B lymphoblastoid cell line C1R-A2.1 has been described previously (65). C1R-A2.1 was transfected via electroporation with a cDNA clone of the M1 gene from Influenza A/PR8 (gift from Dr. Julian Hickling of Cantab Pharmaceuticals Research, Cambridge, U.K.) to generate the line C1R-A2.1.M1, which was maintained in medium containing 300 µg/ml of G418 and 300 µg/ml of hygromycin.

**CTL**

The murine xenospecific CTL clones AT1-15 and AHHII12-2 were derived and grown as described previously (15, 66). Both recognize only human cells expressing HLA-A*0201. The peptide epitope recognized by AHHII12-2 has been identified, but the source protein is unknown (15). Murine CTL lines specific for the peptide YMDGTMSQV derived from the tyrosinase gene (TYR368–376) and the peptide KTWGQYWQV derived from the gp100 gene (gp100 143–150), both presented by HLA-A*0201 on human melanoma cells (67, 68), were generated by i.p. injection of 5 × 108 plaque-forming units of recombinant vaccinia into mice expressing a chimeric MHC class I molecule with the α1 and α2 domains from HLA-A*0201 and the α3 domain from H-2Dd (69). TYR368–376-specific CTL were generated from mice primed with vaccinia encoding the YMDGTMSQV minigene and restimulated weekly with spleenocytes pulsed with 1 µg/ml of the synthetic YMDGTMSQV peptide and IL-2, gp100 134–142-specific CTL were generated from mice primed with vaccinia encoding the full-length gp100 gene and restimulated weekly with spleenocytes pulsed with 1 µg/ml of synthetic KTWGQYWQV peptide and IL-2. The murine CTL clone ILA1, specific for the peptide AIMDKNIIL from the influenza A/PR8 NS1 gene (NS1 122–130) and presented by HLA-A*0201, was previously described (70). All murine lines were grown in RPMI 1640 medium supplemented with 10% FCS, 15 mM HEPES, 50 mM β-ME, 2 mM glutamine, and essential and nonessential amino acids in a humidified 8% CO2 atmosphere at 37°C. The human CTL line LSGM1S, specific for the peptide GILGFVFTL from the influenza A/PR8 M1 gene (M1 104–112) and presented by HLA-A*0201, was previously described (70). Human CTL were grown in RPMI 1640 medium supplemented with 15% FCS and 2 mM glutamine in a humidified 8% CO2 atmosphere at 37°C.

**Acid treatment and flow cytometry**

DM93 cells (20–40 × 10⁵) were washed in cell medium and centrifuged. The pellet was resuspended gently in 1 ml of 300 mM glycine (pH 2.5) and 1% (w/v) BSA and incubated for 3 min at 37°C. The suspension was neutralized by dilution with 40 ml of cell medium and centrifuged. Cells (1 × 10⁶) were aliquoted into 200 µl of cell medium in the presence or the absence of 10 µg/ml of BFA or various concentrations of Lac or LLnL and...
incubated for 5 h at 37°C to allow class I re-expression. For the preincubation experiment, cells were incubated for 2 h at 37°C in the presence or the absence of inhibitors before acid treatment and further incubation with inhibitors. Re-expression was stopped by washing twice in FACS medium (RPMI 1640 supplemented with 2.5% FCS containing SerXtend and 0.08% sodium azide) at 4°C. All cells were then incubated for 1 h at 4°C with the HLA-A*0201-specific mAb PA2.1, washed three times with FACS medium, and incubated for 1 h with FITC-labeled sheep α-mouse IgG (Cappel, Durham, NC). Cells were washed four times with FACS medium, fixed with 2% paraformaldehyde, and analyzed on a Becton Dickinson FACSscan (Mountain View, CA). Ten thousand events were counted, live cells were gated, and the mean fluorescent intensity was determined for cells falling within the gate. PA2.1 recognizes mature, peptide-containing HLA-A*0201 molecules.

Acid treatment and CTL assay

DM93 or C1R.A2.1.M1 cells were acid treated as described above and washed, and 2 × 10^6 cells were aliquoted into 1 ml of cell medium plus 100 μCi of Na^35CrO_4 and incubated for 5 h at 37°C in the presence or the absence of BFA, Lac, or LLnL. Labeled targets were washed four times in cell medium at 4°C and used in a standard 4-h 51Cr release assay. All targets were incubated with 10 μg/ml BFA during the 4-h incubation with CTL to block further egress of class I-peptide complexes and to allow CTL killing to take place in the absence of proteasome inhibitors.

Flu infections and CTL assays

DM93 cells were preincubated for 2 h at 37°C in the presence or the absence of Lac or LLnL, washed twice in serum-free RPMI 1640, resuspended in 20 hemagglutinin units/cell of influenza A/PR8 virus (provided by Dr. T. J. Braciale, University of Virginia, Charlottesville), and placed on ice for 10 min. The mixture was moved to 37°C for 30 min. One milliliter of cell medium plus 100 μCi of Na^35CrO_4 was added, and targets were incubated for 5 h at 37°C in the presence or the absence of Lac or LLnL. Labeled targets were washed four times in cell medium at 4°C and used in a standard 4-h 51Cr release assay. All targets were incubated with 10 μg/ml BFA during the 4-h incubation with CTL to block further egress of class I-peptide complexes and to allow CTL killing to take place in the absence of proteasome inhibitors.

Peptides

Synthetic peptides were made by standard F-moc chemistry using a model AMS422 peptide synthesizer (Gilson, Middleton, WI). All peptides were purified to >98% purity by reverse phase HPLC on a C_8 column (Vydac, Hesperia, CA). Purity and identity were established using a triple quadrupole mass spectrometer (model TSQ-70, Finnigan, San Jose, CA).

Proteasome isolation and digestions

All proteasome preparations were purified from the human B lymphoblastoid cell line 721. Purified 20S proteasomes were isolated as described previously (43). 26S proteasome-enriched fractions were generated as described previously (71). Synthetic M1 24 mer peptide SPLITKGlLGFVFRLTVPSEGR (1 μg) was added to purified proteasomes (0.5 μg) in 50 nM Tris-HCl, 5 mM MgCl_2, 1 mM DTT, and 2 mM ATP at 37°C. Reactions were stopped by the addition of trifluoroacetic acid and acetic acid to final concentrations of 0.2 and 10%, respectively.

Mass spectrometry

Aliquots of proteasome digests corresponding to 1 pmol of the 24 mer initially added were loaded onto a POROS microcapillary column (75 μm i.d. × 12 cm) and gradient eluted using acetonitrile and 0.1 M acetic acid, with the concentration of acetonitrile increasing at 6.6%/min into a triple quadrupole mass spectrometer (model TSQ-70, Finnigan) equipped with an electrospray ion source. Scans were acquired every 1.5 s over a mass range m/z 300 to 1400. Peptides were sequenced by recording collision-activated dissociation (CAD) using argon as the target gas. Five hundred femtolarves of the synthetic peptide YLEPGPVTA (m/z 946) was spiked into each sample before analysis to allow for standardization between sample runs.

Results

Proteasome inhibitors block some, but not all, HLA-A*0201 expression

We investigated the effects of the highly specific proteasome inhibitor Lac as well as the peptide aldehyde LLnL, on the generation of HLA-A*0201 epitopes that were constitutively expressed within cells. Cells expressing HLA-A*0201 epitopes were briefly exposed to a pH 2.5 buffer to denature and remove surface class I–peptide complexes. Acid-stripped cells were then allowed to re-express their class I–epitope complexes in the presence or the absence of proteasome inhibitors.

We first measured the effects of proteasome inhibition on the re-expression of HLA-A*0201 after acid treatment by FACS. Acid treatment decreased surface expression of HLA-A*0201 to undetectable levels as measured by FACS. Re-expression to approximately 50% of normal levels occurred within 5 h, and this recovery was inhibitable by BFA (Fig. 1A). BFA inhibits egress of all proteins through the secretory pathway at the level of the cis-Golgi, including newly generated class I–peptide complexes (62–64). Lac inhibited 40% of the re-expression of class I, with maximum inhibition seen at a concentration of 100 μM. Saturating amounts of LLnL (250 μM) inhibited HLA-A*0201 by approximately 50% (Fig. 1A). One possible source of the class I expressed in the presence of inhibitors shown in Figure 1A is an internal pool of class I binding peptides that could have been generated by proteasomes before inhibition but were not yet expressed on the cell surface during the acid treatment. We therefore pretreated cells with proteasome inhibitors for 2 h to permit any intracellular peptides generated by proteasomes before inhibition to be expressed on the
surface before acid washing. Pretreatment with inhibitors, acid washing, and subsequent incubation with inhibitors did not significantly affect overall class I recovery (Fig. 1B). The inhibitor-resistant expression of HLA-A*0201 cannot therefore be accounted for by a large internal pool of proteasome-generated peptides. Figure 1 establishes the concentrations of inhibitors necessary to obtain maximum inhibition in vivo. It further demonstrates that proteasomes play a significant role in the generation of peptide epitopes associated with HLA-A*0201. However, the majority of HLA-A*0201 expression persists despite saturating amounts of the proteasome inhibitor Lac.

Specific HLA-A*0201 epitopes are re-expressed in 5 h after acid treatment

We next used this acid wash/re-expression system to evaluate the proteasome dependence of specific HLA-A*0201-associated epitopes recognized by CTL. The epitopes recognized by the xenogeneic, HLA-A*0201-specific CTL clones AHIII12-2 and AT1-15 epitopes are TAP independent and dependent, respectively (15, 66). TYR368–376 and gp100154–162 are generated from the membrane proteins tyrosinase and gp100 commonly expressed in melanomas (67, 68). The TYR368–376 epitope is TAP dependent (72), but the TAP dependence of the gp100154–162 epitope is unknown. M158–66 is a TAP-dependent epitope generated from the cytosolically derived M1 protein of the influenza A/PR8 virus. Recognition of each of the epitopes recovered after acid treatment to 50 to 100% of normal levels in 5 h (Fig. 2). In each case, re-expression was dramatically inhibited in the presence of BFA. The residual recognition of acid-treated targets in the presence of BFA could be due to incomplete removal of endogenously expressed Ag by the acid, incomplete inhibition by BFA, or the presence of preformed epitope not yet on the cell surface but past the level of BFA blockade at the cis-Golgi. These possibilities seem unlikely given the overall completeness of acid washing, BFA inhibition, and lack of intracellular pool of peptides in the FACS experiment shown in Figure 1. However, residual killing in the presence of BFA may result from low level nonspecific killing by CTL and/or the possibility that CTL may be more sensitive than FACS to low levels of epitope expression. Whatever the reason for the residual CTL killing shown in Figure 2 after acid treatment plus BFA, the difference in CTL recognition of targets incubated in the absence and the presence of BFA provided a window in which to ask whether the proteasome inhibitors Lac and LLnL blocked the re-expression of the particular epitopes of interest.

Proteasome inhibitors did not block the expression of a TAP-independent epitope

We next addressed the ability of Lac and LLnL to inhibit expression of the TAP-independent epitope recognized by the xenogeneic CTL clone AHIII12-2. Since this epitope appears to be generated from a protein that is cotranslationally translocated into the ER and processed entirely within the ER lumen (11), it was predicted that Lac would not inhibit its expression. Indeed, generation of this epitope was independent of saturating levels of Lac (Fig. 3A). Importantly, the lack of inhibition of AHIII12-2 demonstrates that Lac does not generally block the expression of class I or other molecules necessary for CTL recognition. In addition, expression
of this epitope was insensitive to the addition of saturating levels of LLnL. (Fig. 3B). While LLnL inhibits proteasomes at a concentration of 10 μM, it also inhibits the presentation of some TAP-independent epitopes at a concentration of 250 μM, presumably by blocking one or more ER resident proteases (58). The failure of LLnL to inhibit expression of the AHIII12-2 epitope again demonstrates that this inhibitor does not affect class I expression or CTL recognition nonspecifically and also indicates that generation of this epitope in the ER proceeds via the action of proteases distinct from those implicated in the generation of other TAP-independent peptides.

**Proteasome inhibitors do block the formation of a TAP-independent epitope**

We next looked at the ability of Lac and LLnL to inhibit the re-expression of the HLA-A*0201-presented TAP-dependent peptide epitope recognized by the xenospecific CTL clone AT1-15. Incubation of acid-treated target cells with either LLnL or Lac inhibited re-expression of the AT1-15 epitope by 73 and 55%, respectively, relative to BFA treatment at an E:T cell ratio of 11:1 (Fig. 3C). Collectively with the AHIII12-2 data presented above, these results indicate that Lac, and in these cases LLnL, are working at the level of peptide supply in the cytosol. Both block the expression of the TAP-dependent epitope, but when peptide is supplied directly in the ER, as with the AHIII12-2 epitope, class I processing and epitope presentation are not affected.

**Proteasome inhibitors block the expression epitopes generated from membrane proteins**

We next looked at the necessity of proteasome function in the generation of the TAP-dependent TYR 368–376 epitope and the gp100 154–162 epitope from the membrane proteins tyrosinase and gp100, respectively. The mechanism by which membrane proteins enter the class I pathway in a TAP-dependent manner is currently unknown. Incubation of acid-treated target cells with Lac inhibited re-expression of the TYR 368–376 epitope by >74% relative to BFA treatment at an E:T cell ratio of 13:1 (Fig. 4A) and the gp100 154–162 epitope by >83% relative to BFA treatment at an E:T cell ratio of 20:1 (Fig. 4B). This demonstrates that proteasomes are involved in the generation of both these membrane-derived proteins.

**Proteasome inhibitors enhance rather than block expression of the TAP-dependent influenza M1 58–66 epitope**

The final epitope examined was the well-defined HLA-A*0201-restricted epitope from the M1 protein of the influenza A/PR8 virus. M1 is a cytosolic protein made on free polyribosomes, and the M1 58–66 epitope is TAP dependent (73). We therefore expected that M1 58–66 would be sensitive to both Lac and LLnL. Surprisingly, the expression of this epitope detected with the specific CTL line LSGM1S was instead significantly enhanced by blocking proteasome function with either Lac or LLnL (Fig. 5A). This increase was observed at all concentrations of Lac between 10 and 100 μM, indicating that it was not a consequence of failing to fully inhibit proteasome activity (Fig. 5B). We also evaluated recognition by a second M1 58–66–specific clone, HAM42, which detects M1 58–66 expression on peptide-pulsed and influenza-infected cells, but not on the stable transfected used here because of its low level of M1 expression. However, Lac treatment of the C1R-A2.1.M1 transfected led to recognition by HAM42 (Fig. 5C). This provides further evidence for a significant increase in M1 58–66 epitope expression after inhibition of proteasome activity.

The increase in the expression of the M1 58–66 epitope in cells treated with proteasome inhibitors contrasted with a recent report that M1 58–66 expression was inhibited by Lac (57). The experiments presented in that study differed from those shown in Figure 5 in that the target cells expressed the epitope after influenza A virus infection rather than after stable transfection. Consequently, we evaluated the sensitivity of M1 58–66 expression to proteasome inhibitors in cells infected with influenza A/PR8 virus. As was observed with stable transfectants, treatment of virus-infected cells with 10 μM Lac led to a significant increase in the expression of M1 58–66 epitope as detected by LSGM1S CTL (Fig. 6A). However, in contrast to the observations with the stable transfectants, expression of the M1 58–66 epitope was inhibited in virus-infected cells treated with 25 and 50 μM Lac. Similarly, LLnL treatment of influenza-infected targets caused an increase in M1 58–66 expression at 10 μM and a decrease in expression at 250 μM (data not shown). Interestingly, the lower doses of proteasome inhibitors that resulted in an increased expression of the M1 58–66 epitope blocked the expression of another HLA-A*0201-restricted influenza epitope, N51 122–130, in the same target cells (Fig. 6B). We conclude that high doses of Lac and LLnL interfere with an aspect of influenza virus expression that is not directly related to Ag processing and presentation. However, treatment of virus-infected cells with low doses of proteasome inhibitors and treatment of stable transfectants with either low or high doses both demonstrate the selective enhancement of M1 58–66 epitope expression.

One explanation for the increase in M1 58–66 expression seen with Lac and LLnL is that these inhibitors have nonspecific enhancing effects on HLA-A*0201 protein levels, TAP function, or other aspects of the class I processing pathway. However, this is
unlikely based on the lack of increase in expression of the proteasome insensitive AHIII 12–2 epitope; the blocking effects seen with AT1–15, TYR368–376, and gp100154–162; and the dissimilar chemical structures and modes of action of these two compounds. Another possibility is that M1 epitope generation is not dependent on proteasomes, but blocking proteasome function decreases the number of other peptides that compete with the M15 8–6 6 epitope for HLA-A*0201 binding. However, a similar effect was not seen for the AHIII 12-2 epitope, and therefore, this explanation also seems unlikely. A third possible explanation for the enhancing effects of the protease inhibitors on expression of the M15 8–6 6 epitope is that proteasomes destroy a substrate that is used by another protease within the cell to generate the epitope. To address the possibility that proteasomes destroy the M15 8–6 6 epitope in vivo, we performed in vitro proteasome digests of a long synthetic peptide sequence from the M1 protein.

In vitro proteasome digests destroy the M158–66 epitope

We further addressed the role of proteasomes in M1 epitope formation by digestion by a synthetic 24 mer of the M1 sequence with highly purified 20S proteasomes isolated from the cytoplasmic fraction of the B lymphoblastoid cell line 721 and analyses of the products by tandem mass spectrometry. The naturally processed epitope from M1 has been previously demonstrated to be the nonamer M158–66 (74). This nonamer epitope was centrally located within the 24 mer sequence to minimize end effects of internal cleavages. Digestion was allowed to proceed for 0.5, 3, 6, and 12 h. Preliminary analysis demonstrated that approximately 70% of the starting 24 mer peptide remained intact after 3 h, and later time points showed continued digestion, demonstrating there was still excess starting substrate remaining for the proteasomes at 3 h. To determine what peptides were generated by the proteasomal cleavage, the 3-h 20S digestion as well as parallel digestions conducted in the presence of Lac were further analyzed (Fig. 7). The total ion chromatograms in Figure 7 represent the summation of all ion species measured by the spectrometer as they eluted from the HPLC column. The frames above the total ion chromatograms demonstrate the same digestions, but display the relative intensity of only one particular m/z ion. Each ion shown represents the major charge state of a particular peptide whose sequence was confirmed via CAD and is written in the upper right corner of the frame. The predominant digestion products reveal that the major proteasome cleavage sites of the M1 24 mer were after the phenylalanines, shown by the arrows above the sequence in Figure 7. These cleavage products were decreased by approximately 80%
when the digestion proceeded in the presence of Lac (Fig. 7B).

Similar digests conducted in the presence of LLnL showed the same profile of inhibition (data not shown). Therefore, the major products observed in the proteasome digest were inhibitable by both Lac and LLnL. Further digests of the M1 24 mer were conducted with the 26S-enriched proteasome fractions under the same conditions. The 26S-enriched fractions demonstrated the same major digestion species correlating to destruction of the nonamer epitope as in the purified 20S digestions (data not shown).

Digests were further analyzed for the presence of the intact M1 58–66 epitope. No \( m/z \) species could be found in the digest that corresponded to any charge state of the nonamer sequence. We then wished to determine whether there were longer peptide species in the digest that might contain the intact nonamer sequence internally. The \( m/z \) was calculated for each charge state of all the potential digestion products of any length that could contain the intact nonamer epitope, and the digest was analyzed for the presence of these predicted ions. Although one candidate mass (536) was found in the digest, sequencing by CAD demonstrated that it was not the potential epitope-containing species. Therefore, the purified 20S digest did not generate a detectable species of any length up to 23 mer that contained the intact nonamer epitope (data not shown). At 3 h, the proteasome had digested a significant portion of the starting peptide (30%), yet was still in substrate excess. Therefore, it seems unlikely that an epitope-containing species would be made at later time points. Further analysis of the sample digested for 0.5 h demonstrated the same digestion products as those in the 3 h sample (data not shown). Thus, it did not appear that the epitope was made early and then destroyed. Finally, no new digestion products that contained the nonamer epitope could be found in the digests performed in the presence of Lac (data not shown). This inability of proteasomes to generate the nonamer epitope or a longer species containing it is consistent with the digestion products observed in Figure 7. The nonamer epitope was never generated by any proteasome population in vitro. Instead, proteasomes destroyed the epitope, and that destruction was inhibitable by Lac.

Discussion

We used two different proteasome inhibitors, Lac and LLnL, to study the role of proteasomes in the generation of HLA-A*0201-associated peptides constitutively expressed within cells. We found that neither inhibitor was able to completely inhibit surface
The novel peptide vinyl sulfone (Z-L 3 VS) at 100 μM inhibitions than that seen with LLnL alone (Fig. 1). Saturating levels of both inhibitors do not give greater inhibitory effects observed on total HLA-A*0201 surface expression. The ability of both lactacystin and LLnL to inhibit HLA-A*0201 expression by FACS clearly implicates proteasomes in the generation of at least some of the peptides associated with this class I molecule. However, saturating levels of both inhibitors fail to block the majority of HLA-A*0201 expression. Furthermore, the inhibitor-independent expression of HLA-A*0201 cannot be accounted for by a large internal pool of proteasome-generated peptides (Fig. 1B). These data are consistent with a recent report that Lac and LLnL partially inhibit the intracellular maturation of HLA-A*0201 molecules to endoglycosidase H-sensitive forms (53).

One explanation for the partial inhibition seen is that neither LLnL nor Lac inhibits all the activities of the proteasome. While we cannot formally rule out such a possibility, we believe that several lines of evidence support the contention that saturating levels of inhibitors fully inhibit proteasome activity. Recent reports demonstrate that Lac binds to all the predicted catalytically active β subunits of the proteasome in vivo (52). Preincubation with inhibitors before acid treatment does not further decrease the overall level of class I expression (Fig. 1B). Lac and LLnL also seem to inhibit the same proteasome activities, since coincubating cells with saturating levels of both inhibitors does not give greater inhibition than that seen with LLnL alone (Fig. 1B). Furthermore, the novel peptide vinyl sulfone (Z-L 3 VS) at 100 μM gives results similar to those observed with Lac (unpublished observation). Like Lac, the peptide vinyl sulfone has been shown to fully inhibit the in vitro activity of proteasomes at 100 μM as well as to bind to all known active subunits of the proteasome in vivo (75). We have gone on to observe the proteasome inhibitor-resistant expression of HLA-A*0201 in several different cell lines. While a majority of HLA-A*0201 remained resistant to proteasome inhibitors, two other MHC alleles expressed in the same cell line were fully inhibited, implying that this effect is allele specific (our manuscript in preparation). Again, we can never be sure that we have inhibited all activities of the proteasome, including potential activities as yet undescribed, through the use of proteasome inhibitors. However, based on the reasons stated above, we favor the interpretation that saturating amounts of the specific inhibitors have completely inhibited all the known activities of the proteasome, and that inhibitor-insensitive class I expression derives from peptides not generated by proteasomes.

A likely source of Lac-insensitive HLA-A*0201 expression is the large TAP-independent pool of peptides that bind to this class I molecule (11–15). These peptides appear to be generated by ER resident proteases and do not require cytosolic localization for their expression. Indeed, the large majority of TAP-independent peptides have been shown to be insensitive to both LLnL and Lac (53, 58). Whether all the inhibitor insensitive expression of HLA-A*0201 can be entirely accounted for by the TAP-independent pathway cannot be addressed by these data.

Our results with specific HLA-A*0201-associated epitopes reinforce the conclusions reached from the FACS data. The lack of inhibition of TAP-independent AHIII12-2 and the sensitivity of the TAP-dependent AT1-15 are consistent with the partial inhibitory effects observed on total HLA-A*0201 surface expression. Furthermore, the epitopes derived from membrane proteins tyrosinase and gp100 were both blocked by proteasome inhibitors. Therefore, some aspects of their processing must occur within the cytosol. Our laboratory has recently demonstrated that generation of the TYR368–376 epitope requires initial translation into the ER, export of the full-length product into the cytosol, processing by proteasomes, and retransport of the processed peptide back into the ER via TAP (72). The linking of initial translation in the ER and retrograde transport into the cytosol for class I processing has been observed by others (76) and presumably describes a class I processing pathway that is generalizable to many TAP-dependent epitopes of membrane proteins. It is unknown whether gp100 follows a similar pathway in the generation of the gp100154–162 epitope.

The most surprising result of the present study was the demonstration that proteasome inhibitors do not inhibit, but rather significantly increase, surface expression of the M158–66 epitope. This observation was confirmed with two different CTL and through the use of both influenza-infected cells as well as cells stably transfected with the M1 gene. This result appears to directly conflict with another recent report that demonstrated inhibition of expression of this epitope in virus-infected cells (57). We found that the effects of proteasome inhibitors on M158–66 epitope expression in virus-infected cells were dependent on the concentration of inhibitor. Low concentrations increased expression of the epitope in both infected cells and transfectants. However, high doses continued to augment epitope expression in the transfectant while inhibiting expression in virus-infected cells. We conclude that the decrease in the M158–66 epitope observed at high concentrations of inhibitor during viral infection is unrelated to direct inhibition of M158–66 peptide production from the M1 gene, but may indicate interference with some other aspect of viral gene or protein expression. Therefore, M158–66 expression is clearly increased upon specifically blocking proteasome function.

To address the possibility that proteasomes destroy the M158–66 epitope in vivo, we undertook a series of in vitro proteasome digests of a long synthetic sequence from the M1 protein. Digests with both purified 20S proteasome- and 26S proteasome-enriched fractions showed the same major digestion products that resulted in the destruction of the M158–66 epitope. No intact epitope-containing species could be found in the digests. Furthermore, although treatment of the proteasomes with Lac inhibited proteolytic cleavage within the epitope sequence, it did not result in generation of the intact epitope or other epitope-containing digestion products shorter than the starting material. We realize that the in vitro system described above may not be a perfect model for the in vivo digestion of the M1 protein by proteasomes. The form of the proteasome responsible for digestion of any given protein or the length of the natural substrate for proteasome cleavage is not known or easily tested. However, two other groups found that products observed in in vitro digests accounted for the presence and the absence of epitopes observed in vivo (43, 45). Both groups demonstrated in vitro that proteasomes are able to create one class I epitope while destroying another. In our hands, the destruction of the M158–66 epitope seen with both 20S and 26S populations of proteasomes was also consistent with our in vivo results. We have gone on to demonstrate that these same purified proteasome populations are able to generate an HLA-B7-restricted epitope from six different long synthetics representing naturally occurring variants of the HIV-nef protein (manuscript in preparation). We therefore believe that these purified proteasomes are representative of the in vivo population.

Based on the in vivo and in vitro results, we conclude that not only are proteasomes uninvolved in the generation of the M158–66

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epitope, but they participate in its destruction. Blocking the destruction of M₁₂₈₋₆₆ in cells with Lac or LLnL increases the epitope, but they participate in its destruction. We instead propose that the M₁₂₈₋₆₆ epitope is generated by another protease in the cytosol whose identity is currently unknown.

Interestingly, proteasome inhibitor-insensitive class I expression has recently been shown for several murine alleles, some of which do not demonstrate significant TAP-independent expression (55, 59, 77). There have also been reports that the expression of specific TAP-dependent epitopes was unchanged in cells treated with proteasome inhibitors (59, 60), consistent with the idea that these epitopes are generated in the cytosol via the actions of proteases other than the proteasome. In addition, Vintisky et al. (59) observed that the expression of class I epitopes derived from exogenously introduced hemagglutinin protein was enhanced by Lac, although there was little or no effect on the presentation of the hemagglutinin epitope derived from the endogenously synthesized protein. However, our current work expands the proteasome inhibitor studies to the human allele HLA-A*0201 (59). In addition, we have studied the effects of proteasome inhibition in several different pathways of class I Ag processing: TAP dependent and TAP independent as well as membrane and cytosolic proteins. An important aspect of the present study is that the proteasome dependence of Ags was addressed in a system in which the Ag was being constitutively expressed by the APCs. Certainly our data in the influenza infection model suggest that caution is necessary in the use of proteasome inhibitors in viral expression models. Such constitutive expression, as opposed to cytosolic loading, mirrors the class I processing pathway for the majority of cells and eliminates the possible indirect effects the inhibitors might have on viruses. Finally, the use of the in vitro proteasomal digestion system helps to shed light on the mechanism by which an epitope might increase in the presence of proteasome inhibition and provides additional evidence that proteasomes can destroy class I epitopes. The results presented here indicate that the question of which proteases are involved in Ag processing is far from resolved and that studies of additional epitopes and additional restriction elements are warranted.

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