

Use of Proteasome Inhibitors to Examine Processing of Antigens for Major Histocompatibility Complex Class I Presentation

Luis C. Antón, Jack R. Bennink, and Jonathan W. Yewdell

1. Introduction

Proteasomes are multicatalytic proteases present in the nucleus and cytosol of eukaryotic cells. The central catalytic core, the 20S proteasome, consists of four heptameric rings, the central two of which contain the catalytic β -subunits, members of a new family of threonine (Thr)-proteases. The outer rings, made of α -subunits, bind the regulators that control the substrate specificity of the proteasome. The binding of a 19S regulator to each end of the 20S core creates the 26S proteasome, which degrades ubiquitinated substrates in an adenosine triphosphate-dependent manner (1,2).

Proteolytic degradation of cytosolic substrates is the chief source of antigenic peptides that are presented by major histocompatibility complex class I (MHC-I) molecules. The involvement of proteasomes in the generation of class I ligands was suggested by their intracellular distribution and multiple proteolytic activities and by the fact that genes encoding two of the β -subunits are located in the MHC, and are controlled by cytokines in parallel with class I molecules and other proteins associated with antigen (Ag) processing and presentation (3–5). The introduction of proteasome inhibitors to cellular studies enabled the demonstration of the dominant role of this protease in cellular protein turnover and its involvement in the generation of class I ligands (6).

Currently, there are four kinds of commonly used proteasome inhibitors, all of which, through different mechanisms, base their activity on the modification of the gamma oxygen (O γ) on the N-terminal, active residue of Thr in one or more of the catalytic β -subunits (for a review on the mechanisms of the inhibitors, see ref. 7). These are:

From: *Methods in Molecular Biology*, vol. 156: *Antigen Processing and Presentation Protocols*
Edited by: J. C. Solheim © Humana Press Inc., Totowa, NJ

Table 1
Molecular Weights, and Concentrations and Solvents used for Stock Solutions of the Inhibitors and the Negative Control AcLLM

Inhibitor	Mol wt	Solvent	Concentration
zLLL	475.6	DMSO	40 mM
		Ethanol	
AcLLnL	383.5	DMSO	40 mM
		Ethanol	
zLLnV	461.61	DMSO	10 mM
		Ethanol	
AcLLM	401.6	DMSO	25 mM
		Ethanol	
Lactacystin	376.4	DMSO	40 mM
		H ₂ O	
CLβL	213.2	DMSO	
		Acetonitrile	
NLVS	722.6	DMSO	10 mM

1. Tripeptide aldehydes. Used in initial studies, they are not specific for proteasomes, but affect other proteases, particularly calpains (8). They form a reversible hemiacetal covalent bond with N-terminal Thr (9).
2. Lactacystin (10), and its active form, *clasto*-lactacystin β-lactone CLβL (11), a *Streptomyces* sp. natural product, which covalently and irreversibly blocks proteasome activity; this is, so far, the most specific inhibitor of the proteasome, but some inhibitory effects on other proteases have been reported (12).
3. Peptidyl-vinylsulfones, which form a covalent bond with the active group, and are also irreversible (13).
4. Boronic salts, a group of potent inhibitors that only recently has started to be thoroughly studied (14). Their binding to the active site is reversible.

An important limitation to the use of proteasome inhibitors is that none of the inhibitors exclusively affects the proteasome. To ascertain that proteasome inhibition is the cause (but not necessarily the proximal cause, *see below*) of the effect observed, appropriate controls must be performed. For the peptide aldehydes, there are several related compounds that block a similar spectrum of cellular proteases without affecting the proteasome (one of them, N-acetyl-leucyl-leucyl-methioninal [Ac-LLM], is included in **Table 1**). Also, at least two mechanistically different kinds of proteasome inhibitors should yield comparable results.

The first step in using proteasome inhibitors should be to assess the optimal inhibitor concentration, defined as the minimal concentration that completely blocks the cellular degradation of a proteasome substrate. Chimeric proteins,

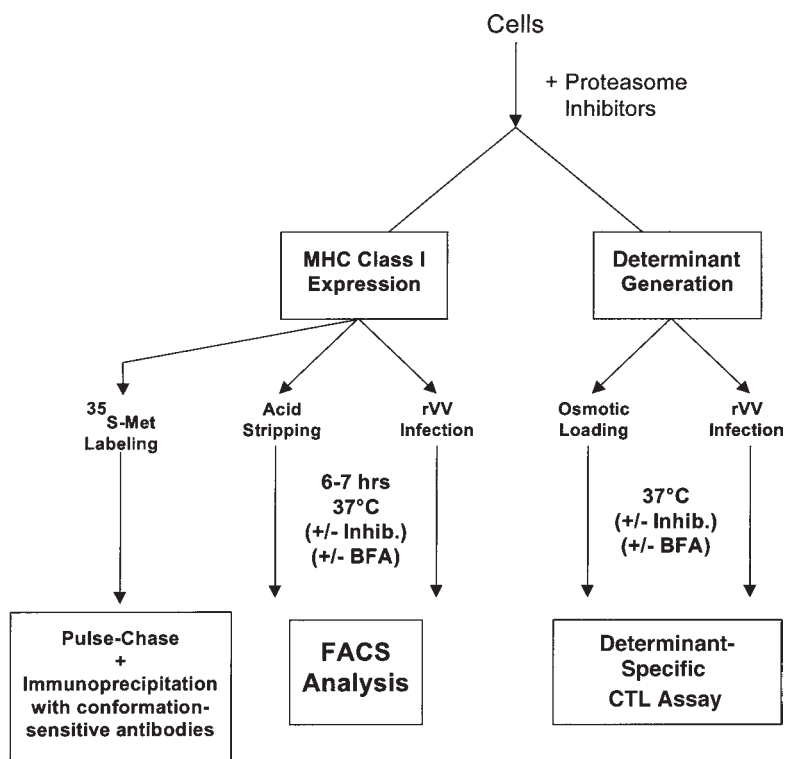


Fig. 1. Scheme of the different methods described to study the effect of proteasome inhibitors on Ag processing for MHC-I presentation.

composed of an N-terminal moiety of ubiquitin, followed by a destabilizing amino acid, according to the N-end rule (*15,16*), and a target protein, are commonly used substrates. The method detailed here uses one of these proteins, UbRNP, in which the nucleoprotein (NP) from influenza virus A/NT/60/68 is preceded by ubiquitin and a destabilizing residue of arginine Arg (*17*). The protein is expressed as a recombinant vaccinia virus (rVV), and pulse-labeling of the infected cells, followed by chases at different times, provides a good estimate of proteasome activity. The same principle could be used with other metabolically unstable proteins expressed in different ways (endogenously expressed, transfected, and so on).

Predominantly there are two different methods of investigating the role of proteasomes in MHC-I presentation (*see Fig. 1*). One way is to examine the effects of proteasome inhibitors on the maturation and cell surface expression of newly synthesized MHC-I molecules, which are peptide binding-dependent. One approach to accomplish this is to pulse-label the cells, in the absence or

presence of inhibitors, and to lyse them after different times of chase. The lysates are then incubated at 37°C, a treatment that renders peptide-receptive (or empty) molecules unable to bind antibodies (Abs) that recognize only folded class I molecules. As a control, synthetic peptides, which bind to the class I allele studied, are added to the lysates before the 37°C incubation. Cells with a compromised peptide delivery to class I molecules in the endoplasmic reticulum (ER) will have fewer molecules recognized by the Ab in the absence of exogenous peptide. Another approach is to estimate by flow cytometry the cell surface expression of newly synthesized class I molecules. This is done either by destroying cell surface molecules by acid treatment, then allowing for new ones to be expressed in the presence or absence of the inhibitors, or by infection with rVV-expressing MHC-I Ags different from those endogenously expressed by the infected cell, and, as before, following their cell surface expression.

The second method is to examine the effects of proteasome inhibitors on the generation of particular class I-peptide complexes, using either peptide-MHC-specific cytotoxic T-lymphocytes (CTL) or monoclonal antibodies (mAbs) specific for these particular complexes (18,19). This requires that the class I-peptide complexes studied are not expressed before proteasome inhibition. There are two chief methods of accomplishing this. The most widely used strategy entails transient expression of the substrate, which is achieved by either viral infection of the target cells or by loading of the purified protein into the cytosol; alternatively, cells constitutively expressing a target Ag are acid-stripped to remove existing complexes, and the effect of proteasome inhibitors on regeneration of peptide-class I complexes is determined.

As alluded to above, one must exercise caution when interpreting results from any experiment using proteasome inhibitors. Even brief inhibition of proteasomes has protean effect on cells, including reduction of ubiquitin pools (20), induction of a stress response (21–23), interference with cell cycle progression (24), and either enhancement or prevention of apoptosis, depending on the cell type (25,26). Cytosolic proteases different from the proteasome may contribute to Ag presentation (27–32). A candidate protease, as well as an inhibitor that blocks this protease (but not proteasomes) have been described, and may prove of great relevance to the field (33,34).

2. Materials

2.1. Inhibitor Stocks

Table 1 shows a list of commercially available proteasome inhibitors commonly used in studies of Ag presentation, solvents, and concentrations of stock solutions. These are carbobenzoxy-leucyl-leucyl-leucinal (zLLL, also known as MG132); N-acetyl-leucyl-leucyl-norleucinal (AcLLnL, calpain inhibitor I); carbobenzoxy-leucyl-leucyl-norvalinal (zLLnV, MG115); AcLLM, calpain

inhibitor II, a control inhibitor which does not affect the proteasome; lactacystin; CL β L, the active component of lactacystin; and 4-hydroxy-5-iodo-3-nitrophenylacetyl-leucyl-leucyl-leucyl-vinylsulfone (NLVS). Stocks should be stored at -20°C or below, and are stable for at least a few months. Aqueous solutions are not recommended for storage, because the half life of the inhibitor is reduced, most dramatically in the case of CL β L. Some useful information about the inhibitors can be obtained on-line from some of the manufacturer's Websites, particularly Calbiochem-Novabiochem (La Jolla, CA [www.calbiochem.com]) or Affinity Research (Exeter, UK [www.affinity-res.com]).

2.2. Cell Lines

There is no cell line specifically recommended for any of the methods described. The choice of cell line will depend mostly on the class I molecule, the determinant studied, and susceptibility to virus infection. Cells commonly used in this kind of studies include P815 (mastocytoma, H-2^d), L929 (fibroblast, H-2^k), EL4 (thymoma, H-2^b), LB27.4 (lymphoblastoid, H-2^{d/b}), or transfectants of the class I-deficient human cell line, H2MY2.C1R. Some of these cells are also available as transfectants expressing other class I molecules. Alternatively, class I molecules can be expressed using rVVs. Most mouse lymphocyte cell lines are resistant to VV infection.

2.3. Determination of the Effective Inhibitor Concentration

All solutions for tissue culture should be sterile.

1. ^{35}S -L-methionine (Met) (10 mCi/mL).
2. Phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) (PBS/BSA).
3. Methionine (Met) starving medium: Met-free, serum-free medium (RPMI or Dulbecco's modified Eagle's medium [DMEM]), containing 20mM HEPES.
4. PBS containing 10 mM L-Met (PBS-Met).
5. Iscove's modified DMEM containing 7.5% fetal calf serum (FCS) and L-Met (I/Met).
6. rVV expressing the chimeric protein UbRNP.
7. Laemmli's sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS/PAGE sample buffer, 2X solution, containing 0.5% (v/v) 2-mercaptoethanol.
8. Protease inhibitor cocktail. Boehringer Mannheim's (Indianapolis, IN) Complete inhibitors work well for this purpose. The cocktail is usually prepared as a 25X stock solution in water.
9. PhosphorImager (Molecular Dynamics, Sunnyvale, CA, or Fuji, Medical Systems, Stamford, CT), with software for quantitation of protein bands.

2.4. Conformational Stability of Newly Synthesized Class I Molecules

1. Same reagents needed for metabolic labeling as indicated in **Subheading 2.3**.
2. Lysis buffer: 50 mM Tris-HCl, pH 7.3, 100 mM NaCl, 1 mM EDTA, and 2% Triton X-100.

3. Protease inhibitors (*see Subheading 2.3.*).
4. Glass fiber filters (Whatman, Clifton, NJ).
5. Trichloroacetic acid (TCA), 10% (w/v).
6. Synthetic peptides with sequences known to bind class I. Prepare a 10 mg/mL stock solution in DMSO, and store at less than -20°C .
7. Protein A- or protein G-agarose (50% suspension). Use 30–40 μL of the 50% suspension per sample.
8. Abs specific for conformationally sensitive epitopes in MHC-I molecules. These Abs fail to recognize class I molecules unfolded after incubation at 37°C . Some Abs that share this characteristic include MA2.1 (ATCC clone no. HB54, specific for HLA-A2 and -B17), B22 (H2-D^b), Y-3 (HB176, H2-K^b), 30-5-7 (HB31, H2-L^d), and 34-5-8S (HB102, H2-D^d).
9. Wash buffers for the immunoprecipitations (35):
 - a. 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and 0.5% Nonidet P-40 (NTE).
 - b. NTE containing 0.5 M NaCl.
 - c. NTE containing 0.15 M NaCl and 0.1% SDS.
 - d. 10 mM Tris-HCl, pH 7.4, and 0.1% Nonidet P-40.
10. PhosphorImager with analysis software.

2.5. Acid Stripping of Class I Molecules and Cytofluorographic Analysis

1. 300 mM Glycine, pH 2.5, containing 1% BSA.
2. Brefeldin A (BFA) stock, 25 mg/mL in methanol (BFA may be purchased least expensively from Sigma, St. Louis, MO).
3. Anticlass I mAbs for use in cytofluorographic analysis. They are available from different manufacturers, unlabeled or conjugated to fluorescein isothiocyanate (FITC), as well as other fluorophores.
4. If the anticlass I Ab is not directly labeled, a FITC-conjugated anti-immunoglobulin Ab, specific for the anticlass I Ab.
5. Ethidium homodimer (Molecular Probes, Eugene, OR). Stock solution in PBS-BSA (100 $\mu\text{g}/\text{mL}$).
6. Flow cytometer.
7. The materials shown would be the same in the case of rVV-expressed class I Ags, but adding those needed for VV infection, and that are included in **Subheading 2.3.**

2.6. Effect of Inhibitors on Presentation of Defined Determinants

2.6.1. Infection of Target Cells with VV

1. All reagents needed for vaccinia virus infection, as described in **Subheading 2.3.**
2. rVV viruses expressing the proteins of interest. Controls, including a wild type VV, or an irrelevant rVV, would be included as well.
3. BFA stock (25 mg/mL) in methanol.
4. Iscove's modified DMEM containing FCS (7.5%).
5. $\text{Na}^{51}\text{CrO}_4$, 10 mCi/mL.

6. Peptide-specific CTLs.
7. γ -counter.

2.6.2. Osmotic Loading of Substrates

1. Hypertonic medium: RPMI containing 0.5 M sucrose, 10% polyethylene glycol 1000 and 10 mM HEPES, pH 7.2. Prepare fresh, and warm to 37°C before adding to the cells. The medium should also contain the substrate protein at high concentration (on the order of 20 mg/mL).
2. Hypotonic medium: 60% RPMI in water. As before, warm it to 37°C before adding.
3. Other reagents as in **Subheading 2.6.1.**

3. Methods

3.1. Determination of Effective Inhibitor Concentration

The method given is based on the reduced half life of the chimeric protein, UbRNP, with an Arg residue between ubiquitin and the NP from influenza virus. The protein, however, is completely stable in the presence of proteasome inhibitors. As a control, the full-length NP or the stable UbMNP, with a stabilizing Met, instead of Arg, can be used. All forms are expressed as rVV (*see Note 1*).

1. Wash the cells with PBS–BSA (318g in a benchtop centrifuge), and resuspend them at 10^7 cells/mL in the same buffer now containing the rVV-expressing UbRNP, at a multiplicity of infection (MOI) of 10 PFU/cell. The high cell concentration enables efficient virus adsorption.
2. After 1 h at 37°C, mixing every 10–15 min, add medium to reach a final cell density of 10^6 cells/mL. Incubate at 37°C for another 30 min.
3. Wash the cells with warm PBS buffer, and resuspend them in Met-free medium, containing the desired concentration of the inhibitor (*see Note 2*), at a density of 5×10^6 cells/mL. Incubate 20 min at 37°C (*see Note 3*).
4. Wash the cells, and resuspend them again in Met-free medium, containing the appropriate amount of inhibitor (final cell density of 10^7 cells/mL). Add 10–20 μ Ci of 35 S-Met per 2×10^6 cells. Incubate for 1 min at 37°C (*see Note 4*).
5. Add ice-cold PBS–Met. Wash the cells, and make aliquots of 2×10^6 each.
6. Separate one aliquot for time 0, and lyse, as described in **step 7**, or freeze immediately on dry ice. The remaining aliquots are resuspended in 1 mL of I/Met, containing the corresponding inhibitors, and incubated for different times before being lysed. For UbRNP, chase times of 10, 30, 60, and 120 min are sufficient to estimate the half-life of the protein.
7. Lyse the cells with 100 μ L boiling sample buffer of SDS-PAGE, containing protease inhibitors, and boil for 5 min (*see Note 5*).
8. Separate the proteins in a 9% SDS-PAGE gel. Fixed and dried gels are exposed to a PhosphorImager screen for an appropriate time, and imaged using the PhosphorImager. For normalization of samples, one of the metabolically stable

VV proteins, which may be seen in the scanned image, can be used as an internal standard in the different chase times.

3.2. Effect of Proteasome Inhibitors on MHC-I Ag Presentation

3.2.1. Conformational Stability of Newly Synthesized Class I Molecules

The method described takes advantage of the conformational instability, detected by mAbs, of empty class I molecules. An alternative method is the analysis of the transport of newly synthesized class I molecules from the ER to the Golgi, which takes place only after peptide binding. It can be detected by resistance of the carbohydrate groups in the class I molecules to digestion by endoglycosydase H, which is acquired in the Golgi.

1. Incubate the cells for 30 min at 37°C in Met-free medium, with the appropriate concentration(s) of inhibitors (*see Note 6*). Use 2×10^6 cells/immunoprecipitation.
2. Pellet the cells, and resuspend them at 10^7 cells/mL in the Met-free medium. Add 200 μ Ci of 35 S-Met/ 2×10^6 cells, and incubate for 15 min at 37°C.
3. Add an excess of ice-cold PBS–Met, wash the cells once, and make the appropriate number of 2×10^6 cell aliquots.
4. Save one aliquot for time = 0, and resuspend the rest in 1 mL I/Met, containing the corresponding inhibitor. Incubate them at 37°C for the desired intervals.
5. Pellet the cells, and lyse them on ice in 100 μ L of lysis buffer, for 30 min at 0°C.
6. Pellet the nuclei by spinning the cells at 15,000g at 4°C for 15 min. Harvest the supernatant.
7. Estimate the amount of radioactivity incorporated in the different samples by spotting 5 μ L of each sample, in triplicate, on glass-fiber filters. Wash filters with 10% TFA (w/v), dry, and place in appropriate vials with biodegradable scintillation fluid, and count the filters in a β -counter.
8. Adjust the volumes of each sample used for immunoprecipitation, so that each sample contains the same amount of incorporated 35 S-Met. Prepare two aliquots with each sample.
9. To one of the aliquots, add a class I-binding synthetic peptide (final concentration 5 μ g/mL). Incubate all the samples, with or without peptide, at 37°C for 2 h, then incubate the extracts on ice.
10. Load control and class I-specific Abs to protein A/G-agarose by rotating the beads (30–40 μ L/sample) with the Ab preparations (~ 20 μ g Ab/sample) for 1 h at 4°C. Wash the beads with PBS. Resuspend in PBS containing 10% lysis buffer (to make approx a 50% slurry).
11. First incubate extracts with beads coupled to the irrelevant Ab, in a shaker, for 2 h at 4°C. This step will clear the lysates from proteins that bind nonspecifically to the Ab-coupled beads. After pelleting, transfer the supernatant to a new tube containing the beads coupled to the conformation-sensitive anti-class I Ab. Incubate for 2 h in a shaker at 4°C.

12. Pellet the beads and harvest (keep the supernatant for use with other anti-class I Abs). Wash the beads with 1 mL of each of the wash buffers, and boil in 100 μ L of 2X sample buffer containing β -mercaptoethanol.
13. Separate the proteins by SDS-PAGE and expose dried gels to PhosphorImager screens and analyze with the PhosphorImager.

3.2.2. Cytofluorographic Analysis of Cell Surface Class I Molecules

3.2.2.1. ACID STRIPPING OF CLASS I-ASSOCIATED PEPTIDES

This method can be used for the analysis of the effects of proteasome inhibitors on the cell surface expression of MHC-I molecules that had been destroyed by acid stripping. It can be used, as well, for class I molecules expressed by rVVs. In this case, cells are infected with the recombinant viruses in the presence of the inhibitors, as in **steps 1 and 2 of Subheading 3.1**. Cells are harvested for analysis at least 7 h after infection. The method described uses fluorescence-activated cell sorting, but a cytotoxicity (CTL) assay may also be performed in some circumstances.

1. Incubate cells with the inhibitor, as in **Subheading 3.2.1**. Include enough cells to have at least 5×10^5 cells per sample per Ab staining.
2. Pellet cells, and resuspend in 300 mM glycine, pH 2.5, containing 1% BSA (use 100 μ L glycine buffer/ 2×10^6 cells).
3. Incubate for 3 min at 37°C, and neutralize immediately with a large excess of medium.
4. Pellet cells, and resuspend in culture medium containing the appropriate inhibitor. A control with BFA (5 μ g/mL), a drug that blocks transport from the ER to the Golgi, and thus blocks class I cell surface expression should be included.
5. Incubate for 5–8 h at 37°C, rotating.
6. Pellet cells. The next steps should all be carried out on ice and in buffers containing 0.02% NaN_3 , to prevent internalization of cell surface molecules (*see Note 7*). Wash cells in ice-cold PBS containing 0.2% BSA.
7. Stain for 1 h on ice with an Ab specific for the class I molecule of interest. A volume of 50 μ L dilution is sufficient for as many as 1×10^6 cells. The Ab can be labeled with FITC, or unlabeled. Incubations should be performed in round bottom 96-well polystyrene plates. Pellet by allowing centrifuge to reach 650 g, then setting timer to 0. Remove liquid by a single hard flick, and tap plates hard to resuspend cells prior to addition of next reagent. Use 270 μ L for washes.
8. If the first Ab is unlabeled, wash cells in PBS–BSA, and incubate with a FITC-labeled Ab, specific for the class I Ab used, and incubate for 1 h.
9. In both cases, i.e., whether using labeled or unlabeled anticlass I Ab, wash the cells with PBS–BSA and finally resuspend in 400 μ L PBS–BSA containing 10 μ g/mL ethidium homodimer (*see Note 8*). Using a multichannel pipetor transfer cells to 1 mL conical tubes arrayed in a 96-well format, and keep on ice.
10. Analyze the cells in a flow cytometer, gating out dead cells as identified by positive staining for ethidium homodimer. Conical tubes are inserted into the stan-

dard tube, and simply flicked out into the biohazard waste when the sample has been analyzed. Ten thousand events, or more, may be counted. The mean channel florescence values can be used to estimate the amount of class I expressed in the cell surface, with the background levels given by the cells incubated in the presence of BFA.

3.2.3. Effect of Inhibitors on Presentation of Defined Determinants

3.2.3.1. INFECTION OF TARGET CELLS WITH rVV

The method described here uses vaccinia as an expression vector, but it can be used with different viruses (*see Note 9*). BFA is added before ^{51}Cr labeling, in order to avoid exposing CTLs to proteasome inhibitors. BFA prevents the presentation of peptides generated after inhibitor removal.

1. Incubate the cells with the appropriate amount of the inhibitors (*see Subheading 3.2.1.*). Include 1.5×10^6 cells per final target cell group. As a negative control, an irrelevant rVV should be included, (*see Note 10*).
2. Wash the cells with PBS-BSA, and proceed with the infections as in **steps 1 and 2** of **Subheading 3.1**. The length of incubation after infection depends on the protein expressed by the rVV and the particular determinants studied. Usually, 3–5 h are required after the initial 1-h infection.
3. Before labeling, add BFA to a final concentration of 10 mg/mL, incubate 5–10 min at 37°C, and pellet the cells. BFA (5 $\mu\text{g/mL}$) will now be included in all the media used in washes, and during the CTL assay.
4. Pellet cells, leaving 20–50 μL medium, and add 100 $\mu\text{Ci } ^{51}\text{Cr}/1 \times 10^6$ cells. Incubate for 1 h at 37°C.
5. Add 5 mL culture medium and pellet cells. Aspirate as much media as possible, resuspend cells in warm medium (with BFA) and incubate for 10–15 min at 37°C. Pellet, and again scrupulously remove the supernatant. Finally, resuspend the labeled cells in medium, containing BFA, at a density of 10^5 cells/mL.
6. Add 100 μL /well (10^4 cells) of the cell suspension in round-bottom 96-well plates. These may contain different numbers of the peptide-specific CTL, in 100 μL medium, in order to have different effector-to-target ratios. The final volume in the wells should be 200 μL . Data points are obtained at least in triplicate.
7. Incubate at least 4–6 h at 37°C in a CO_2 incubator.
8. Harvest 100 μL supernatant, and count the released ^{51}Cr in a γ -counter. The spontaneous release is obtained from target cells incubated in the absence of CTL, and the total release, by incubation in Triton X-100 (final concentration 1% v/v). The specific release is calculated by the formula:

$$\frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Total release} - \text{Spontaneous release}} \times 100$$

3.2.3.2. OSMOTIC LOADING OF SUBSTRATES

The method is essentially identical to the one described in the previous Subheading, replacing the infection with the osmotic loading described below (**36**) (see **Note 11**). Care must be taken to control for the presence of antigenic peptides in the preparation.

1. Incubate cells with the appropriate amount of the inhibitors (see **Subheading 3.2.1.**).
2. Pellet 1.5×10^6 cells and resuspend in 500 μL warm hypertonic medium containing approx 20 mg/mL of the protein substrate. Incubate for 10 min at 37°C .
3. Add 14 mL warm hypotonic medium, and incubate for 3 min at 37°C .
4. Pellet cells, and proceed as in the previous subheading, adding BFA before labeling.

4. Notes

1. A method commonly used in the references relies on the accumulation of ubiquitinated proteins in the presence of proteasome inhibitors, because of their compromised degradation. These modified proteins appear as high-mol-wt smears in Western blots of gels from extracts of treated cells, developed with anti-ubiquitin. The caveat of this method is that it is not a quantitative approach, and it is difficult, if not impossible, to distinguish between partial inhibition of proteasomes and total inhibition.
2. The range of concentrations to test varies with the inhibitor and the cell type used, but is always within the μM range. With lactacystin, a range of 5–100 μM would be recommended, although concentrations as high as 500 μM have been reported. In the case of zLLL, concentrations range from 0.5 to 50 μM (concentrations in the higher range affect protein biosynthesis). AcLLnL has been used at concentrations from 2.5 to 250 μM .
3. In vaccinia infected cells after 2–3h of infection at an MOI of 10, most of the endogenous gene expression has been shut off, and biosynthesized proteins are mostly restricted to early VV gene products. Because rVVs generally use the early–late 7.5K promoter, this enables detection of the NP band in the gels of total cell lysates relatively well separated from other proteins. This is relevant for nuclear proteins, such as flu NP, in which recovery after lysis in nonionic detergents is not complete, making quantitative immunoprecipitation impossible.
4. Longer labeling periods, in the absence of inhibitors, result in a considerable degradation of UbRNP during the labeling time (less than 60% left is sometimes observed after a 5-min pulse). If longer times of labeling are required, this should be taken into account. This is also relevant when UbMNP is used as a control, because some co-translational degradation of the protein is observed, presumably before removal of the ubiquitin moiety, whereas the final product is stable. Such an effect is not observed, however, with the wild type NP.
5. Often, the lysate is too viscous to handle easily. This can be solved by shearing the DNA with a probe sonicator, passing through a 23 G needle, or keeping the lysates overnight at 4°C .

6. The incubation time required to inactivate proteasomes varies with the inhibitor and cell type. A safe estimate would be at least 15 min with peptide aldehydes and 30 min with lactacystin (which must be converted to the CL β L form, which represents both the cell-membrane-permeable and active form (37). For example, with HeLa cells, we have seen that times as short as 5 min with 25 μ M zLLL before pulse are enough to protect UbRNP from proteasomal degradation. For 100 μ M lactacystin, however, more than 15 min were required.
7. The method described here uses live, unfixed cells for flow cytometry analysis. Staining with ethidium homodimer, which stains the nuclei of dead cells, allows gating of live cells. The method can be used with fixed cells, as well, and gating can be performed by incubating with ethidium homodimer prior to the fixation step (cells must be washed thoroughly, to prevent carryover of the dye and postvital staining).
8. VV infection is associated with an increase in staining of live cells, with up to a 10-fold increase in ethidium homodimer or propidium iodide. The cells are still distinguished from nonviable cells, which are at least 10-fold brighter.
9. Flu infections can be very useful here, because the class I-restricted response is well characterized for many alleles. In the particular case of influenza, the infection (20 hemagglutinating U/cell) should be carried out in AIM (MEM, Gibco-BRL, Rockville, MD, 0.1% BSA, and 20 mM HEPES, pH 6.8), instead of PBS-BSA. It is essential to wash cells to remove FCS, which blocks viral adsorption.
10. It is advisable, whenever feasible, to control for the effect of the inhibitors on gene expression (vaccinia or any other gene, viral or not). A seemingly ideal tool are rVVs expressing minigenes that code for the presented peptide. However, the number of peptide-MHC complexes this generates far exceeds the sensitivity threshold of the CTL. Therefore, variations in the amount of complexes in the cell surface may not be detected, a limitation that is often overlooked. The use of Abs specific for MHC-peptide complexes, or extraction and quantitation of the presented peptides, can solve this problem. The effect on gene expression can also be tested by FACS analysis of cell surface expression of virus-encoded proteins. In influenza virus infections, for example, cell surface expression of neuraminidase was mostly reduced, compared to nontreated controls, when cells were treated overnight with 10 μ M lactacystin, and could not even be detected when cells were treated with 100 μ M lactacystin.
11. An alternative is electroporation of the protein into the cell. Exogenous loading suffers from the difficulty of obtaining abundant and consistently pure substrate protein. Ovalbumin, commercially available, has been successfully used by different groups in this kind of experiment, but one should be cautious as to whether the results can be extrapolated to other substrates, particularly endogenous Ags. In this sense, viral infections offer much more flexibility.

References

1. Baumeister, W., Walz, J., Zühl, F., and Seemüller, E. (1998) Proteasome: paradigm of a self-compartmentalizing protease. *Cell* **92**, 367–380.

2. Hoffman, L., and Rechsteiner, M. (1996) Regulatory features of multicatalytic and 26S proteasomes. *Curr. Top. Cell Regul.* **34**, 1–32.
3. Yewdell, J. W. and Bennink, J. R. (1992) Cell biology of antigen processing and presentation to Major Histocompatibility Complex class I molecule-restricted T lymphocytes. *Adv. Immunol.* **52**, 1–123.
4. Goldberg, A. L. and Rock, K. L. (1992) Proteolysis, proteasomes and antigen processing. *Nature* **357**, 375–379.
5. Nandi, D., Marusina, K., and Monaco, J. J. (1998) How do endogenous proteins become peptides and reach the endoplasmic reticulum. *Curr. Top. Microbiol. Immunol.* **232**, 15–47.
6. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* **78**, 761–771.
7. Bogoy, M., Gaczynska, M., and Ploegh, H. L. (1997) Proteasome inhibitors and antigen presentation. *Biopolymers* **43**, 269–280.
8. Vinitsky, A., Michaud, C., Powers, J. C., and Orlowski, M. (1992) Inhibition of the chymotrypsin-like activity of the pituitary multicatalytic proteinase complex. *Biochemistry* **31**, 9421–9428.
9. Löwe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W., and Huber, R. (1995) Crystal structure of the 20S proteasome from the Archaeon *T. Acidophilum* at 3.4 Å resolution. *Science* **268**, 533–539.
10. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* **268**, 726–731.
11. Dick, L. R., Cruikshank, A. A., Grenier, L., Melandri, F. D., Nunes, S. L., and Stein, R. L. (1996) Mechanistic studies on the inactivation of the proteasome by lactacystin. A central role for clasto-lactacystin β -lactone. *J. Biol. Chem.* **271**, 7273–7276.
12. Ostrowska, H., Wojcik, C., Omura, S., and Worowski, K. (1997) Lactacystin, a specific inhibitor of the proteasome, inhibits human platelet lysosomal cathepsin A-like enzyme. *Biochem. Biophys. Res. Commun.* **234**, 729–732.
13. Bogoy, M., McMaster, J. S., Gaczynska, M., Tortorella, D., Goldberg, A. L., and Ploegh, H. L. (1997) Covalent modification of the active site threonine of proteasomal β subunits and the *Escherichia coli* homolog HslV by a new class of inhibitors. *Proc. Natl. Acad. Sci. USA* **94**, 6629–6634.
14. McCormack, T., Baumeister, W., Grenier, L., Moomaw, C., Plamondon, L., Pramanik, B., et al. Active site inhibitors of *Rhodococcus* 20 S proteasome. Kinetics and mechanism. *J. Biol. Chem.* **272**, 26,103–26,109.
15. Bachmair, A., Finley, D., and Varshavski, A. (1986) In vivo half-life of a protein is a function of its amino-terminal residue. *Science* **234**, 179–186.
16. Varshavsky, A. (1996) N-end rule: functions, mysteries, uses. *Proc. Natl. Acad. Sci. USA* **93**, 12,142–12,149.
17. Townsend, A., Bastin, J., Gould, K., Brownlee, G., Andrew, M., Coupar, B., Boyle, D., Chan, S., and Smith, G. (1988) Defective presentation to class I-restricted

- cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen. *J. Exp. Med.* **168**, 1211–1224.
18. Porgador, A., Yewdell, J. W., Deng, Y., Bennink, J. R., and Germain, R. N. (1997) Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity* **6**, 715–726.
 19. Andersen, P. S., Stryhn, A., Hansen, B. E., Fugger, L., Engberg, J., and Buus, S. (1996) Recombinant antibody with the antigen-specific, major histocompatibility complex-restricted specificity. *Proc. Natl. Acad. Sci. USA* **93**, 1820–1824.
 20. Mimnaugh, E. G., Chen, H. Y., Davie, J. R., Celis, J. E., and Neckers, L. (1997) Rapid deubiquitination of nucleosomal histones in human tumor cells caused by proteasome inhibitors and stress response inducers: effects on replication, transcription, translation, and cellular stress. *Biochemistry* **36**, 14,418–14,429.
 21. Zhou, M., Wu, X., and Ginsberg, H. N. (1996) Evidence that a rapidly turning over protein, normally degraded by proteasomes, regulates *hsp72* gene transcription in HepG2 cells. *J. Biol. Chem.* **271**, 24,769–24,775.
 22. Bush, K. T., Goldberg, A. L., and Nigam, S. K. (1997) Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. *J. Biol. Chem.* **272**, 9086–9092.
 23. Kawazoe, Y., Nakai, A., Tanabe, M., and Nagata, K. (1998) Proteasome inhibition leads to the activation of all members of the heat-shock-factor family. *Eur. J. Biochem.* **255**, 356–362.
 24. Hershko, A. and Ciechanover, A. (1998) The ubiquitin system. *Ann. Rev. Biochem.* **67**, 425–479.
 25. Grimm, L. M. and Osborne, B. A. (1999) Apoptosis and the proteasome. *Results Probl. Cell Differ.* **23**, 209–228.
 26. Meriin, A. B., Gabai, V. L., Yaglom, J., Shifrin, V. I., and Sherman, M. Y. (1998) Proteasome inhibitors activate stress kinases and induce Hsp72. Diverse effects on apoptosis. *J. Biol. Chem.* **273**, 6373–6379.
 27. Yellen-Shaw, A. J. and Eisenlohr, L. C. (1997) Regulation of class I-restricted epitope processing by local or distal flanking sequence. *J. Immunol.* **158**, 1727–1733.
 28. Vinitsky, A., Antón, L. C., Snyder, H. L., Orłowski, M., Bennink, J. R., and Yewdell, J. W. Generation of MHC class I-associated peptides is only partially inhibited by proteasome inhibitors. Involvement of nonproteasomal proteases in antigen processing? *J. Immunol.* **159**, 554–564.
 29. Cerundolo, V., Benham, A., Braud, V., Mukherjee, S., Gould, K., Macino, B., Neefjes, J., and Townsend, A. (1997) Proteasome-specific inhibitor lactacystin blocks presentation of cytotoxic T lymphocyte epitopes in human and murine cells. *Eur. J. Immunol.* **27**, 336–341.
 30. Antón, L. C., Snyder, H. L., Bennink, J. R., Vinitsky, A., Orłowski, M., Porgador, A., and Yewdell, J. W. (1998) Dissociation of proteasomal degradation of biosynthesized viral proteins from generation of MHC class I-associated antigenic peptides. *J. Immunol.* **160**, 4859–4868.

31. Benham, A., Grommé, V., and Neefjes, J. (1998) Allelic differences in the relationship between proteasome activity and MHC class I peptide loading. *J. Immunol.* **161**, 83–89.
32. Luckey, C. J., King, G. M., Marto, J. A., Venketeswaran, S., Maier, B. F., Crotzer, V. L., et al. (1998) Proteasomes can either generate or destroy MHC class I epitopes: evidence for nonproteasomal epitope generation in the cytosol. *J. Immunol.* **161**, 112–121.
33. Glas, R., Bogyo, M., McMaster, J. S., Gaczynska, M., and Ploegh, H. L. (1998) A proteolytic system that compensates for loss of proteasome function. *Nature* **392**, 618–622.
34. Geier, E., Pfeifer, G., Wilm, M., Lucchiari-Hartz, M., Baumeister, W., Eichmann, K., and Niedermann, G. (1999) Giant protease with potential to substitute for some functions of the proteasome. *Science* **283**, 978–981.
35. Williams, D. B., Swiedler, S. J., and Hart, G. W. (1985) Intracellular transport of membrane glycoproteins: two closely related histocompatibility antigens differ in their rates of transit to the cell surface. *J. Cell Biol.* **101**, 725–734.
36. Moore, M. W., Carbone, F. R., and Bevan, M. J. (1988) Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell* **54**, 777–785.
37. Dick, L. R., Cruikshank, A. A., Destree, A. T., Grenier, L., McCormack, T. A., Melandri, F. D., et al. (1997) Mechanistic studies on the inactivation of the proteasome by lactacystin in cultured cells. *J. Biol. Chem.* **272**, 182–188.



<http://www.springer.com/978-0-89603-745-8>

Antigen Processing and Presentation Protocols

Solheim, J.C. (Ed.)

2001, IX, 270 p., Hardcover

ISBN: 978-0-89603-745-8

A product of Humana Press