THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 20, Issue of May 16, pp. 13437–13445, 1997

Printed in U.S.A.

Lactacystin and *clasto*-Lactacystin β-Lactone Modify Multiple Proteasome β-Subunits and Inhibit Intracellular Protein Degradation and Major Histocompatibility Complex Class I Antigen Presentation*

(Received for publication, August 22, 1996, and in revised form, November 25, 1996)

Abie Craiu‡, Maria Gaczynska§, Tatos Akopian§, Colette F. Gramm‡, Gabriel Fenteany¶, Alfred L. Goldberg§, and Kenneth L. Rock‡‖**

From the ‡Division of Lymphocyte Biology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115, the §Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, the ¶Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138, and the ‖Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

The antibiotic lactacystin was reported to covalently modify β-subunit X of the mammalian 20 S proteasome and inhibit several of its peptidase activities. However, we demonstrate that [^{3}H]lactacystin treatment modifies all the proteasome's catalytic β-subunits. Lactacystin and its more potent derivative β-lactone irreversibly inhibit protein breakdown and the chymotryptic, tryptic, and peptidylglutamyl activities of purified 20 S and 26 S particles, although at different rates. Exposure to these agents for 1 to 2 h reduced the degradation of short- and long-lived proteins in four different mammalian cell lines. Unlike peptide aldehyde inhibitors, lactacystin and the β-lactone do not inhibit lysosomal degradation of an endocytosed protein. These agents block class I antigen presentation of a model protein, ovalbumin (synthesized endogenously or loaded exogenously), but do not affect presentation of the peptide epitope SIINFEKL, which does not require proteolysis for presentation. Generation of most peptides required for formation of stable class I heterodimers is also inhibited. Because these agents inhibited protein breakdown and antigen presentation similarly in interferon-γ-treated cells (where proteasomes contain LMP2 and LMP7 subunits in place of X and Y), all β-subunits must be affected similarly. These findings confirm our prior conclusions that proteasomes catalyze the bulk of protein breakdown in mammalian cells and generate the majority of class I-bound epitopes for immune recognition.

MHC[1] class I molecules typically bind 8–9-residue peptides derived from cellular or viral proteins. Most of these peptides are generated by protein breakdown in the cytosol and are transported by the transporter associated with antigen presentation transporter into the endoplasmic reticulum (1). Here the peptide, a MHC class I heavy chain, and a β_2-microglobulin molecule associate, and the complex is then transported through the Golgi apparatus to the plasma membrane (1). This process allows T lymphocytes to screen for cells that are synthesizing foreign or abnormal proteins. The mechanisms responsible for the generation of the class I-presented peptides had been unclear until recently. However, a variety of recent evidence has suggested that the proteasome plays a primary role in this process and that during the turnover of cytosolic and nuclear proteins a fraction of the peptides generated by the proteasome are utilized for MHC class I presentation (1–3).

Proteasomes are found in the nucleus and cytosol of all cells and are essential components of the ATP-ubiquitin-dependent pathway for protein degradation. The 20 S proteasome is a 700-kDa particle with multiple peptidase activities, including a chymotryptic-, tryptic-, and peptidylglutamyl-like activity (4–6). It is a cylindrical-shaped structure composed of four rings, the outer two each contain seven α-subunits and the inner two each contain seven β-subunits (7, 8). Proteolysis occurs in the central chamber of this particle and is catalyzed through a nucleophilic attack on the peptide bond by the N-terminal threonine hydroxyl groups on certain β-subunits, named X (ε), Y(δ), Z (HCO), and their homologues LMP2, LMP7, and LMP10 (MECL-1) (9–12). The 20 S particle functions as the proteolytic core of a larger 26 S (2000 kDa) ATP-dependent proteasome complex which selectively degrades proteins that are modified by conjugation to multiple ubiquitin molecules (6, 13, 14). Although the ubiquitin-proteasome pathway is clearly essential for the rapid degradation of short-lived or highly abnormal proteins and polypeptides in yeast (15) and mammalian cells (16–19), its role in degradation of the bulk of cell proteins, which are long-lived, is uncertain (16, 20).

Several lines of evidence had suggested that the proteasome was also responsible for the generation of some class I-presented peptides. Two of the proteasome's catalytic β-subunits, LMP7 and LMP2, are encoded in the MHC region (21, 22), and the absence of these subunits in mutant cells or mice decreases the efficiency of presentation of certain antigens (23–26). Furthermore, inhibiting ubiquitin conjugation in a TS mutant decreased antigen presentation of a model protein (27). On the other hand, modifications of a protein that stimulate its ubiquitinylation and degradation by 26 S proteasomes enhance its rate of MHC class I presentation (28). Finally, when 20 S proteasomes are incubated with a protein for extended periods, they can generate some class I-binding peptides, although such experiments involve highly nonphysiological conditions (29–31).

More definitive evidence for the proteasome's general importance in antigen presentation *in vivo* requires methods to spe-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed. Present address: Department of Pathology, University of Massachusetts Medical Center, Worcester, MA 01655. Tel.: 508-856-1090; Fax: 508-856-1094.

[1] The abbreviations used are: MHC, major histocompatibility complex; IFN-γ, interferon-γ; LLnL, *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal; FITC, fluorescein isothiocyanate; KLH, keyhole limpet hemocyanin; PAGE, polyacrylamide gel electrophoresis; AMC, 7-amino-4-methylcoumarin.

cifically inhibit proteasome function in intact cells. Recently, certain peptide aldehydes (such as *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal, LLnL, and *N*-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norvalinal, MG115) have been shown to strongly inhibit multiple peptidase activities of proteasomes and to reduce protein hydrolysis (3). Moreover, these agents can enter cells and block the degradation of most cellular proteins and the generation of the majority of class I-presented peptides (3). Although these peptide aldehydes can also inhibit the cysteine proteases found in lysosomes and calpains (3), several findings argued that the inhibition of protein degradation and antigen presentation was due to effects on the proteasome. For example, the rank order of potencies of different peptide aldehydes in inhibiting the proteasome was the same as for blocking protein degradation and antigen presentation and did not correlate with efficacy against cysteine proteases (3). Also, inhibition of these cysteine proteases did not affect protein breakdown or antigen presentation. Nevertheless, more selective proteasome inhibitors are needed to establish definitively a major role for the proteasome in these processes.

A chemically distinct type of proteasome inhibitor is the antibiotic lactacystin which was isolated from *Streptomyces* by Omura and colleagues (32) and synthesized by Corey and colleagues (33). Fenteany *et al.* (11) have shown that [^{3}H]lactacystin bound covalently to a polypeptide identified as proteasome β-subunit X in bovine brain cells and that lactacystin could irreversibly inhibit the chymotryptic- and tryptic-like peptidase activity and reversibly inhibit the post-acidic activity of purified proteasomes. Lactacystin spontaneously hydrolyzes into *clasto*-lactacystin β-lactone, which appears to be the active inhibitor that reacts with the N-terminal threonine of subunit X (34). Because lactacystin and the β-lactone appeared to be highly specific inhibitors that do not affect cysteine or serine proteases, they are potentially very useful research tools. We therefore examined whether they inhibit proteasomes that contain β-type subunits not expressed in neurons (*e.g.* LMP7, LMP2, and MECL-1), whether they reduce protein hydrolysis *in vitro* and in cells, and whether they can block MHC class I antigen presentation.

EXPERIMENTAL PROCEDURES

Reagents—Chicken ovalbumin and keyhole limpet hemocyanin (KLH) were purchased from Sigma. The SIINFEKL peptide was synthesized by the molecular biology core of the Dana-Farber Cancer Institute (Boston, MA).

Monoclonal antibodies were purified from culture supernatants of hybridomas Y3 (anti-class I K^b heterodimers) (35), B22 (anti-D^b heterodimers) (36), and S19.8.503 (anti-β_2-microglobulin) by affinity chromatography on protein A-Sepharose. Rabbit anti-K^b exon 8 was provided by Dr. Ian York.

LLnL was purchased from Sigma. Lactacystin and *clasto*-lactacystin β-lactone were kindly provided by ProScript (Cambridge, MA) and Dr. K. Omura (Kitasato Institute, Tokyo, Japan). [^{3}H]Lactacystin was prepared as described (11).

Recombinant human IFN-γ was a kind gift from Biogen Inc. (Cambridge, MA). FITC-casein was prepared as described previously (37). Synthetic substrates for peptidase assays were purchased from Bachem (Switzerland).

Cell Lines and Culture Conditions—LB27.4 B lymphoblasts (38), E36.12.4 hamster carcinoma cells (27), RF33.70 (OVA 257–264 plus K^b-specific) T-T hybridomas (39), RMA T lymphoblasts (40), U937 monocytoid cells, and A3.1A7 murine macrophage cells (41) have been described previously. 143B.TK− human osteosarcoma cells were kindly provided by J. Yewdell and J. Bennink. The human lymphoblastoid cell lines 721 and .174 were kindly provided by R. DeMars (42). A retroviral-transduced B16 melanoma line, which constitutively secretes murine IFN-γ, was a gift from Dr. Glenn Dranoff (Dana-Farber Cancer Institute, Boston, MA). DAP34.8 cells are mouse L cells stably transfected with class I K^b and CD54.

For electroporation loading experiments LB27.4 cells were grown in Opti-MEM (Life Technologies, Inc.) supplemented with normal mouse serum (1%). In all other experiments cells were grown in RPMI 1640 or Dulbecco's modified Eagle's medium (Irving Scientific, Santa Ana, CA) supplemented with fetal calf serum (10%) and antibiotics. Transfected cells were passaged with geneticin (Life Technologies, Inc.).

LB27.4 and DAP34.8 cells were incubated in the presence or absence of murine IFN-γ (20% supernatant from confluent cultures of IFN-γ-transduced B16 cells, containing approximately 10 units/ml IFN-γ) for 3 to 4 days at 37 °C. 721 cells and U937 human macrophages were treated with 3000 units/ml recombinant human IFN-γ for 3 or 7 days, respectively.

Proteasome Isolation and Assays—20 S and 26 S proteasome-rich cytosolic fractions ("proteasome fractions") were isolated from cultured cells as described previously (43, 44), and 20 S and 26 S particles were purified from rabbit skeletal muscle as described in Ref. 6.

Activity against the fluorogenic peptide substrates was determined by continuously monitoring the fluorescence of 7-amino-4-methylcoumarin (excitation wavelength 380 nm, emission wavelength 460 nm) on a McPherson Spectrofluorimeter FL-750 connected to a Waters 740 Data Module. The assays were performed in 50 mM Tris-HCl buffer (pH 7.5) in a 0.75-ml final reaction volume. All peptide stocks were prepared and stored in Me_2SO, and the final concentration of the solvent in the assay never exceeded 2%, which did not affect proteasome activity. The rates of substrate hydrolysis were determined at 25 °C from the initial linear portions of curves (2–5 min). The peptide substrate concentrations used were Suc-Leu-Leu-Val-Tyr-AMC, 67 μM, Boc-Leu-Arg-Arg-AMC, 100 μM, and Z-Leu-Leu-Glu-AMC, 100 μM. Enzyme concentration was 0.1–0.5 nM. Duplicate assays were performed for every time point.

The continuous assay of proteasome proteinase activity was performed in 50 mM Tris-HCl (pH 7.5) containing 7.5 μg of FITC-casein in a 0.75-ml volume. The mixture was equilibrated in the cuvette at 25 °C until a steady fluorescence intensity was observed (generally after 5 min). 1–3 μg of enzyme was then added, and the linear increase of fluorescence was recorded for up to 30 min at 490 nm excitation and emission of 521 nm. A blank without proteasome was run in parallel.

The rate of inactivation of proteasome by inhibitors was calculated under pseudo first-order conditions (*i.e.* [I] $\gg$ [*E*]). Apparent rate constants (k_{obs}) were determined from semi-logarithmic plots of ln V_t/V_0 against incubation time of inhibitor with enzyme (V_0 is enzyme activity in the absence of inhibitor and V_t is residual activity in the presence of inhibitor). Apparent second-order rate constant k_a is calculated from $k_a = k_{obs}/[I]$. The half-life of the free enzyme is given by $t_{1/2} = 0.693/k_{obs}$. The irreversibility of inhibitors was tested by measuring the enzyme activity after dilution of reaction 1000 times.

Virus and Plasmids—vTF7-3 vaccinia virus, encoding T7 RNA polymerase, was obtained from ATCC (VR-2153) (45). Viral stocks (approximately 10^8 plaque-forming units per ml) were prepared from infected 143B.TK$^-$ cells.

The plasmid pBS.OVA was constructed by cloning ovalbumin cDNA into pBluescript SK (Promega, Madison, WI), using *Hin*dIII and *Xba*I (Life Technologies, Inc.), such that the orientation of the ovalbumin gene was under the control of the T7 RNA polymerase promoter. The plasmid pBS.miniOVA was constructed from the primers 5′-AGCTTCACCATGTCTATAATAAACTTTGAGAAGTTATAGTGACCATGGG-3′ and 5′-AGTGGTACAGATATTATTTGAAACTCTTCAATATCACTGGTACCCTTAA-3′. Primers were phosphorylated with polynucleotide kinase and then annealed and ligated into pBluescript SK, using *Hin*dIII and *Eco*RI (Life Technologies, Inc.). The gene expressed under control of the T7 RNA polymerase promoter encoded the SIINFEKL peptide with an initiating N-terminal Met and contained an *Nco*I site near the 3′ end for selection after ligation and transformation. cDNAs were sequenced at the Dana-Farber Cancer Institute molecular biology core facility.

Assays for Intracellular Protein Degradation—Cells (E36.12.4, LB27.4, RMA, or A3.1A7) were incubated with [^{3}H]tyrosine (5 μCi/ml) for 6 h at 37 °C to assay degradation of long-lived proteins and for 1 h to assay degradation of short-lived proteins. Assays for the measurement of degradation of such proteins have been previously described in Ref. 3.

To measure lysosomal degradation of exogenous proteins, keyhole limpet hemocyanin (KLH) was used as a substrate. 200 mg of KLH (Sigma) was reacted with [^{125}I]sodium iodide (3 mCi) to a specific activity of 3 μCi/mg by the IODO-GEN method. Assays for the uptake and catabolism of ^{125}I-KLH were then performed according to the method of Gray *et al.* (46). ^{125}I-KLH-pulsed A3.1A7 cells were incubated in triplicate (4×10^6 per group) for 4 h at 37 °C in the presence or absence of inhibitors. Culture medium and cell pellets were treated with trichloroacetic acid, and radioactivity in supernatants and pellets was measured. Protein breakdown was evaluated by measuring the

TABLE I
Rate constants for inactivation of proteasome activity by lactacystin and clasto-lactacystin β-lactone

20 S and 26 S proteasomes (0.1–0.5 nM) from rabbit skeletal muscle were assayed for activity against the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC (67 μM) in the presence of different concentrations of inhibitor. Activity of the 26 S proteasome against two other substrates (Boc-Leu-Arg-Arg-AMC and Z-Leu-Leu-Glu-AMC) was also determined in the absence and presence of 10 μM *clasto*-lactacystin β-lactone. Activity and kinetics were determined by monitoring fluorescence of 7-amino-4-methylcoumarin over time, as described under "Experimental Procedures." $t_{1/2} = 0.693/k_{obs}$, where k_{obs} is determined from semilogarithmic plots of ln V_t/V_0 against incubation time of inhibitor with enzyme. V_0 is enzyme activity in the absence of inhibitor, and V_t is residual activity in the presence of inhibitor. Apparent second-order rate constant $k_a = k_{obs}$/inhibitor concentration.

Substrate and enzyme	Inhibitor	$t_{1/2}$	k_{obs}	k_a
	μM	*min*	$s^{-1} \times 10^{-3}$	$M^{-1}\ s^{-1}$
Suc-Leu-Leu-Val-Tyr-AMC (chymotryptic) with 20 S proteasome	Lactacystin			
	10	11.5	1.08	108
	25	5.8	2.05	80
	50	3.0	3.90	72
	β-Lactone			
	0.5	7.2	1.60	3,200
	1.0	5.0	2.38	2,380
	2.0	1.8	6.66	3,300
Suc-Leu-Leu-Val-Tyr-AMC (chymotryptic) with 26 S proteasome	Lactacystin			
	10	5.5	2.10	210
	25	3.2	3.60	144
	50	2.3	6.66	130
	β-Lactone			
	0.25	5.8	2.00	8,000
	0.50	3.1	3.70	7,400
	0.75	1.9	6.10	8,100
Boc-Leu-Arg-Arg-AMC (tryptic) with 26 S proteasome	β-Lactone			
	10	3.1	3.50	350
Z-Leu-Leu-Glu-AMC (peptidyl-glutamyl) with 26 S proteasome	β-Lactone			
	10	10.1	1.15	115

conversion of radiolabeled protein to trichloroacetic acid-soluble form appearing in culture medium.

Antigen Presentation Assays—To assay for the presentation of endogenously synthesized antigen, E36.12.4 cells or DAP34.8 cells were seeded overnight in 6-well plates to a density of ≈10^6 cells/well. Cells were then preincubated for 30 min in the presence or absence of inhibitors, in Opti-MEM medium. vTF7-3 virus was then added to wells at 10 plaque-forming units/cell for 30 min. Media containing vTF7-3 was then removed and plasmid (pBS.OVA (5 μg) or pBS.miniOVA (0.7 μg)) in Lipofectin (Life Technologies, Inc.) was added in 1 ml of Opti-MEM, in the presence or absence of inhibitors. Cells were incubated for 140 min (E36.12.4 cells) or 200 min (DAP34.8 cells) at 37 °C and then fixed in 1% paraformaldehyde for 10 min at room temperature. The presence of peptide-K^b complexes on the surface of these cells was measured by quantifying the amount of interleukin-2 produced by the ovalbumin-K^b-specific T-T hybridoma RF33.70, after stimulation with antigen-presenting cells in duplicate cultures, as described previously (39).

To assay for presentation of antigen introduced into the cytosol by electroporation, LB27.4 cells or DAP34.8 cells were permeabilized as described (3) in the presence or absence of inhibitors, with ovalbumin (30 mg/ml) or SIINFEKL peptide (0.5 μg/ml) in electroporation buffer. Cells were either fixed with paraformaldehyde immediately after electroporation or were incubated for 2 h at 37 °C and then fixed. The inhibitors were present where appropriate during the 0.5-h pretreatment, electroporation, and 2-h incubation steps. The presence of SIINFEKL·K^b complexes on the cell surface was determined using RF33.70 T-T hybridomas as described above and in Ref. 47.

Electrophoretic Methods and Western Blotting—Fractions from proteasome-rich cells or purified proteasomes were incubated with [^{3}H]lactacystin (100 μM) for 3 h at 37 °C. Since proteasomes from different cell types, or cells treated with IFN-γ, differ in their 20 S subunit composition, control and IFN-γ-treated macrophages or lymphoblasts were used as sources of proteasomes to ensure that all 20 S subunits would be present in detectable amounts in the preparations. Two-dimensional PAGE (O'Farrell system) was used to detect and identify proteasomal subunits modified by [^{3}H]lactacystin. Proteins from the two-dimensional gels (12.5%) were transferred to nitrocellulose, and the filters were incubated with specific antibodies to β-subunits (kindly provided by K. Tanaka and K. Hendil) and then with ^{125}I-protein A. β-Subunits of the 20 S proteasome recognized by antibodies were visualized on the filters using PhosphorImaging (Molecular Dynamics). Antibody-^{125}I-protein A complexes were then stripped from the filters until no detectable ^{125}I remained. The filters were reprobed with other anti-β-subunit antibodies and ^{125}I-protein A, and finally stripped filters were treated with Amplify enhancer (Amersham Corp.), dried, and exposed to Kodak AR film to obtain fluorograms with visualized proteins labeled by [^{3}H]lactacystin. The subunits that [^{3}H]lactacystin covalently modifies were identified on the fluorograms obtained from two-dimensional gels and compared with Western blot phosphorimages.

The assembly of MHC class I molecules was measured as described previously in Ref. 3.

TABLE II
Effects of lactacystin and clasto-lactacystin β-lactone on proteasome-mediated hydrolysis of FITC-casein

20 S and 26 S rabbit muscle proteasomes were assayed for hydrolysis of FITC-casein in the absence or presence of inhibitors (preincubated with enzyme for 30 min). Activity was determined continuously, as described under "Experimental Procedures." One unit of enzyme activity is defined as the amount of enzyme that generates a fluorescence signal equivalent to that of 1 pmol of FITC, under the assay conditions. % inhibition was determined as the percent decrease in degradation rate in the presence of inhibitor.

Inhibitor	20 S proteasomes		26 S proteasomes	
	activity	inhibition	activity	inhibition
μM	*units*	%	*units*	%
Lactacystin	65.0	0	41.6	0
50	50.7	22	31.2	25
β-Lactone				
1	57.2	12	34.1	18
5	50.1	23	22.9	45
10	29.2	55	13.3	68

RESULTS

Inhibition of Peptide and Protein Hydrolysis by Proteasomes—The 20 S proteasome contains distinct activities that cleave model peptides after hydrophobic, basic, and acidic residues (4–6), and Fenteany *et al.* (11) demonstrated that lactacystin blocks these activities in crude extracts. Both lactacystin and the β-lactone rapidly inactivated the chymotryptic activity of 20 S and 26 S proteasomes purified from skeletal muscle (Table I). The β-lactone appeared approximately 20-fold more effective than lactacystin, in accord with prior findings that lactacystin must first be converted to the lactone to inhibit proteasomes (34). Unlike the peptide aldehydes, these agents were more potent against the 26 S particles than 20 S. The lactone also inhibits the tryptic and peptidylglutamyl-hydrolyzing activities, but these inhibitory reactions are much slower than inhibition of the chymotryptic activity and require higher concentrations of the β-lactone.

In vivo, the primary function of the proteasome is the hydrolysis of proteins into oligopeptides (6). Since the effect of these inhibitors on this process had not been examined, we tested their ability to inhibit the hydrolysis of FITC-conjugated casein (Table II). This protein substrate is degraded in an ATP-dependent reaction by the 26 S complex without ubiquitinylation and in an ATP-independent fashion by the 20 S particles. The β-lactone, and to a lesser extent lactacystin, inhibited casein breakdown by both the 20 S proteasomes and 26 S complexes. However, the concentrations necessary to inhibit

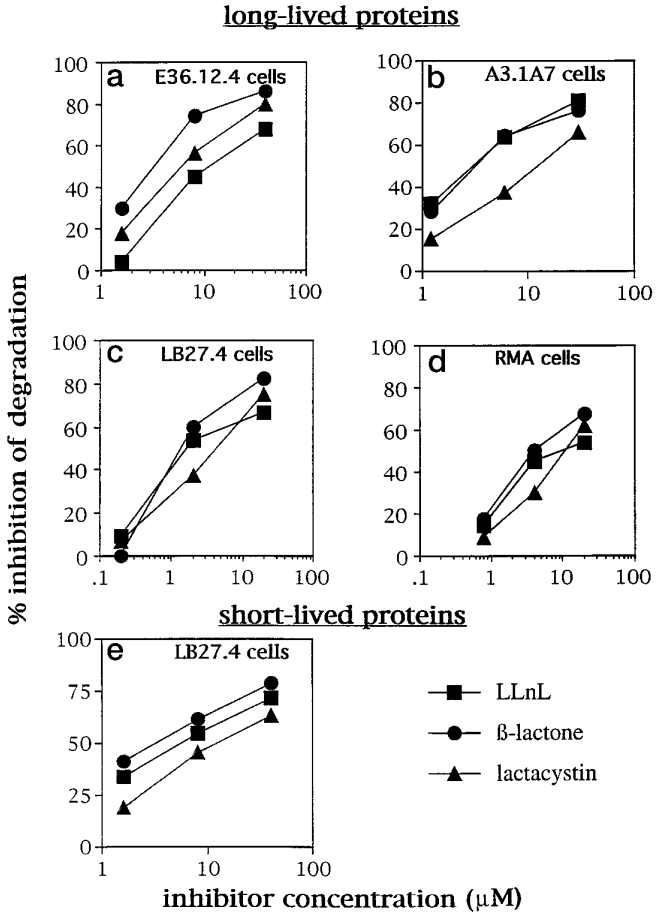

FIG. 1. **Effects of lactacystin and *clasto*-lactacystin β-lactone on protein degradation in different cell types.** Representative experiments showing the effects of lactacystin (*triangles*), *clasto*-lactacystin β-lactone (*circles*), and LLnL (*squares*) on the degradation of long-lived proteins in hamster E36.12.4 cells (*A*), murine A3.1A7 (*B*), LB27.4 (*C*), or RMA cells (*D*) and on short-lived proteins in LB27.4 cells (*E*). The labeling of cells and measurement of protein degradation were performed as described in Ref. 3. The incorporation of [^{3}H]tyrosine into trichloroacetic acid-insoluble fractions was as follows: *A*, 320,000; *B*, 400,000; *C*, 320,000; *D*, 220,000; and *E*, 80,000 cpm, per group.

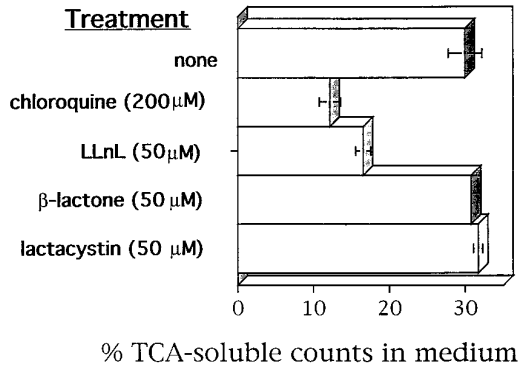

FIG. 2. **Unlike LLnL, lactacystin and *clasto*-lactacystin β-lactone do not inhibit lysosomal degradation of exogenous ^{125}I-KLH.** ^{125}I-KLH was incubated with A3.1A7 cells for 1 h at 4 °C to allow surface binding of protein. Cells were then centrifuged through fetal calf serum to remove unbound radioactivity and incubated for 4 h at 37 °C in the absence or presence of chloroquine (200 μM), LLnL (50 μM), lactacystin (50 μM), or *clasto*-lactacystin β-lactone (50 μM). Culture medium was removed, and trichloroacetic acid (*TCA*)-soluble and -precipitable counts in the medium and resuspended cell pellets were determined. Total radioactivity per sample group was between 6,000 and 12,000 cpm.

protein breakdown were consistently higher than to reduce peptide hydrolysis.

Inhibition of Cellular Protein Degradation—Because lactacystin and its β-lactone derivative inhibit protein breakdown by proteasomes but do not affect other classes of proteases, they appeared very useful to test further the importance of the proteasome in protein turnover. We examined the effects of these agents on nonlysosomal degradation of long-lived proteins in four different cell types as follows: hamster lung carcinoma cells (E36.12.4) (Fig. 1*A*), a murine macrophage cell line (A3.1A7) (Fig. 1*B*) and murine B (LB27.4) (Fig. 1*C*), and T (RMA) lymphoblastoid cells (Fig. 1*D*). Cells treated with the inhibitors remained fully viable for the duration of the experiment (8 h) (*i.e.* they excluded vital dyes and showed no reduction in protein synthesis). In all three cell types lactacystin and the β-lactone inhibited protein degradation in a concentration-dependent fashion. The β-lactone was consistently two to four times as effective as lactacystin, in accord with findings on purified proteasomes (Tables I and II) (11). Generally, after a 2-h exposure 50% inhibition was obtained with inhibitors at 1–10 μM. These agents were slightly more potent in the LB27.4 cell line (K_i of 1–4 μM) (Fig. 1*C*.) than in A3.1A7, E36.12.4, and RMA cells (K_i of 3–10 μM) (Fig. 1, *A*, *B*, and *D*). Maximal inhibition was generally reached in these assays at concentrations above 20 μM. Under these conditions, lactacystin and the β-lactone generally had a similar potency to the peptide aldehyde LLnL in the murine cell lines (Fig. 1, *B*, *C*, and *D*) but were more active than LLnL in the hamster line (Fig. 1*A*), possibly due to differences in the entry or metabolism of the agents or in their activity against the proteasomes in the different cell types.

These agents were also found to be effective in inhibiting the degradation of short-lived proteins in LB27.4 cells, and again the β-lactone appeared two to four times more potent than lactacystin (Fig. 1*E*). Thus, the nonlysosomal degradation of the bulk of cellular proteins can be reduced by these highly specific inhibitors of the proteasome.

To verify that these agents did not inhibit other processes *in vivo*, we assayed their effects on the intralysosomal protein degradation of an endocytosed protein, ^{125}I-labeled keyhole limpet hemocyanin (KLH), in murine A3.1A7 cells, a macrophage cell line active in lysosomal process. At concentrations of lactacystin and the β-lactone (50 μM) that maximally inhibit protein degradation in A3.1A7 cells (Fig. 1*B*), there was no inhibition of the degradation of ^{125}I-KLH over 4 h (Fig. 2). In contrast, the peptide aldehyde inhibitor of the proteasome, LLnL, at 50 μM also inhibits lysosomal degradation of ^{125}I-KLH by approximately 50%, presumably due to its inhibition of lysosomal cysteine proteases. As expected, the weak base chloroquine, which raises intralysosomal pH and thereby inhibits lysosomal protein degradation, also reduced degradation of ^{125}I-KLH. These results clearly demonstrate that lactacystin and the β-lactone are more specific inhibitors of proteasomes *in vivo* than the peptide aldehydes.

Inhibition of MHC Class I Antigen Presentation—Since lactacystin and the β-lactone selectively inhibit proteasome function *in vivo*, we used these agents to investigate the importance of the proteasome in the generation of peptides presented on MHC class I molecules. Initial experiments examined their ability to inhibit presentation of ovalbumin expressed transiently (Fig. 3). Antigen-presenting cells were first treated with or without the inhibitors and then infected with a recombinant vaccinia virus (vTF7-3) that expresses high levels of T7 RNA polymerase, and subsequently transfected with a plasmid containing ovalbumin cDNA under the control of a T7 promoter. After allowing time for antigen presentation, cells were fixed and tested for the presence of surface K^b MHC class I molecules

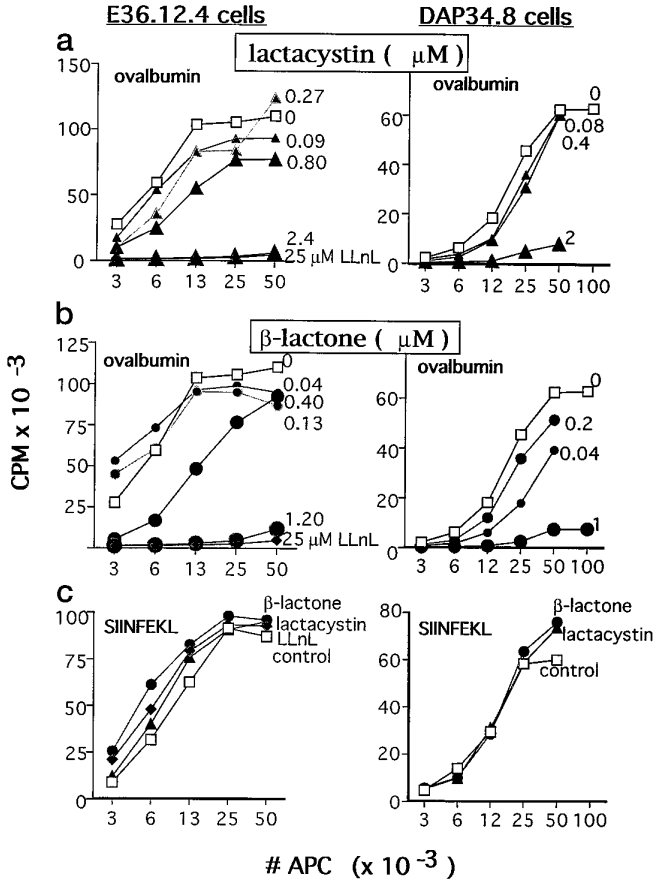

FIG. 3. **Lactacystin and *clasto*-lactacystin β-lactone inhibit MHC class I presentation of endogenously synthesized ovalbumin in E36.12.4 and DAP34.8 cells.** Hamster E36.12.4 cells (*left*) or murine DAP34.8 cells (*right*) were incubated with the indicated concentrations (μM) of lactacystin (*triangles*), *clasto*-lactacystin β-lactone (*β-lactone*) (*circles*), or LLnL (*diamonds*) for 30 min. Cells were next infected with vTF7-3 (10 plaque-forming units/cell) for 30 min and then transfected with 5 μg of pBS.OVA (*ovalbumin*) (*A* and *B*) or 0.7 μg of pBS.miniOVA (SIINFEKL) (*C*) in cationic liposomes. *C*, 2 μM lactacystin, 1 μM *clasto*-lactacystin β-lactone, and 25 μM LLnL were used; *control* refers to an untreated group. Cells were incubated for 2.3 h (E36.12.4) or 3 h (DAP34.8) at 37 °C in the presence or absence of inhibitors and then fixed and assayed for cell surface SIINFEKL-K^b complexes, using RF33.70 T-T hybridomas. Data represent the mean of duplicate cultures. In the absence of antigen, background responses were <1500 cpm. # *APC* refers to number of antigen-presenting cells.

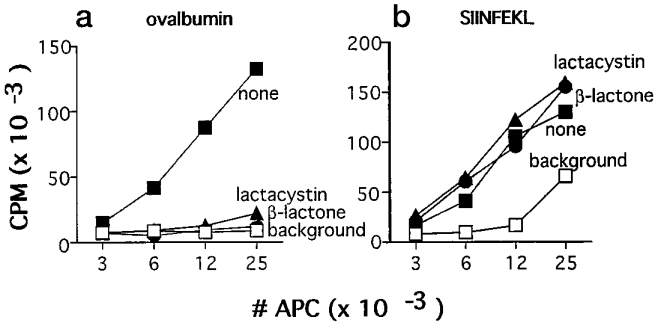

FIG. 4. **Lactacystin and *clasto*-lactacystin β-lactone inhibit MHC class I presentation of ovalbumin loaded into the cytoplasm.** LB27.4 cells were incubated with 36 μM lactacystin (*triangles*) or 24 μM *clasto*-lactacystin β-lactone (*circles*) for 1 h and then 20 mg of ovalbumin (*A*) or 0.5 μg of SIINFEKL peptide (*B*) was introduced into the cytosol by electroporation. Cells were then either fixed immediately (*background*) (*open squares*) or after a 2-h incubation at 37 °C in the presence or absence of inhibitors. Cells were assayed for cell surface SIINFEKL-K^b complexes, using RF33.70 T-T hybridomas.

containing the ovalbumin-derived peptide SIINFEKL, using the antigen-specific T-T hybridoma RF33.70. Since murine lymphoid cells are resistant to vaccinia infection, we used the hamster lung carcinoma cell line E36.12.4 and the murine L cell line DAP34.8 (Fig. 3, *left* and *right panels*, respectively) as the antigen-presenting cells; both are stably transfected with murine K^b and ICAM-1 and support vaccinia infection.

Both lactacystin and the β-lactone inhibited the presentation of the ovalbumin-derived peptide in a concentration-dependent fashion in both hamster (E36.12.4) and murine (DAP34.8) cell lines (Fig. 3, *A* and *B*). These agents completely blocked this process at 1–2 μM, which is 10 to 20 times lower than the concentration of LLnL necessary for complete inhibition. The β-lactone (Fig. 3*B*) was consistently twice as effective as lactacystin (Fig. 3*A*) in achieving a similar inhibition of ovalbumin presentation.

To determine whether these inhibitors blocked the proteolytic generation of SIINFEKL from ovalbumin or some other step in antigen presentation, we examined their effect on the presentation of SIINFEKL expressed in the cell from a minigene (Fig. 3*C*). A cDNA encoding SIINFEKL, with an initiating Met, was placed under the control of a T7 promoter in a plasmid vector and transfected into antigen-presenting cells expressing T7 RNA polymerase. Presentation of this epitope was not affected by concentrations of lactacystin or the β-lactone that completely inhibited the presentation of ovalbumin. Since the cDNAs for ovalbumin and SIINFEKL were both expressed from the same plasmids, the differential inhibition was not due to interference with expression of the transfected gene (also see below). Therefore, these agents inhibit only the generation of peptide epitopes from a protein and do not affect other steps in the class I pathway.

To further test these conclusions, we studied whether lactacystin and the β-lactone could inhibit antigen presentation when ovalbumin or the SIINFEKL peptide was delivered directly into the cytosol of murine B lymphoblastoid cells (LB27.4) by electroporation (Fig. 4). LB27.4 cells were grown in serum-free medium to minimize the binding of peptides to cell surface class I MHC due to xenogeneic β_2-microglobulin in serum. Cells fixed immediately after electroporation of either ovalbumin or SIINFEKL (p316) did not present antigen, as expected (Fig. 4, *open squares*). After a 2-h incubation both ovalbumin-loaded and SIINFEKL-loaded cells presented antigen (Fig. 4, *closed squares*). Lactacystin and the β-lactone inhibited presentation from ovalbumin (Fig. 4*A*) but did not affect presentation after SIINFEKL loading (Fig. 4*B*). These results provide further evidence that the inhibitors block the proteolytic step required for generation of peptide epitopes from a protein antigen. For reasons that are unclear, 20–40-fold higher concentrations of these compounds were required to cause a maximal inhibition of the presentation of electroporated ovalbumin than were required to inhibit presentation from transfected plasmids. This difference in sensitivity to the inhibitors was observed even in comparisons of transfection *versus* electroporation in the same cell line (DAP34.8 cells) in the same experiment (data not shown).

To determine whether most peptides presented on MHC class I are generated by the proteasome, as suggested previously (3), we determined the effects of lactacystin and the β-lactone on the assembly of stable MHC class I heterodimers in the endoplasmic reticulum. The formation of stable class I·β_2-microglobulin complexes requires peptide binding (48, 49); therefore, agents that reduce the generation of antigenic peptides should inhibit this process. Murine T lymphoblastoid cells (RMA) were labeled with [^{35}S]methionine in the presence or absence of inhibitors, and class I heterodimers were then immunoprecipitated with a conformation-sensitive antibody that

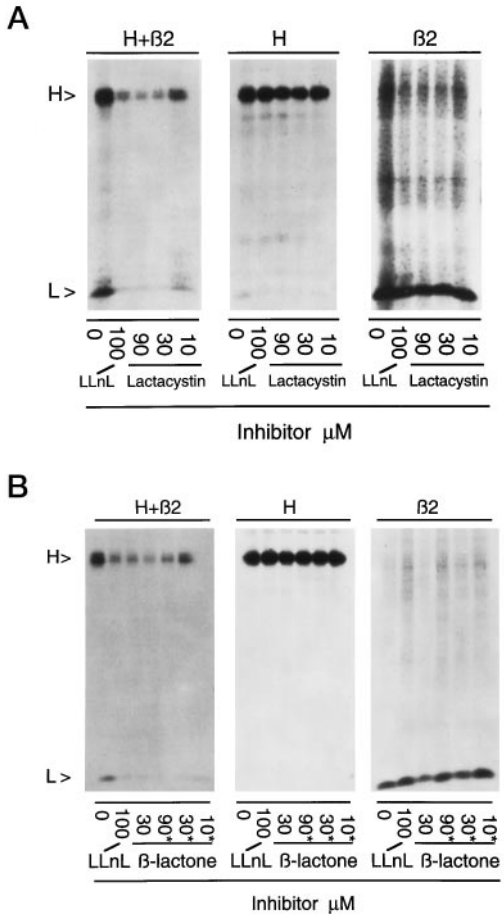

FIG. 5. **Lactacystin and *clasto*-lactacystin β-lactone inhibit the assembly of K^b class I heterodimers.** Autoradiograms of immunoprecipitated K^b heterodimers ($H+\beta_2$), free heavy chains (H), or free light chains (β_2) from [^{35}S]methionine-labeled RMA cells treated with or without different concentrations of inhibitors: lactacystin (*A*), *clasto*-lactacystin β-lactone (β-lactone) (*B*) or LLnL. RMA cells (15×10^6) were treated with or without inhibitors for 2.5 h, with [^{35}S]methionine (0.7 mCi/ml) added for the last 30 min of incubation. Because the β-lactone is relatively unstable in aqueous solution, this inhibitor was added to some groups three times (*asterisk*) over the incubation period to the final concentration indicated. Detergent lysates were precleared and immunoprecipitated sequentially with Y3 (anti-K^b heterodimers), rabbit anti-exon 8 (anti-K^b H chain), and S19.8.503 (anti-β_2) antibodies and analyzed on SDS-PAGE gels. The positions of the class I heavy chains and β_2-microglobulin on the autoradiograms are indicated by H and L, respectively.

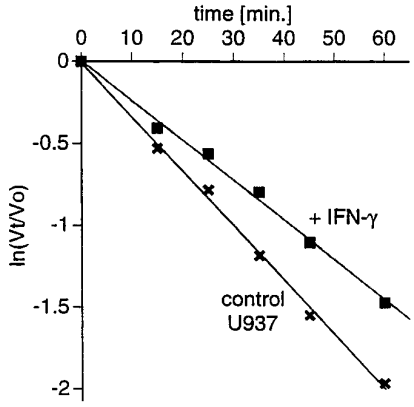

FIG. 6. **Kinetics of inhibition of the chymotrypsin-like activity of proteasomes by lactacystin in IFN-γ-treated and untreated cells.** Proteasomes were isolated from control (*cross*) and IFN-γ-treated (*square*) U937 macrophages and incubated with Suc-LLVY-MCA. 10 μM lactacystin was preincubated with the proteasomal fractions (0.5 mg of protein per ml) for $t = 0-60$ min at 37 °C. $k_a = k_{obs}/[I]$, where k_{obs} is the pseudo first-order association rate constant; $\ln(V_t/V_0) = -k_{obs}\ t$. I = concentration of an inhibitor. V_t = velocity of the degradation reaction at time = t; V_0 = velocity at time = 0. Values of k_a were 55 $M^{-1}\ s^{-1}$ for control U937 and 40 $M^{-1}\ s^{-1}$ for IFN-γ-treated U937.

reacts only with assembled heterodimers (Fig. 5). Treatment of labeled cells with either inhibitor (Fig. 5, *A* and *B*) markedly reduced the amount of class I heterodimers (Fig. 5, *left panels*) but did not reduce the amount of free class I heavy chain or β_2-microglobulin synthesized (Fig. 5, *middle* and *right panels*). Therefore, these agents caused a concentration-dependent inhibition of the assembly of these complexes. Maximal effects were obtained at concentrations similar to those required to maximally inhibit protein degradation and the presentation of electroporated ovalbumin. Class I assembly was inhibited almost completely, as was also found with LLnL. Together, these findings prove that the great majority of peptides presented are generated by the proteasome.

The Activities of LMP-containing Proteasomes Are Inhibited by Lactacystin—Lactacystin was previously reported to modify mainly the N-terminal threonine on subunit X, and also a residue on the Z-subunit, in proteasomes from bovine brain (11). However, the ability of this compound to inhibit three major peptidase activities of proteasomes (Table I) suggested that it might react with additional active sites. We therefore tested whether lactacystin inhibits the peptidase activities of proteasomes from U937 monocytes cultured in IFN-γ for 7 days, which have very low levels of subunits X, Y, and Z, and high levels of LMPs and MECL-1. Lactacystin was found to be a potent inhibitor of proteasomes whether or not they contained the X-subunit (Fig. 6). For proteasomes isolated from IFN-γ-treated U937 cells, the k_a value appeared 30% lower than for control preparations (31 ± 3 and 45 ± 3 $M^{-1}\ s^{-1}$; $n = 4$, $p < 0.05$). Similarly, a mutant lymphoblast that lacks LMPs was also sensitive to inhibition of its chymotryptic activity by lactacystin (data not shown). These findings indicate that lactacystin does not only react with X (and Z)-subunits.

We therefore investigated the ability of lactacystin and the β-lactone to affect the function of LMP-containing proteasomes in cells treated with IFN-γ. In IFN-γ-treated LB27.4 cells (Fig. 7*B*) protein degradation was found to be equally, or slightly more, susceptible to these agents than in nontreated cells (Fig. 7*A*). Similar findings were obtained for RMA cells treated with IFN-γ (data not shown). Both inhibitors were also slightly more potent in blocking antigen presentation of electroporated ovalbumin after IFN-γ treatment (Fig. 8). These results demonstrate that lactacystin and the β-lactone inhibit protein degradation and class I epitope generation mediated by LMP2-, 7-, and 10 (MECL-1) -rich proteasomes and that subunit X is not the sole target for their effects *in vivo*.

Lactacystin Covalently Modifies Multiple Proteasome β-Subunits—To confirm that lactacystin can inactivate multiple β-subunits (and not just X), we examined whether radiolabeled lactacystin could covalently modify subunits of proteasomes isolated from cells treated with or without IFN-γ. Crude cell extracts, proteasome fractions, or purified 20 S and 26 S particles were incubated with [^{3}H]lactacystin. SDS-PAGE and fluorography revealed multiple radioactive bands in the 20–30-kDa region of the gel, where the subunits of the 20 S proteasome migrate (not shown). To identify the lactacystin-modified proteins, proteasome preparations were incubated with [^{3}H]lactacystin, and then subunits were resolved on two-dimensional PAGE and identified by Western blotting with specific anti-β subunit antibodies and fluorography. Six different subunits of the 20 S proteasome were definitively identified as labeled by [^{3}H]lactacystin: LMP2, LMP7, Y (δ), X (ϵ), Z, and MECL-1 (Fig. 9). All these subunits, which were visualized and

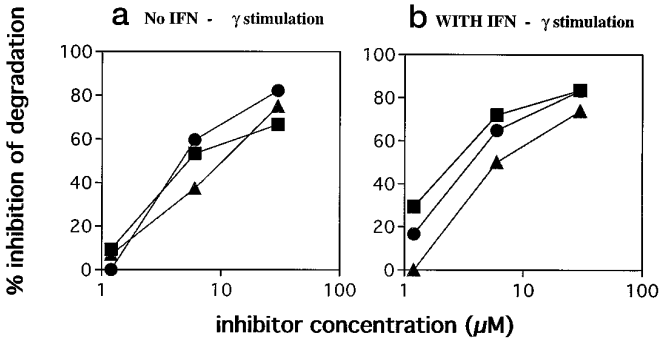

FIG. 7. **Lactacystin and *clasto*-lactacystin β-lactone inhibit protein degradation in IFN-γ-stimulated cells.** LB27.4 cells were grown in the absence (*A*) or presence (*B*) of murine IFN-γ for 72 h. Effects of the inhibitors were then determined as in Fig. 1. *Squares*, LLnL; *circles*, *clasto*-lactacystin β-lactone; *triangles*, lactacystin.

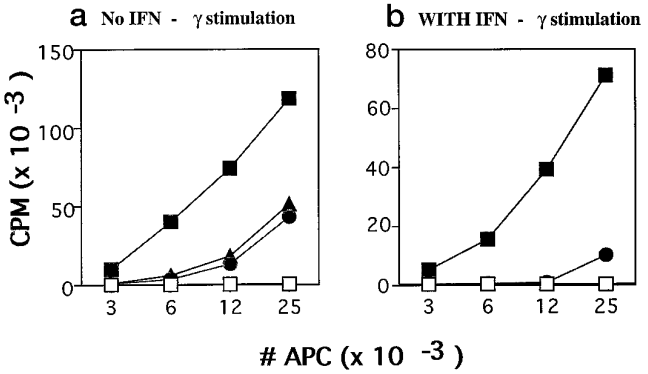

FIG. 8. **Lactacystin and *clasto*-lactacystin β-lactone inhibit antigen presentation in IFN-γ-stimulated cells.** DAP34.8 cells were grown in the absence (*A*) or presence (*B*) of murine IFN-γ for 72 h. Antigen presentation was then assayed using ovalbumin electroporated into the cytosol as described in Fig. 4. In this experiment, presentation of SIINFEKL from pBS.miniOVA was not blocked by the inhibitors (data not shown). Background (*open squares*) refers to groups fixed immediately after electroporation. *Dark squares,* no inhibitor; *dark circles,* 32 μM *clasto*-lactacystin β-lactone; *dark triangles,* 48 μM lactacystin.

identified on at least two fluorogram/Western blot pairs, are of β-type. Thus, lactacystin reacts with all of the β-subunits that are believed to be catalytically active and is highly specific for only these subunits. No other proteins were found on the two-dimensional fluorograms to be reproducibly labeled by [^{3}H]lactacystin.

DISCUSSION

Role of the Proteasome in Protein Degradation—The present studies have used the highly specific proteasome inhibitors lactacystin and its derivative β-lactone to examine the role of the proteasome in intracellular protein degradation. As reported earlier for 20 S proteasomes (11), we found that these agents inhibit the multiple peptidase activities of 20 S and 26 S proteasomes, and also the LMP-containing "immuno-proteasome," and demonstrate for the first time that these inhibitors also reduce protein hydrolysis by the 20 S and 26 S particle. Interestingly, for both 20 S and 26 S particles, the concentrations and incubation times required for inactivation differed for chymotryptic, tryptic, and peptidylglutamyl activities. These activities must therefore be mediated by distinct sites that differ in their affinities and/or rate of reaction with these compounds. Substantially higher concentrations of the inhibitors and longer incubation times were required to inhibit casein degradation than to block the chymotryptic-like activity, as was previously observed using peptide aldehyde inhibitors (3). Presumably, multiple peptidase activities, including some with lower affinity for the lactone than the chymotryptic-like site, participate in protein degradation and must be inactivated to block this process.

In a variety of mammalian cells, these inhibitors blocked degradation of both long-lived and short-lived proteins similarly. Inhibition of 75–95% of protein degradation was achieved within 1-h exposure to ≥20 μM of the inhibitors. Therefore, these agents inhibit the nonlysosomal pathway for protein degradation in cells, which, as shown here and previously, is the major degradative pathway in cells. Nevertheless, these reagents did not reduce cell viability or protein synthesis for at least several hours (data not shown). These results provide strong further evidence that the proteasome is responsible for degrading most proteins in mammalian cells. Previous experiments with different peptide aldehydes had suggested that the proteasome was responsible for most protein degradation (3). However, since peptide aldehydes can inhibit thiol proteases (*e.g.* lysosomal proteases) and, as shown here, can reduce breakdown of endocytosed protein, the prior findings could not exclude the possibility that some unknown cellular proteases also contributed to this process. However, very similar results were found here with lactacystin and the β-lactone which react with proteasome β-subunits, do not inhibit any other known proteases aside from the proteasome (11), and do not interfere with lysosomal protein degradation. Both classes of inhibitor appear very useful for studying the role of the proteasome in physiological processes, although studies with peptide aldehydes require controls with other inhibitors to rule out the involvement of lysosomal or calcium-dependent proteases (3, 6).

Our results with various cell types indicate a greater contribution of the proteasome to overall protein breakdown than previously estimated from studies with temperature-sensitive ubiquitin conjugation mutants (16–18, 27). Such studies had failed to demonstrate a significant effect of ubiquitin on degradation of long-lived cellular proteins. It is possible that ubiquitin conjugation was not completely inactivated in the ubiquitin mutant studies. Alternatively, a significant component of intracellular degradation may involve proteasomes but not ubiquitin conjugation.

Role of the Proteasome in MHC Class I Antigen Presentation—As had earlier been found with peptide aldehydes, lactacystin and the β-lactone both strongly inhibited presentation of the dominant class I epitope from ovalbumin. The proteasome thus is responsible for the endoproteolytic step that liberates the appropriate peptide from ovalbumin. These agents did not inhibit the class I presentation of the peptide epitope alone (whether synthesized endogenously or loaded). Therefore lactacystin and the β-lactone inhibit protein hydrolysis of the whole protein into peptides, without affecting any other steps in antigen presentation or cell viability during the experiments. By reducing the supply of presentable peptides required for assembly of the complex, the inhibitors also prevented the stable assembly and movement to the cell surface of MHC class I molecules. Proteasomes must be the source of the great many peptides presented on MHC class I. The assembly of MHC class I molecules was not fully inhibited by these agents, either because the proteasome was not completely inhibited or a small fraction of antigenic peptides was being generated by other proteinases in cells (as is well established for the peptides generated from signal sequences released from secreted proteins in the endoplasmic reticulum (50)).

Interestingly, the concentration of lactacystin required to inhibit the presentation of ovalbumin loaded into the cytosol was 10–20-fold higher and the concentration of the peptide aldehyde was up to 4-fold higher than was required when

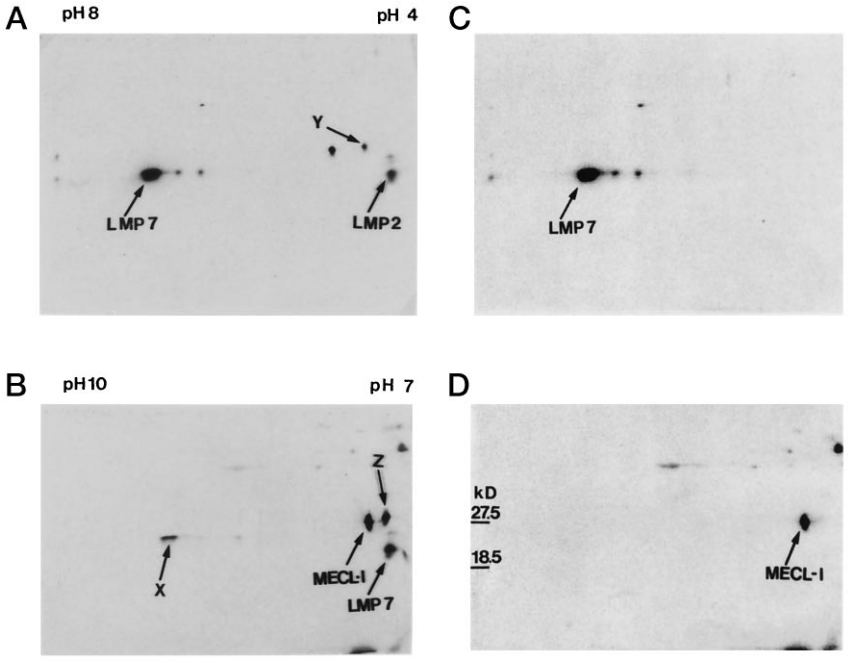

FIG. 9. **[^{3}H]lactacystin interacts with multiple proteasome β-subunits.** Two-dimensional polyacrylamide gels of [^{3}H]lactacystin-treated proteasome fractions isolated from 721 (*A* and *C*) and IFN-γ-stimulated 721 lymphoblasts (*B* and *D*) were transferred to nitrocellulose. Fluorograms of the filters were analyzed (*A* and *B*), or Western blotting with specific antibodies was performed (*C* and *D*). Representative Western blots for LMP7 (*C*) and MECL-1 (*D*) are shown. Data for other β-subunits is not shown but permitted the identification of radioactive spots in the fluorogram as labeled. Although subunit Z was not visualized in *A*, it was seen in other experiments. Isoelectric points for the range of ampholytes used in two-dimensional fluorograms are noted: 3–10/6–8 in *A* and *C*, and 6–8/8–10 in *B* and *D*.

ovalbumin was expressed in the vaccinia transfection system. Typically, near maximal inhibition of antigen presentation from exogenous ovalbumin required 20 μM lactacystin or the β-lactone, which was similar to the concentrations required to inhibit protein degradation and peptide-dependent assembly of MHC complexes. The reason why the presentation of endogenously expressed ovalbumin is more sensitive to the inhibitors remains unclear. We have previously found that the presentation of native ovalbumin loaded into the cytosol is inhibited by temperature-sensitive mutations that block ubiquitin conjugation, whereas the presentation of transfected ovalbumin is not (51). This may reflect differences in the folding of the two substrates or their subcellular localization. Possibly, ubiquitinated molecules are more resistant to inhibition. These differences cannot be explained by involvement of different species of proteasome, since lactacystin is more effective against 26 S and the aldehyde against the 20 S particle.

Lactacystin Modifies Multiple Proteasome β-Subunits—Lactacystin was previously shown to modify subunits X and Z in brain extracts (11). However, we found here that six β-subunits (LMP2, LMP7, MECL-1, X, Y, and Z) can react with lactacystin, all of which have N-terminal threonines, whose hydroxyl groups catalyze the nucleophilic attack on the substrate (10, 52, 53). Thus, all the β-subunits that are capable of forming the threonine-based active sites are shown here to be covalently modified by lactacystin. Accordingly, lactacystin was found to inhibit protein degradation as well as the presentation of ovalbumin by both control cells and IFN-γ- stimulated cells, in which X-, Y-, and Z-subunits are largely replaced by LMP2, LMP7, and MECL-1.

The difference between these findings and the specificity for the subunit X noted previously in studies of brain extracts (11) may be due to the variation in LMP expression in different tissues. Unlike the proteasomes studied here, brain proteasomes normally contain very low levels of LMP2 and LMP7 β-subunits compared with liver or spleen (25), presumably because brain also has much lower levels of MHC molecules and is less active in antigen presentation (54). Consequently, any reaction of lactacystin with the three IFN-γ-induced subunits would have been missed in the earlier studies.

REFERENCES

1. York, I. A. & Rock, K. L. (1996) *Annu. Rev. Immunol.* **14,** 369–396
2. Goldberg, A. L. & Rock, K. L. (1992) *Nature* **357,** 375–379
3. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D. & Goldberg, A. L. (1994) *Cell* **78,** 761–771
4. Orlowski, M. (1990) *Biochemistry* **29,** 10289–10297
5. Rivett, J. (1993) *Biochem. J.* **291,** 1–10
6. Coux, O., Tanaka, K. & Goldberg, A. L. (1996) *Annu. Rev. Biochem.* **65,** 801–847
7. Grziwa, A., Baumeister, W., Dahlmann, B. & Kopp, F. (1991) *FEBS Lett.* **290,** 186–192
8. Lowe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W. & Huber, A. (1995) *Science* **268,** 533–539
9. Zwickl, P., Kleinz, J. & Baumeister, W. (1994) *Nat. Struct. Biol.* **1,** 765–771
10. Seemüller, E., Lupas, A., Stock, D., Löwe, J., Huber, R. & Baumeister, W. (1995) *Science* **268,** 579–582
11. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J. & Schreiber, S. L. (1995) *Science* **268,** 726–731
12. Brannigan, J. A., Dodson, G., Duggleby, H. J., Moody, P. C., Smith, J. L., Tomchick, D. R. & Murzin, A. G. (1995) *Nature* **378,** 416–419
13. Hershko, A. & Ciechanover, A. (1992) *Annu. Rev. Biochem* **61,** 761–807
14. Rechsteiner, M., Hoffman, L. & Dubiel, W. (1993) *J. Biol. Chem.* **268,** 6065–6068
15. Seufert, W. & Jentsch, S. (1992) *EMBO J.* **11,** 3077–3080
16. Gropper, R., Brandt, R. A., Elias, S., Bearer, C. F., Mayer, A., Schwartz, A. L. & Ciechanover, A. (1991) *J. Biol. Chem.* **266,** 3602–3610
17. Ciechanover, A., Finley, D. & Varshavsky, A. (1984) *Cell* **37,** 57–66
18. Kulka, R. G., Raboy, B., Schuster, R., Parag, H. A., Diamond, G., Ciechanover, A. & Marcus, M. (1988) *J. Biol. Chem.* **263,** 15726–15731
19. Deveraux, Q., Ustrell, V., Pickart, C. & Rechsteiner, M. (1994) *J. Biol. Chem.* **269,** 7059–7061
20. Ciechanover, A. (1994) *Cell* **79,** 13–21
21. Monaco, J. J. & McDevitt, H. O. (1986) *Hum. Immunol.* **15,** 416–426
22. Monaco, J. J. (1992) *Immunol. Today* **13,** 173–179
23. Fehling, H. J., Swat, W., Laplace, C., Kuhn, R., Rajewsky, K., Muller, U. & von Boehmer, H. (1994) *Science* **265,** 1234–1237
24. Cerundolo, V., Kelly, A., Elliott, T., Trowsdale, J. & Townsend, A. (1995) *Eur. J. Immunol.* **25,** 554–562
25. Van Kaer, L., Ashton-Rickardt, P. G., Eichelberger, M., Gaczynska, M., Nagashima, K., Rock, K. L., Goldberg, A. L., Doherty, P. C. & Tonegawa, S. (1994) *Immunity* **1,** 533–541
26. Sibille, C., Gould, K. G., Willard-Gallo, K., Thomson, S., Rivett, A. J., Powis, S., Butcher, G. W. & DeBaetselier, P. (1995) *Curr. Biol.* **5,** 923–930
27. Michalek, M. T., Grant, E. P., Gramm, C., Goldberg, A. L. & Rock, K. L. (1993) *Nature* **363,** 552–554
28. Grant, E. P., Michalek, M. T., Goldberg, A. L. & Rock, K. L. (1995) *J. Immunol.* **155,** 3750–3758
29. Boes, B., Hengel, H., Ruppert, T., Multhaup, G., Koszinowski, U. H. & Kloetzel, P.-M. (1994) *J. Exp. Med.* **179,** 901–909
30. Dick, L. R., Aldrich, C., Jameson, S. C., Moomaw, C. R., Pramanik, B. C., Doyle, C. K., DeMartino, G. N., Bevan, M. J., Forman, J. M. & Slaughter, C. A. (1994) *J. Immunol.* **152,** 3884–3894
31. Niedermann, G., Butz, S., Ihlenfeldt, H. G., Grimm, R., Lucchiari, M., Hoschützky, H., Jung, G., Maier, B. & Eichmann, K. (1995) *Immunity* **2,** 289–299
32. Omura, S., Matsuzaki, K., Fujimoto, T., Kosuge, K., Fuzuya, T., Fujita, S. &

Nakagawa, A. (1991) *J. Antibiot.* (*Tokyo*) **44,** 113–118
33. Fenteany, G., Standaert, R. F., Reichard, G. A., Corey, E. J. & Schreiber, S. L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 3358–3362
34. Dick, L. R., Cruikshank, A. A., Grenier, L., Melandri, F. D., Nunes, S. L. & Stein, R. L. (1996) *J. Biol. Chem.* **271,** 7273–7276
35. Jones, B. & Janeway, C. A. J. (1981) *Nature* **292,** 547–549
36. Lemke, H., Hammerling, G. J., Hohmann, C., and Rajewsky, K. (1978) *Nature* **271,** 249–251
37. Akopian, T. W., Kisselev, A. F. & Goldberg, A. L. (1997) *J. Biol. Chem.* **272,** 1791–1798
38. Kappler, J., White, J., Wegmann, D., Mustain, F. & Marrack, P. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 3604–3607
39. Rock, K. L., Rothstein, L. & Gamble, S. (1990) *J. Immunol.* **145,** 804–811
40. Kärre, K., Ljunggren, H. G., Piontek, G. & Kieesling, R. (1986) *Nature* **319,** 675–678
41. Kovacsovics-Bankowski, M. & Rock, K. L. (1994) *Eur. J. Immunol.* **24,** 2421–2428
42. DeMars, R., Chang, C. C. & Rudersdorf, R. A. (1983) *Hum. Immunol.* **8,** 123–139
43. Gaczynska, M., Rock, K. L. & Goldberg, A. L. (1993) *Nature* **365,** 264–267
44. Gaczynska, M., Rock, K. L., Spies, T. & Goldberg, A. L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 9213–9217
45. Fuerst, T. R., Niles, E. G., Studier, F. W. & Moss, B. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8122–8126
46. Grey, H. M., Colon, S. M. & Chesnut, R. W. (1982) *J. Immunol.* **129,** 2389–2395
47. Rock, K. L. & Benacerraf, B. (1983) *J. Exp. Med.* **157,** 1618–1634
48. Townsend, A. R., Bastin, J., Gould, K. & Brownlee, G. G. (1986) *Nature* **324,** 575–577
49. Townsend, A., Elliott, T., Cerundolo, V., Foster, L., Barber, B. & Tse, A. (1990) *Cell* **62,** 285–295
50. Henderson, R. A., Michel, H., Sakaguchi, K., Shabanowitz, J., Appella, E., Hunt, D. F. & Engelhard, V. H. (1992) *Science* **255,** 1264–1266
51. Michalek, M. T., Grant, E. P. & Rock, K. L. (1996) *J. Immunol.* **157,** 617–624
52. Löwe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W. & Huber, R. (1995) *Science* **268,** 533–539
53. Goldberg, A. L. (1995) *Science* **268,** 522–523
54. Chamberlain, J. W., Vasavada, H. A., Ganguly, S. & Weissman, S. M. (1991) *Mol. Cell. Biol.* **11,** 3564–3572