The Protease Inhibitor, N-Acetyl-L-Leucyl-L-Leucyl-L-Norleucinal, Decreases the Pool of Major Histocompatibility Complex Class I-binding Peptides and Inhibits Peptide Trimming in the Endoplasmic Reticulum

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Summary

N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL), which inhibits proteasomes in addition to other proteases, was found to prolong the association of major histocompatibility complex class I molecules with the transporters associated with antigen processing (TAP), and to slow their transport out of the endoplasmic reticulum (ER). LLnL induced a reversible accumulation of ubiquitinated proteins and changed the spectrum of peptides bound by class I molecules. These effects can probably be attributed to proteasome inhibition. Unexpectedly, in the TAP-deficient cell line .174, the rate of intracellular transport of human histocompatibility leukocyte antigen (HLA) A2 was also reduced by LLnL, and the generation of most HLA-A2-associated signal sequence peptides was inhibited. The inhibition of HLA-A2 transport in .174 cells was found to be less sensitive to LLnL than in wild-type cells, and a similar difference was found for a second protease inhibitor, benzoyloxycarbonyl-L-leucyl-L-leucyl-L-phenylalanilyl. These data suggest that under some conditions such inhibitors can block trimming of peptides by an ER peptidase in addition to inhibiting cytosolic peptide generation.

Newly synthesized MHC class I heavy chain β2-microglobulin (β2m) dimers bind peptides in the endoplasmic reticulum (ER) before their transport to the cell surface. The vast majority of the associated peptides are derived from cytosolic proteins. These peptides are transported into the ER from the cytosol in an ATP-dependent fashion by the transporters associated with antigen processing (TAP; for a review see reference 1), which physically associate with peptide-free class I-β2m dimers via the TAP.1 subunit (2-4). Peptide binding to the class I molecules triggers their release from TAP, allowing their transport to the cell surface. Certain MHC class I alleles also bind peptides derived from the signal sequences of a small number of secreted or type I transmembrane proteins (5-7). In these cases, peptide loading is usually, though not always (8), independent of TAP.

The predominant protease responsible for the generation of cytosolic peptide has thought to be the proteasome, a large (20S) multisubunit protease. The 20S proteasome can degrade proteins in vitro, but in vivo it predominantly exists as the nucleus of a larger (26S) ATP-dependent complex (9, 10). The 26S proteasome is responsible for the degradation of ubiquitinated proteins as well as at least one nonubiquitinated protein, ornithine decarboxylase (11, 12). Two subunits of the proteasome, LMP2 and LMP7, are encoded in the MHC (13). Mice with targeted disruption of either of these genes exhibit some deficiency in CTL development, and LMP7-deficient mice exhibit a reduction in expression of class I MHC molecules (14, 15). Additionally, treatment of antigen-loaded target cells with proteasome inhibitors prevents their recognition by class I-restricted CTL (16). It has also been argued that ubiquitination of cytosolic protein antigens is important for their recognition by CTL, because mutant cell lines temperature sensitive for a key step in ubiquitination exhibit reduced sensitivity to CTL (17), although this finding is not universally accepted (18).

One of the inhibitors used by Rock et al. (16) to implicate the proteasome in HMC class I peptide generation in living cells was the peptide aldehyde N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL), which has recently been
shown to bind to the active sites of the archebacterial proteasome (19). The mammalian proteasome has been shown to have at least five different proteolytic activities, and LLnL inhibits them to varying degrees (20, 21). In this study, we set out to examine the effects of proteasome inhibition on TAP-class I association and on the rate of egress of class I–peptide complexes from the ER. In addition to finding anticipated effects, we made the surprising observation that transport of HLA-A2 molecules in TAP-negative cells was slowed by LLnL, and by a second proteasome inhibitor, benzoyloxycarbonyl-L-leucyl-L-leucyl-L-phenylalanine (Z-LLF-CHO), and found that the profile of associated signal sequence peptides was also affected by LLnL. The implication of these findings for potential ER processing of class I–associated peptides is discussed.

Materials and Methods

Cell Lines. Transfectants of the HMY2.C1R cell line, C1R.A2, C1R.B7, and C1R.B27 and the TAP-negative mutant cell line C1R.BT, and CIR.B27 and the TAP-negative mutant cell line .174 have been previously described (22, 23). All cell lines were maintained in IMDM (GIBCO BRL, Gaithersburg, MD) with 5% calf serum (Hyclone Laboratories Inc., Logan, UT) and gentamicin at 20 μg/ml.

Antibodies. The mAbs 4E (anti-HLA-B locus), BB7.2 (anti-HLA-A2), and 1G12 (antitransferrin receptor) were previously described (24–26). Affinity-purified anti-TAP.1 rabbit serum RRING4C generated against a COOH-terminal peptide from TAP.1, (2), and the rat mAb 3B10.7 (anti-class I) were also previously described (27). Immunoblots were probed with a rabbit anti-ubiquitin serum generously provided by Dr. Arthur L. Haas (Medical College of Wisconsin, Milwaukee, WI).

Inhibitors. The protease inhibitor LLnL or Calpain Inhibitor 1 was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA) and prepared as a 25-nM (100X) stock solution in DMSO. The inhibitor Z-LL-F-CHO was also prepared in DMSO at 25 mM and was a kind gift from Dr. Marian Orlowski (Mount Sinai School of Medicine, New York, NY).

Metabolic Labeling. 8 × 10⁶ cells were incubated in methionine-free medium containing 6% dialyzed FCS (Hyclone) with LLnL at 250 μM or the DMSO solvent as control, for 1 h at 37°C. The cells were pulse labeled with 0.5 mCi [35S]methionine (ICN Biochemicals, Inc., Costa Mesa, CA) for 15 min in fresh methionine-free medium in the continued presence of LLnL or DMSO alone and chased with a 15-fold excess of unlabeled methionine at 37°C for the indicated times. Labeling was stopped by diluting the cells in cold PBS. For experiments involving immunoprecipitation, 2 mCi of [35S]methionine was used.

Immunoprecipitations and Endoglycosidase H Treatment. Labelled cells were pelleted and lysed in 10 mM Tris, 150 mM NaCl (Tris-buffered saline [TBS]), pH 7.4, 1% Triton X-100 (Sigma Chemical Co., St. Louis, MO), or 5% digitonin (Wako Pure Chemical Industries, Ltd., Richmond, VA), containing 0.5 mM PMSF, 0.1 mM N-tosyl-L-lysyl-chloromethyl ketone (TLCK), and 5.0 mM iodoacetamide (IAA). Postnuclear supernatants were precleared for 1 h with normal rabbit serum and protein A–Sepharose and then incubated with 4E, BB7.2, or 1G12 and protein A–Sepharose for 1 h. Endoglycosidase H (endo H) digests were performed as described previously (28). To detect TAP–associated class I molecules in RRING4C immunoprecipitates from digitonin extracts, the protein A beads were heated at 100°C for 5 min in 2% SDS, 2 mM dithiothreitol in TBS, diluted 10-fold in 1% Triton X-100 in TBS with 10 mM IAA, and allowed to incubate at room temperature for 30 min. After cooling to 4°C, released class I heavy chains were precipitated as above with 3B10.7 and protein G–Sepharose.

Immunoblots. Blots were performed as described (29). Briefly, 10⁶ cells were lysed in 100 μl 1% Triton X–100 in TBS as above. Postnuclear supernatants were diluted with reducing sample buffer, separated by a 5–20% gradient SDS-PAGE, and electroblotted onto an Immobilon membrane (Millipore Corp., Bedford, MA). The membrane was blocked for 1 h in PBS containing 0.05% Tween 20 and 5% dehydrated milk, rinsed in PBS, and incubated overnight at 4°C with the rabbit anti-ubiquitin serum diluted in PBS containing Tween 20 and dehydrated milk. Bands were visualized with horseradish peroxidase–conjugated secondary goat anti-rabbit IgG antibody and chemiluminescence (ECL) substrate (Amersham Corp., Arlington Heights, IL).

Analysis of 3H-labeled Class I–associated Peptides. Cells (6–8 × 10⁶) in log phase growth were washed in PBS and incubated for 1 h at 37°C in Leu-free, Lys-free RPMI-1640 (GIBCO BRL) at 10⁷ cells/ml supplemented with 3% dialyzed FCS and 10 mM Heps (GIBCO BRL) with or without 25 mM LLnL added, as a 100X stock. Control experiments contained equal concentrations of DMSO (0.1% vol/vol). 1 mCi each of [3,4,5-3H]leucine and [3,4,5-3H]lysine (Amersham Corp.) in 10 mM Trs, 50 mM NaCl, pH 7.4, with PMSF, TLCK, and IAA. The postnuclear supernatants were cleared by centrifugation for 1 h at 100,000 g and applied to affinity columns. Affinity columns were packed with Biogel A5ma beads (Bio-Rad Laboratories, Hercules, CA) coupled to the mAbs 4E or MA2.1. Bound class I molecules were eluted and denatured by adding 10% acetic acid, and low molecular weight species were separated from class I heavy chain and β2m by filtering through a Centricon 10 (Amicon, Beverly, MA). Filtrates were resolved on a reversed-phase (RP) column (μBondapak C18) using a HPLC system (Waters Chromatography Division, Milford, MA). Gradients were generated using an increasing concentration of acetonitrile in 0.1% hydrochloric acid. Flow was 0.5 ml/min and 1.0-mI fractions were collected.

Quantitation of Gel Bands. The ratio of endo H–resistant to endo H–sensitive forms of class I was determined by exposing the dried SDS-PAGE gel to a low intensity phosphorus screen and scanning by a GS-250 Molecular Imager (Bio-Rad Laboratories). The bands were quantitated using the program Molecular Analyst Version 2.0.1 (Bio-Rad Laboratories) run on a Macintosh 8100/80 (Apple, Inc., Cupertino, CA).

Peptide Synthesis and Retention Times. Peptides used to determine the retention times of HLA-A2 signal sequences were synthesized and purified by the Keck Foundation Biotechnology Resource Laboratory (Yale University). Retention times were determined by loading and eluting 10 μg of each peptide individually using the column and gradient conditions described above.

Results

LLnL Causes a Reversible Accumulation of Ubiquitinated Proteins. Ubiquitin–dependent proteolysis is believed to be the major nonlysosomal proteolytic pathway (30, 31). Ubiqu-
proteasome of this complex by LLnL disrupts the degradation of ubiquitinated proteins, as evidenced by the general suppression of peaks with the exception of a single peak in fraction numbers 70-74 (Fig. 4 B). The most dramatic effect on bound peptides was seen with HLA-B7 (Fig. 4, C and D). In this example, equal numbers of cells were treated, and again, less than half the amount of class I molecules was recovered. Here the profiles were normalized to account for the difference in the amounts of recovered heavy chain and showed a striking difference in the amounts of recovered heavy chain and showed a striking difference in the amounts of recovered heavy chain and showed a striking difference in the amounts of recovered heavy chain and showed a striking
Figure 2. LLnL slows the transport of MHC class I molecules but not a peptide-independent molecule, the transferrin receptor. Cells were preincubated for 1 h with 250 μM LLnL or solvent alone at 37°C, pulsed for 15 min with 0.5 mCi of [35S]methionine, and chased for 2 h. Immunoprecipitates were treated with endo H and were separated on a 10.5% SDS-PAGE gel. The ratio of endo H-resistant bands to endo H-sensitive bands are shown in the respective graphs. Cells and precipitating antibodies are as follows: (A) C1R.A2, BB7.2; (B) CIR..B7, 4E; (C) C1P,.B27, 4E; and (D) C1P,.B7, 1G12 (control, antitransferrin receptor). Controls (lanes Con) used isotype-matched antibodies. (Filled arrowheads) Endo H-resistant bands; (unfilled arrowheads) endo H-sensitive bands.

Enhancement of peaks in fractions 100 and 125 in the LLnL-treated population (note the change in the y-axis). A similar but less dramatic effect was seen for HLA-B27-bound peptides, also normalized for the amount of recovered heavy chain (Fig. 4, E and F). For HLA-B27, as in the previous examples, peaks at 75, 90, and 110 were enhanced. The augmentation of a single peak in the presence of LLnL was also demonstrated with HLA-A3 (data not shown). The peptides that are enhanced by LLnL may represent peptides translated at this length in the cytoplasm requiring no proteolytic cleavage, they may be peptides whose generation is unaffected by the inhibitory action of LLnL, or they may be signal sequence peptides generated in the ER whose generation is also unaffected by LLnL. Both HLA-A2 and HLA-B7 have been shown to bind signal sequence-derived peptides (5–7). To determine the origin of these peptides, it would be necessary to sequence them. Unfortunately, the time during which cells can be treated with LLnL is limited to 10 h, after which they begin to die (data not shown). This would make it difficult to accumulate sufficient quantities of the peptides for sequencing.

LLnL Inhibits Peptide Generation in the ER. The prolonged TAP association and slower transport of MHC class I molecules in LLnL-treated cells was assumed to result from a reduction in the supply of cytosolically generated peptides. HLA-A2 in .174 and T2 cells binds signal sequence peptides, and its transport should therefore not be affected by LLnL. Unexpectedly, however, the rate of egress of HLA-A2 from the ER in .174 was found to be greatly diminished in the presence of the inhibitor (Fig. 5 A). To determine if the inhibi-
tion of signal sequence peptide generation was reducing HLA-A2 assembly and transport. HPLC profiles of peptides bound by HLA-A2 in .174, with and without LLnL treatment, were generated (Fig. 5 B). The major doublet peak 3 and the minor peak 1 were decreased in the presence of LLnL, whereas peaks 2 and 4 were unaffected. These peaks, in the cell line T2 (a fusion product of .174 and similarly deficient in TAP expression), were previously found

Figure 3. LLnL enhances and extends the association of MHC class I molecules with TAP 1. Cells were preincubated for 1 h in 250 μM LLnL or solvent alone at 37°C, pulsed for 15 min with 2.0 nCi of [35S]methionine, chased for 4 h and extracted in 1% digitonin. TAP molecules were immunoprecipitated using purified anti-TAP. 1 rabbit antibodies. Associated class I heavy chains were released by SDS denaturation and reprecipitated using the mAb 3B10.7 (see Materials and Methods). Cells were as follows: (A) C1R.A2; (B) C1R.B7; and (C) C1R.B27.

Figure 4. LLnL reduces the amount of assembled MHC class I molecules and changes the profile of peptides bound. Cells were preincubated with 250 μM LLnL or solvent alone for 1 h at 37°C, then labeled with 1 mCi each of L-[3,4,5-3H]leucine and L-[4,5-3H]lysine for 5.5 h. MHC class I molecules were isolated by affinity purification and the bound peptides were separated by HPLC (see Materials and Methods). Cells and affinity columns were as follows: (A–B) C1R.A2, MA2.1 (peptides loaded on HPLC were not normalized for class I recovery); (C–D) C1R.B7, 4E (peptides loaded were normalized for class I recovery); and (E–F) C1R.B27, 4E (peptides loaded were normalized for class I recovery).
to be peptides derived from signal sequence peptides (5, 6). The six signal sequence peptides isolated from HLA-A2 in T2 were synthesized, and their retention times in RP-HPLC were determined (Table 1). These peptides included fragments of the signal sequences of calreticulin, the γ-interferon inducible protein (IP-30), and the signal sequence receptor α subunit. The synthetic peptides coeluted with the peptides isolated from .174, although the requirement for collecting fractions to detect the 3H-labeled peaks made it impossible to resolve three of the peptides, which are grouped as peak 3 in Fig. 5 and Table 1. However, only peaks 2 and 4 were unaffected by LLnL. Peak 2 corresponds to the longest peptide (12 residues) derived from IP-30 and terminates in a COOH-terminal glutamine residue. Peak 4 corresponds to a part of the calreticulin signal sequence and ends in glycine. Peak 1 and all the potential components of peak 3 terminate in valine or alanine. Thus LLnL in this case may be inhibiting a peptidase with specificity for an aliphatic residue. Because signal sequences are cleaved in the ER, and the TAP deficiency of .174 precludes the reentry of peptides into the ER after cytosolic trimming, it seems most likely that the affected enzyme is an ER peptidase.

### Cytosolic and ER Proteolysis Have Distinct Sensitivities to LLnL and Z-LLF-CHO.

To determine the relative sensitivities of the proteases affecting class I assembly in C1R.A2 and .174 to LLnL and a more potent proteasome inhibitor (Z-LLF-CHO; 20), we titrated their effects on the intracellular transport of HLA-A2 by pulse–chase analysis (Fig. 6). Inhibition of HLA-A2 transport by LLnL and Z-LLF-CHO in C1R.A2 was detectable at 2.0 and 0.08 μM, respectively (Fig. 6, A and C). However, in .174, 50.0 μM LLnL and 2.0 μM Z-LLF-CHO were required for an observable effect (Fig. 6, B and D). These findings are consistent with the idea that the responsible protease in C1R and the responsible protease inhibited in .174 are different, and that the protease in C1R, presumably the proteasome, is significantly more sensitive. Z-LLF-CHO was also found to affect the peptide profile of CIR.B7 in a similar fashion to LLnL (data not shown).

### Discussion

Many studies have focused on the role of the 26S protease complex (and its core subunit, the 20S proteasome) in...
Retention times and source of HLA-A2–associated peptides in .174 (see Fig. 5). Indicated references are Wei and Cresswell (6) and Henderson et al. (5).

the generation of class I bound peptides. These studies have used LMP7 and LMP2 knockout mice (14, 15), cells expressing a temperature-sensitive ubiquitination phenotype (17, 18), and inhibitors of the proteasome (16). Proteasome inhibitors, many being peptide aldehydes, have been found to inhibit the proteolytic activity of the 20S proteasome in vitro (20, 21), to bind to the active site in the crystal structure of the 20S proteasome (19), and to block the generation of peptides from cytoplasmic proteins and prevent the subsequent expression of peptides on the cell surface in conjunction with MHC class I (16). To further characterize the effect of proteasome inhibitors on the processing and loading of MHC class I molecules, we investigated one of the most widely used cell-permeable inhibitors, LLnL.

<table>
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<th>Peak</th>
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<th>Peptide source and reference</th>
<th>Retention time</th>
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<td>1</td>
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<td>IP-30; Wei and Henderson</td>
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</tr>
<tr>
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<td>IP-30; Wei</td>
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<tr>
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<tr>
<td></td>
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<td>107.51</td>
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<tr>
<td></td>
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Figure 6. HLA-A2 transport in .174 is less sensitive to both LLnL and Z-LLF-CHO than in the wild-type cell CIR.A2. CIR.A2 (A and C) and .174 (B and D) cells were preincubated in the indicated concentration of LLnL (A and B) or Z-LLF-CHO (C and D), metabolically labeled for 15 min, and chased for 2 h in the continued presence of the inhibitor. HLA-A2 was immunoprecipitated with BB7.2 at the indicated times and the ratios of endo H–resistant to endo H–sensitive class I determined (see Materials and Methods).
LLnL treatment produced all of the predicted effects on class I processing that would result from a peptide-deficient state in the cell. First, the rate at which class I molecules were transported from the ER was slowed in the presence of LLnL. Second, the association of class I and TAP molecules in the ER was enhanced and extended by treatment with LLnL. Third, the amount of peptides bound by class I molecules was decreased. LLnL caused a reversible accumulation of ubiquitinated proteins, normally degraded by the 26S protease complex. Rock et al. (16) showed that the ability of a range of peptide aldehyde inhibitors, including LLnL, to inhibit proteasome function, correlated with their ability to block MHC class I-restricted antigen processing. Thus, although the precise role of ubiquitination in antigen processing remains in question, the combination of evidence strongly argues that the proteasome is the major protease involved.

In addition to reducing the overall yield of MHC class I molecules and associated peptides (Fig. 4, A and B), LLnL also induced changes in the profile of peptides bound. This is particularly evident in Fig. 4, C and D, where two HLA-B7-associated peaks are dramatically increased. These and similar peaks must correspond to peptides either unaffected by LLnL and better represented because of an overall reduction in the available competing pool of peptides, or to peptides actively enhanced by LLnL treatment. For example, polypeptides might normally be cleaved within the peptide sequences enhanced in LLnL-treated cells. This could occur either in the cytosol, perhaps mediated by the chymotryptic-like activity most strongly inhibited by LLnL (21), or even in the ER after TAP-mediated translocation.

That peptide cleavage can occur in the ER is clearly shown by the effects of LLnL on the signal sequence-derived peptides associated with HLA-A2 in the TAP-negative .174 cell line (Fig. 5). LLnL treatment unexpectedly slowed the egress of HLA-A2 from the ER in .174, as did a second inhibitor, Z-LLF-CHO (Fig. 6). The inhibitors had no effect on the transport rate of transferrin receptors in .174 cells, arguing for an effect specific to class I molecules (data not shown). HLA-A2 escapes the ER in TAP-negative cell lines because it binds peptides generated from a number of hydrophobic signal sequences. We found that LLnL inhibited the generation of the majority of signal sequence-derived peptides that bind to the HLA-A2 allele and propose that the decrease in HLA-A2-specific peptides reduces the number of properly assembled, transport-competent HLA-A2 molecules. Effects on signal sequence degradation are unlikely to result from proteasome inhibition because the proteasome is confined to the cytosol and nucleus (33). Although evidence exists for an ATP-dependent mechanism for peptide translocation from the ER to the cytosol (34), any peptides trimmed in the cytosol presumably would require TAP to reenter the ER. Thus, in .174, any peptidase involved in generating class I-associated peptides and affected by LLnL must reside in the ER. The data shown in Fig. 5 B and Table 1 suggest that the peptidase inhibited by LLnL may cleave COOH-terminal to aliphatic amino acids because the unaffected peptides (peak 2 and 4, Fig. 5 B) terminate in a glutamate residue and glycine residue, respectively. However, with the limited number of peptides available for study it would be premature to make this a firm conclusion.

Transport of HLA-A2 molecules was found to be affected at lower concentrations of LLnL and Z-LLF-CHO in C1R cells than in .174 cells (Fig. 6). Nevertheless, it seems likely that some of the effects of LLnL on class I peptide loading in wild-type cells could result from inhibition of ER peptidases. When such inhibitors are used to investigate the origin of individual peptides that serve as T cell epitopes, as opposed to studies of the general process of MHC class I-restricted peptide generation, this possibility clearly must be borne in mind. Trimming of certain peptides in the ER, first suggested by Falk et al. (35) and for which reasonable evidence now exists (36, 37), may be inhibited by LLnL or other peptide aldehyde inhibitors that also inhibit proteasome-mediated degradation. Even more specific proteasome inhibitors, such as the recently described Streptomyces-derivative lactacystin (38), must be evaluated for possible effects on ER-mediated proteolysis before their use in antigen-processing studies can be properly evaluated. Clearly, inhibitors that specifically affect ER peptidases would be extremely useful in investigating the mechanisms involved in generating MHC class I-associated peptides. Effects on signal sequence degradation, measured using HLA-A2 in .174 cells to "trap" the degradation intermediates as shown in Fig. 5, might provide a useful assay for such inhibitors.

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