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Distinct Proteolytic Processes Generate the C and N Termini of MHC Class I-Binding Peptides[1]

X. Y. Mo,* Paolo Cascio,† Kristen Lemerise,* Alfred L. Goldberg,† and Kenneth Rock[2]*

Most of the MHC class I peptides presented to the immune system are generated during the course of protein breakdown by the proteasome. However, the precise role of the proteasome, e.g., whether this particle or some other protease generates the carboxyl (C) and amino (N) termini of the presented 8- to 10-residue peptides, is not clear. Here, we show that presentation on D^b of ASNENMETM, a peptide from influenza nucleoprotein, and on K^b of FAPGNYPAL, a peptide from Sendai virus nucleoprotein, was blocked by the proteasome inhibitor, lactacystin. Using plasmid minigene constructs encoding oligopeptides of various lengths, we found that presentation of ASNENMETM from C-terminally extended peptides that contain this antigenic peptide plus three or five additional amino acids and presentation of FAPGNYPAL from a peptide containing FAPGNYPAL plus one additional C-terminal residue required the proteasome. In contrast, the proteasome inhibitor did not reduce presentation of cytosolically expressed ASNENMETM or FAPGNYPAL or N-terminally extended versions of these peptides, suggesting involvement of aminopeptidase(s) in trimming these N-extended variants. Accordingly, when the N termini of these 3N-extended peptides were blocked by acetylation, they were resistant to hydrolysis by cellular aminopeptidases and pure leucine aminopeptidase. Moreover, if introduced into the cytosol, Ag presentation of these peptides occurred to a much lesser extent than from their nonacetylated counterparts. Thus, the proteasome is essential for the generation of ASNENMETM and FAPGNYPAL peptides from the full-length nucleoproteins. Although it generates the C termini of these presented peptides, distinct aminopeptidase(s) can trim the N termini of these presented peptides to their proper size. *The Journal of Immunology,* **1999, 163: 5851–5859.**

The immune system continually screens for viral infections and cancers by monitoring whether cells are synthesizing foreign or mutant proteins. This surveillance process depends on the presence of MHC class I molecules that bind and display to CTL 8- to 10-residue peptides that are derived from the spectrum of proteins expressed in the cell (1). Most of these peptides are generated in the cytosol during protein degradation by proteasomes (2, 3). These peptides are then translocated via the TAP transporter into the endoplasmic reticulum (ER)[3] (4, 5), where they bind to the MHC class I molecules. The resulting peptide-MHC class I complexes are then transported to the cell surface.

Our understanding of how antigenic peptides are generated remains incomplete. The active form of the proteasome, which appears to degrade most cellular proteins, is the 26S proteasome (3). This complex is formed by the association of the 19S regulatory complex with each end of the core 20S proteasome. This 2-MDa structure degrades ubiquitinated and some nonubiquitinated proteins in an ATP-dependent manner (2). Proteins are cleaved within the 20S (700 kDa) core proteasome, which is composed of four stacked rings. The two inner β-rings contain six proteolytic sites, which differ in substrate specificity; two have chymotrysin-like specificity, two trypsin-like, and two postacidic-cleaving activities (2, 3). These active sites all have a novel proteolytic mechanism, in which the active nucleophile is the hydroxyl group of the amino terminal threonine residue on several of the β-subunits (2, 3). Proteasome inhibitors have been described that interfere with this catalytic mechanism (3). The most selective inhibitor is the antibiotic lactacystin and its active derivative, β-lactone, which acylates the amino terminal threonine residues of the β-subunits and thereby prevent catalytic activity (6, 7).

It is now well established that the ubiquitin-proteasome pathway is involved in the generation of the majority of class I-presented peptides (3, 8). For example, proteasome inhibitors, such as lactacystin or peptide aldehydes, can block the processing and presentation of specific antigenic peptides and markedly reduce the overall supply of peptides to class I molecules (8–12). In addition, genes encoding the alternative proteasome β-subunits, LMP-2 and LMP-7, are encoded in the MHC-gene region (13, 14), and cell lines or mice that lack these subunits have defects in Ag presentation (15–18). On the other hand, IFN-γ, which promotes Ag presentation (3), causes induction of these MHC-encoded subunits and thus alters proteasomal peptidase activities, apparently to favor generation of peptides with C termini appropriate for MHC binding (19, 20). Furthermore, in mutant cell lines with a temperature-sensitive ubiquitin-activating enzyme (E1), the inactivation of ubiquitin conjugation at the nonpermissive temperature blocks generation of antigenic peptides from the OVA (21, 22). However, because the presentation of some antigenic peptides is not reduced by proteasome inhibitors, other proteases might also produce some class I-presented peptides (23–25).

In those cases where the proteasome clearly is essential for the generation of class I-presented peptides, it is uncertain whether it makes the final cleavages that yield the presented peptides or if

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[3] Abbreviations used in this paper: ER, endoplasmic reticulum; NP, nucleoprotein.

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Table I. *Peptides or translated products of expressed genes from constructed plasmids or vaccinia constructs*

Name	Amino Acid Sequence[a]	Length (amino acid)
Plasmid		
p.ASNENMETM	ASNENMETM	9
p.5+ASNENMETM	RGVQIASNENMETM	14
p.ASNENMETM+1	ASNENMETME	10
p.ASNENMETM+2	ASNENMETMES	11
p.ASNENMETM+3	ASNENMETMESS	12
p.ASNENMETM+4	ASNENMETMESST	13
p.ASNENMETM+5	ASNENMETMESSTL	14
p.FAPGNYPAL	FAPGNYPAL	9
p.FAPGNYPAL+1	FAPGNYPALW	10
p.5+FAPGNYPAL	PVHGEFAPGNYPAL	14
Vaccinia construct		
V-FluNP	Full-length NP of influenza A PR/8/34	506
V-SVNP	Full length NP of Sendai virus	524
Peptide		
3+ASNENMETM	VQIASNENMETM	12
3+FAPGNYPAL	HGEFAPGNYPAL	12
3+SIINFEKL	QLESIINFEKL	11
acetyl-3+ASNENMETM	Acetyl-VQIASNENMETM	12
acetyl-3+FAPGNYPAL	Acetyl-HGEFAPGNYPAL	12
acetyl-3+SIINFEKL	Acetyl-QLESIINFEKL	11

[a] ASNENMETM is encoded by codon 366-374 of cDNA for influenza A/PR/8/34 (H0N1) NP protein, so is FAPGNYPAL by codon 324-332 of cDNA for NP protein of Sendai virus, and SIINFEKL by codon 257-264 of cDNA for OVA.

other proteases are needed to trim further the proteasomal products. To bind to class I molecules with high affinity, peptides must be of a precise length, either 8, 9, or 10 residues, depending on the class I molecule (26, 27). The peptides produced by purified 20S and 26S proteasomes during protein degradation range in size from 4 to 24 residues, but two-thirds are too short to serve in Ag presentation (<8 residues) (28, 29). However, most such studies with isolated proteasomes have used highly unphysiological conditions, and therefore it is uncertain whether such results can be extrapolated to the in vivo situation (3).

We have recently shown in intact cells that proteasomes were responsible for generating the correct C terminus of SIINFEKL from a longer peptide, but other protease might generate its proper N terminus (30). Furthermore, in cell extracts, aminopeptidases, including the IFN-γ-inducible enzyme (31), leucine aminopeptidase, can trim N-terminally extended versions of SIINFEKL to generate the correct octomer (32). In an effort to define the proteolytic activities that make the final carboxyl- and amino-terminal cleavages that generate natural viral antigenic peptides, we have extended our studies to the D^b-restricted influenza peptide nucleoprotein (NP) 366–374 (ASNENMETM) and the K^b-restricted Sendai $NP_{324–332}$ (FAPGNYPAL) peptides. By expressing N- and C-terminally extended versions of these peptides, we have tested whether proteasomes function directly in these final steps in the production of antigenic peptides.

Materials and Methods

Vaccinia constructs

Vaccinia constructs containing the full-length sequences for NP of influenza virus A/Puerto Rico/8/34 (PR8) strain (V-FluNP) (a kind gift from Dr. J. W. Yewdell, National Institute of Allergy and Infectious Diseases, Bethesda, MD) or NP of Sendai virus (V-SVNP) (provided by Dr. D. Kolakofsky, University of Geneva School of Medicine, Switzerland), or T7 RNA polymerase (vTF7-3) (obtained from American Type Culture Collection, Manassas, VA) (VR-2153) were all propagated in thymidine kinase-deficient human 143BTK$^-$ osteosarcoma cells (CRL 8303; American Type Culture Collection).

Cell lines and hybridomas

E36.17.3 (E36/D^b) and E36.12.4 (E36/K^b) APC lines used in this study were derived from E36 cells (hamster lung carcinoma cells), which were stably transfected with murine H-2D^b or H-2K^b, and ICAM-1 molecules (21) and maintained in RPMI 1640 medium (10% FCS) with G418. Another APC, LB27.4 B, (33) is a lymphoblastoid-presenting cell line, expressing H-2K^b molecules, and was grown in OptiMem (Life Technologies, Grand Island, NY) with 1% normal mouse serum. T cell hybridomas 12.33, specific for D^b-restricted $NP_{366–374}$ ASNENMETM of PR 8, and B3.4D8, specific for K^b-restricted $NP_{324–332}$ FAPGNYPAL of Sendai virus, were kind gifts from Dr. D. Woodland (St. Jude Children's Research Hospital, Memphis, TN) (34, 35). All the above cell lines (except LB27.4 B), and the human cervical carcinoma cell line HeLa S3, were maintained in RPMI 1640 medium with 10% FCS (Atlanta Biologicals, Norcross, GA). The T cell Hybridoma RF33.70 specific for $OVA_{257–264}$ was described previously (36).

Reagents

The proteasome inhibitor lactacystin was obtained from Dr. E.J. Corey (Harvard University, Cambridge, MA), and β-lactone was a kind gift from Dr. Julian Adams (ProScript, Cambridge, MA). The proteasome inhibitors were dissolved at 10 μM in DMSO and stored at -80°C.

The peptides VQIASNENMETM (influenza $NP_{363–374}$), HGEFAPGNYPAL (Sendai $NP_{321–332}$), QLESIINFEKL ($OVA_{254–264}$), and their N-terminal acetylated counterparts (Table I) were synthesized and purified by HPLC by the Peptide Facility at the University of Massachusetts Medical School (Worcester, MA).

Plasmid constructs

Synthetic minigenes encoding the antigenic peptides from influenza virus $NP_{366–372}$ (ASNENMETM) or Sendai $NP_{324–332}$ (FAPGNYPAL) with or without natural N- or C-terminal flanking residues were constructed and cloned into pBluscript SK under the control of the T7 RNA promoter as follows. Coding oligonucleotides were designed to consist of a Kozak consensus sequence, a start codon, the relevant DNA sequences encoding the oligopeptide (see Table I) followed by two stop codons, and an internal *Nco*I site (to facilitate the identification of recombinant plasmids). In addition, these oligonucleotides were flanked at both ends by *Eco*RI and *Hin*dIII sites for cloning into the multiple cloning site of the pBluscript SK plasmid. The oligonucleotides were phosphorylated with T4 polynucleotide kinase, annealed, and ligated into pBluscript SK. The recombinant plasmids were transformed into DH5α (Life Science Biolab, Gaithersburg, MD). Clonies were picked and plasmids isolated using Wizard plus minipreps kit (Promega, Madison, WI). The correct plasmid was identified by the presence of *Nco*I site and sequenced. All the enzymes used in these constructions were purchased from New England Biolabs (Beverly, MA).

Ag presentation assay

When Ags were expressed by recombinant vaccinia virus infection, the APCs (E36/D^b or E36/K^b cells) were first seeded onto a 6-well plate and

incubated for 24 h. They were then infected with vaccinia virus V-FluNP or V-SVNP at a multiplicity of infection of 10 for 5 h at 37°C, after which time they were harvested and fixed. Alternatively, when Ags were expressed from transfected plasmids, E36 APCs were first infected with vTF7-3 (multiplicity of infection of 10) for 30 min in OptiMem medium. The vTF7-3-containing media was then removed and replaced with various plasmids that had been incubated with liposomes (lipofectin; Life Technologies, Grand Island, NY) in OptiMem medium. E36 cells were then incubated at 37°C for 5 h before fixation. When Ags were introduced into the cytosol by electroporation, LB27.4 or E36Db cells were permeablized in electroporation buffer (0.4 M mannitol, 10 mM HEPES in PBS) as described (8) in the presence of N terminus-modified or unmodified peptides (0.2–40 μg) (Table I). Cells were then either fixed immediately after electroporation or after a further 2 h incubation at 37°C.

For fixation, APCs were incubated with 0.0025% glutaldehyde (Sigma, St. Louis, MO) for at least 10 min at room temperature. Cells were then washed with PBS three times before being plated onto 96-well plate with T cell hybridomas (1×10^5/well). T cell responses were evaluated by their production of IL-2 in the supernatant using the CTLL assay (37).

In experiments using proteasome inhibitors, E36 cells were pre-incubated with specified concentrations of proteasome inhibitors for 30 min before viral infection, and inhibitors were continually present in the culture medium during all subsequent incubation before fixation.

In vitro degradation of oligopeptides

Cytosolic extracts were prepared from HeLa S3 cells as previously described (32). Briefly, cells were homogenized with glass beads, and cytosolic extracts were prepared by centrifugation of the homogenates for 20 min at 10,000 $\times$ *g* and 1 h at 100,000 $\times$ *g* and for an additional 6-h centrifugation at 100,000 $\times$ *g* for removal of proteasomes. The residual proteasome activity in these extracts was <15%, and this was completely inhibited by the addition of the proteasome inhibitor MG132 (100 μM) for 15 min at room temperature. Next, 15 nmol of various oligopeptide substrates (Table I) were incubated with 2 μg of the proteasome-free cell extracts or with 0.5 μg of leucine aminopeptidase (Sigma) for 30 min at 37°C in 50 mM Tris-HCl buffer, pH 8.5. Reactions were terminated by adding 0.4% TCA and incubated for 15 min on ice. The peptide-containing supernatant was then subjected to reverse-phase HPLC on a 4.6 $\times$ 250-mm Vydac C18 column (Vydac, Hesperia, CA) in 0.06% TCA with a flow rate of 1 ml/min. Elution was performed with 30 min linear gradient from 4 to 48% acetonitrile, and the eluting peptides were detected by measuring absorbance at 214 nm. The relative concentrations for each eluted peptide were calculated by integration of the peptide peaks on the chromatograms. To study the effects of bestatin, the extracts were preincubated with the inhibitor for 30 min at room temperature.

Results

Ag presentation from full-length NPs is blocked by proteasome inhibitors

To investigate whether the generation of the antigenic peptide ASNENMETM requires the proteasome, vaccinia constructs that encoded full-length NP of influenza A Puerto Rico/8/34 strain were used to express the antigenic protein in E36/Db APCs in the presence or absence of the proteasome inhibitor, lactacystin. Peptide presentation was assayed by measuring the responses of a T hybridoma specific for influenza $NP_{366-374}$ (ASNENMETM) presented on D^b molecules. As shown in Fig. 1*A*, the presentation of this influenza NP-derived epitope was completely inhibited by 2 μM lactacystin. Similarly, the presentation of NP in cells infected with influenza virus was also inhibited by 2 μM lactacystin (data not shown). In contrast, the presentation of the antigenic peptide ASNENMETM expressed from a minigene, which does not require further proteolytic cleavage, was not significantly affected by this proteasome inhibitor (Fig. 1*B*).

The effect of proteasome inhibitors on the generation of another viral protein-derived peptide was also examined. Vaccinia encoding the full-length NP of Sendai virus was used to introduce the Ag in E36/Kb APCs, and the presentation of FAPGNYPAL on K^b was evaluated by the response of the T hybridoma B3.4D8. The presentation of this peptide from Sendai NP was completely inhibited by 5 μM of lactacystin (Fig. 1*C*). In contrast, the presentation of

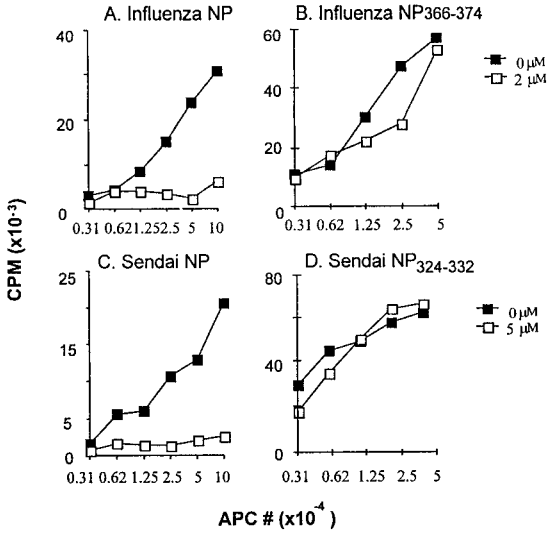

FIGURE 1. Effect of lactacystin on the presentation of ASNENMETM or FAPGNYPAL from full-length NPs. *A* and *C*, APCs (E36/D^b for *A* and *B*; E36/K^b for *C* and *D*) were treated with or without lactacystin for 30 min, then infected with vaccinia constructs that expressed full-length NP of influenza PR8 (*A*) or Sendai virus (*C*) for 5 h before fixation. *B* and *D*, Similar to *A* and *C*, except that APCs were infected with vTF7-3 and then transfected with 1 μg of plasmid p. ASNENMETM for expression of Flu $NP_{366-374}$ (*B*) or p. FAPGNYPAL for expression of SV $NP_{324-332}$ (*D*). MHC class I Ag presentation was then evaluated by incubating the fixed APCs with the T cell hybridoma 12.33 (*A* and *B*) or B3.4D8 (*C* and *D*) for 20 h, and the IL-2 content in the supernatant was measured as described in *Materials and Methods*. The results were representative of at least five independent experiments.

the antigenic peptide, FAPGNYPAL, expressed from a minigene was not inhibited under the same conditions (Fig. 1*D*).

The failure of lactacystin to block the presentation of the antigenic peptides from minigenes was anticipated, because the encoded oligopeptides do not require further proteolytic cleavage. Similar findings with other antigenic peptides and other proteasome inhibitors have been reported previously (8, 10, 24). Moreover, this result demonstrates that the proteasome inhibitor does not affect other steps in the Ag presentation pathway. Therefore, the ability of lactacystin to prevent presentation of influenza NP-derived ASNENMETM and Sendai NP-derived FAPGNYPAL from full-length proteins indicates that the proteasome is required to generate these presented peptides. However, this result does not resolve whether the proteasome directly produces the 9-residue presented peptide or whether other proteases may also be participating in this process, perhaps after the proteasome-dependent step.

Proteasomes are required for cleavage at the C termini of antigenic peptides

To examine whether the proteasome was responsible for the cleavages that yield the C terminus of the influenza-derived peptide, we tested whether ASNENMETM presentation from a construct with a C-terminal extension could be blocked by a proteasome inhibitor. A plasmid containing a minigene-encoding ASNENMETM plus the 5 aa that normally flank its C terminus (p.ASNENMETM + 5) was constructed under the control of a T7 promoter (Table I). This construct was expressed by transfection into APCs that were infected with a vaccinnia recombinant-encoding T7 polymerase (vTF7-3). The presentation of the extended oligopeptide was then studied in the presence or absence of a proteasome inhibitor.

As shown in Fig. 2*A*, ASNENMETM could be presented on MHC class I molecules from the C-terminally extended construct,

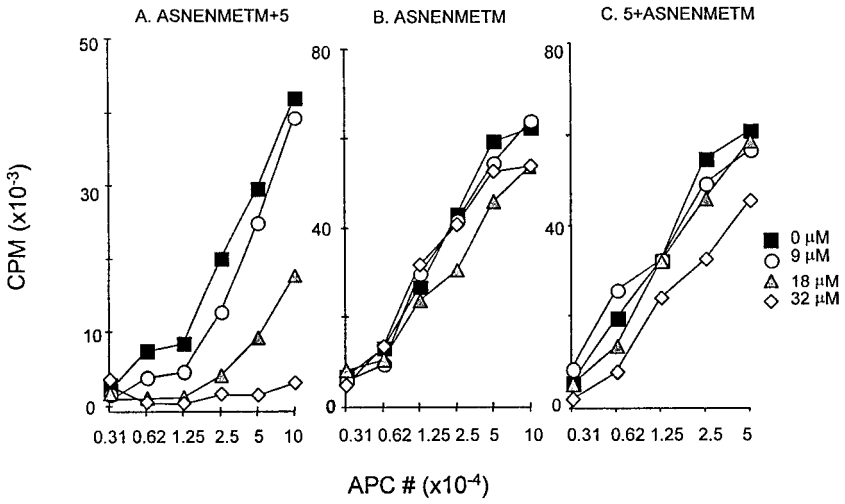

FIGURE 2. Proteasome inhibitor blocked the presentation of ASNENMETM with a C-terminal extension, but not with a N-terminal extension. E36/D^b APCs were treated with or without the indicated doses of clasto-lactacystin, β-lactone for 30 min, and were then infected with vTF-7 for another 30 min. APCs were then transfected with ASNENMETM-containing plasmids p. ASNENMETM + 5 (*A*), p. ASNENMETM (*B*), or p.5 + ASNENMETM (*C*) for 5 h before fixation. Ag presentation was then measured by IL-2 production by hybridoma 12.33 stimulated with the APCs. These results are from one representative experiment that was repeated three times.

ASNENMETM + 5. This presentation was inhibited in a dose-dependent manner by the proteasome inhibitor, β-lactone, although much higher concentrations of this agent were required than for the full-length construct. For example, the presentation was not inhibited by β-lactone at 9 μM, but was partially blocked at 18 μM and completely inhibited at 36 μM. This effect of the inhibitor on the presentation of ASNENMETM + 5 was specific, because β-lactone did not block the presentation of the expressed minimal antigenic peptide, ASNENMETM, at any of the concentrations tested (Fig. 2*B*). Therefore, the proteasome is required to cleave within the five carboxyl-terminal flanking residues of the ASNENMETM construct.

The finding that the proteasome could cleave off the C-terminal extension led us to study the influence of C-terminal extensions of different lengths on antigenic presentation. Plasmids were constructed with extensions of either one, two, three, or four additional amino acids at the C terminus of ASNENMETM based on the amino acid sequence of influenza NP (p.ASNENMETM + 1, +2, +3, and +4, respectively, Table I). Then the relative extent of presentation of ASNENMETM from these different expressed peptides was examined. As shown in Fig. 3, Ag presentation from ASNENMETM + 3 (Fig. 3*C*) and ASNENMETM + 4 (Fig. 3*D*) was apparent even when only 0.35 μg of the plasmids were transfected, and the presentation increased with greater amounts of plasmid in a dose-dependent manner. Surprisingly, no presentation occurred with ASNENMETM + 1 (Fig. 3*A*) and ASNENMETM + 2 (Fig. 3*B*), even when the dose of plasmids used was increased to 8.1 μg or when the length of time after the transfection was increased to 24 h (data not shown). This result was confirmed with other recombinant clones made independently and with different preparations of plasmids. Because presentation was observed with ASNENMETM and ASNENMETM + 3, but not for ASNENMETM + 1 or ASNENMETM + 2, the removal of the P1′ and P2′ residues cannot occur readily in the cytosol by carboxypeptidase trimming. This result is in accord with our prior failure to find cytosolic carboxypeptidase activity (32). Instead, these C-terminal cleavages must involve an endopeptidase, perhaps the proteasome, whose activity required minimally the presence of the P3′ residue. Presumably, the protease that makes the correct cleavage to generate ASNENMETM can only cleave off the C-terminal residues if the extended C-terminal sequence is sufficiently long or contains a specific residue in the P3′ position.

To test whether the cleavage of ASNENMETM + 3 to generate the presented peptide depended on the proteasome, Ag presentation from this minigene construct was studied in the presence or absence of the proteasome inhibitor. As show in Fig. 4, Ag presentation from ASNENMETM + 3 (Fig. 4*A*) was blocked by β-lactone at 30 μM. Thus, the proteasome was responsible for the removal of C-terminal tripeptide from ASNENMETM and there was lack of other activities that could efficiently remove these residues.

Because the proteasome was also involved in the generation of Sendai NP, we performed a similar analysis with a carboxyl-terminal extended version of FAPGNYPAL expressed from minigene. Unlike the findings with ASNENMETM + 1, Ag presentation from FAPGNYPAL + 1 was efficient. Moreover, the presentation of FAPGNYPAL + 1 was inhibited by β-lactone (Fig. 4*B*). Therefore, the proteasome was required to remove the C-terminal residue to generate the FAPGNYPL peptide.

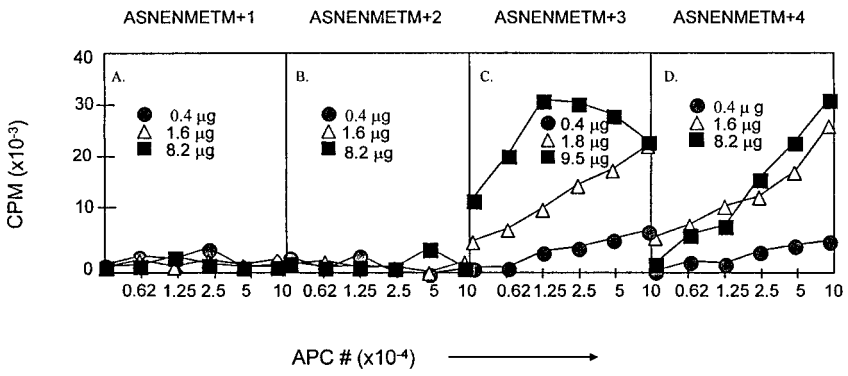

FIGURE 3. Ag presentation of ASNENMETM from constructs with C-terminal flanking residues of different lengths. APCs were infected with vTF-7 for 30 min and then transfected with the indicated dose of plasmids p. ASNENMETM + 1 (*A*), p. ASNENMETM + 2 (*B*), p. ASNENMETM + 3 (*C*), and p. ASNENMETM + 4 (*D*) for 5 h. Presentation of ASNENMETM were then measured with the 12.33 hybridoma. Data in *A*, *C*, and *D* were from the same experiment, and data in *B* were from an independent experiment. All the data were repeated at least three times.

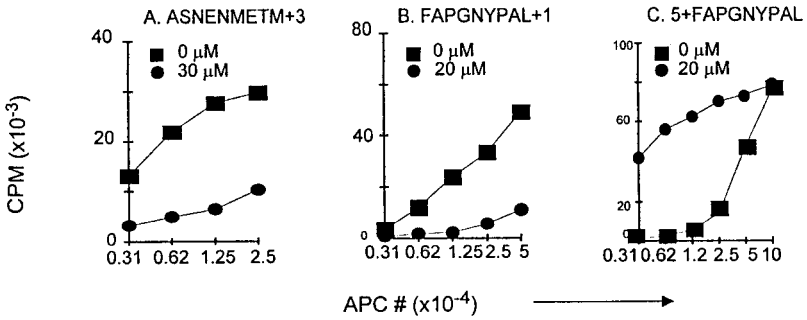

FIGURE 4. β-Lactone inhibited Ag presentation from antigenic peptides with C-terminal flanking residues. APCs (E36/D^b for *A*, E36/K^b for *B* and *C*) either with or without preincubation with β-lactone were infected with vTF-7 and then transfected with plasmids that encoded ASNENMETM with three additional amino acids (*A*) at the C terminus or with plasmids that encoded FAPGNYPAL with one additional residue at the C terminus (*B*) or with 5 additional amino acids at the N terminus (*C*). Ag presentation was then measured with the 12.33 (*A*) and B3.4D8 (*B* and *C*) T hybridomas. *A* was representative of three independent experiments. *B* and *C* were from the same experiment that was repeated twice.

Proteasome inhibitors do not block the N-terminal trimming of extended peptides

To examine the possible involvement of the proteasome in making the cleavages that define the amino termini of the presented peptides, we studied the effect of proteasome inhibitors on the presentation of constructs with N-terminal flanking residues. When we expressed a construct encoding the influenza NP epitope ASNENMETM plus a five-residue extension at its amino terminus (5 + ASNENMETM), corresponding to the sequence in influenza NP, ASNENMETM was efficiently presented on D^b. In contrast to the C-terminally extended peptide, Ag presentation from this N-terminally extended form of ASNENMETM was not blocked by β-lactone (Fig. 2*C*) at a concentration that did block presentation from the NP protein (Fig. 1*A*) and from the C-terminally extended constructs (Fig. 2*A*) in these cells. Moreover, the presentation of 5 + ASNENMETM was also resistant to the highest concentrations of β-lactone tested (72 μM, data not shown). A similar resistance to the proteasomal inhibitors was observed when a N-terminally extended version of the Sendai NP epitope, FAPGNYPAL, was expressed from a minigene. This peptide was efficiently presented from a 5 + FAPGNYPAL construct on K^b, and its presentation was not inhibited by concentrations of β-lactone that block the presentation of the C-terminally extended construct, FAPGNYPAL + 1 (Fig. 4*C*). In fact, the presentation of 5 + FAPGNYPAL was actually enhanced by β-lactone (Fig. 4), as has been described by other research groups (23, 24, 38). These data indicate that the trimming of N-terminal flanking residues is by a protease that is resistant to the proteasome inhibitor.

N-terminally extended oligopeptides are trimmed by aminopeptidases in cell extracts

The finding that Ag presentation from N-terminally extended constructs was not blocked by the proteasome inhibitor (Figs. 2*C* and 4*C*) suggested that other cellular proteases could remove these flanking residues. We previously showed that aminopeptidases could trim amino-terminal flanking residues from an extended OVA-derived construct, QLESIINFEKL, and we identified the major aminopeptidase responsible for this activity in HeLa cells as leucine aminopeptidase (32). Therefore, we further examined whether the N-terminally extended influenza and Sendai NP peptides could also be trimmed by cellular aminopeptidases. For these experiments, we synthesized oligopeptides corresponding to the influenza (VQIASNENMETM), Sendai (HGEFAPGNYPAL), and OVA (QLESIINFEKL) epitopes with three naturally occurring N-terminal flanking residues. When incubated with purified leucine aminopeptidase, all three peptides were rapidly degraded (Table II). Similarly, when these extended peptides were incubated with HeLa cell extracts that were depleted of proteasomes, the peptides were degraded. This process was inhibited to a significant (but varying) extent by the aminopeptidase inhibitor, bestatin. Thus,

Table II. *Degradation of N-terminally extended peptides by Leucine aminopeptidase or by bestatin sensitive aminopeptidase in cell extracts*[a]

	Leucine Aminopeptidase	Cell Extracts		
			Peptide Degraded by Aminopeptidase (nmol/30 min)	
Substrate	Peptide Degrade (nmol/30 min)	Peptide Degraded (nmol/30 min)	nmol/30 mins	% of total degraded peptides
QLESIINFEKL	2.0	2.2	1.0	46
Acetyl-QLESIINFEKL	0	2.8	0	0
HGEFAPGNYPAL	7.0	3.8	2.6	68
Acetyl-HGEFAPGNYPAL	0	2.0	0	0
VQIASNENMETM	3.6	2.1	0.6	29
Acetyl-VQIASNENMETM	0	1.8	0	0

[a] A total of 15 nmol of each peptide were incubated with 10 μg of HeLa extract or with 0.5 μg of pure porcine leucine aminopeptidase for 30 min at 37°C in 100 μl of buffer 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 8.5. Reactions were stopped by adding 100 μl 0.4% TFA, and the samples were subjected to reverse-phase HPLC on a 4.6 × 250 mm Vydac C18 column. The fraction degraded by aminopeptidase was defined as the amount whose hydrolysis was sensitive to bestatin. Bestatin was used at a final concentration of 200 μM to block aminopeptidase activity.

cytosolic aminopeptidases can trim the N-terminal flanking residues on all three antigenic peptides studied. Although bestatin inhibits almost completely the hydrolysis of simple amino acid-AMC substrates by leucine aminopeptidase, this agent inhibits only partially (by about 50%) the trimming of an 11-mer peptide by this enzyme (32), presumably because the longer peptide has a much higher affinity for the enzyme. Therefore, the bestatin-resistant degradation of these oligopeptides in the cytosolic extracts may represent incomplete inhibition of the aminopeptidases. Alternatively, these substrates may be degraded by other types of peptidases which are not sensitive to bestatin.

To be degraded by aminopeptidases, substrates must have a free α-amino group. Therefore, to test whether the extended antigenic peptides were being degraded by such enzymes, N-terminally extended oliogopeptides were synthesized with an acetylated N-terminal residue (Table I). As shown in Table II, the acetylated peptides were completely resistant to digestion by purified leucine aminopeptidase (Table II) and were not hydrolyzed by the bestatin-sensitive component in cytosolic extracts. These results indicate that additional cellular aminopeptidases were able to trim these N-terminally extended peptides in vitro, and the acetyl-group modification at the N terminus blocked this process.

Ag presentation of oligopeptides with blocked N termini is impaired

Because acetylation of the α-amino group of the N-terminally extended ligopeptides blocked their digestion by aminopeptidases in vitro, we investigated whether this modification would affect their presentation in vivo. The blocked and unmodified N-terminally extended oligopeptides (Table I) were introduced into the cytosol of APCs by electroporation, and their presentation was evaluated using the T cell hybridoma assay. Immediately after electroporation, none of the constructs were presented, indicating that peptides were not binding directly to class I molecules on the cell surface (Fig. 5, backgrounds). However, after a 2-h incubation, peptides were presented from all three unmodified constructs, VQIASNENMETM, HGEFAPGNYPAL, and QLESIINFEKL (Fig. 5). In contrast, presentation of acetyl-VQIASNENMETM was not detectable when 3 μg of peptide was used (Fig. 5*A*) and remained undetectable even when a larger amount of the peptide was used (Fig. 5*B*). Similarly, acetylation of the N termini of HGEFAPGNYPAL and QLESIINFEKL markedly inhibited their presentation (Fig. 5, *C–F*). The results strongly suggest that aminopeptidases play a major role in the trimming of N termini flanking sequences of these antigenic peptides in vivo.

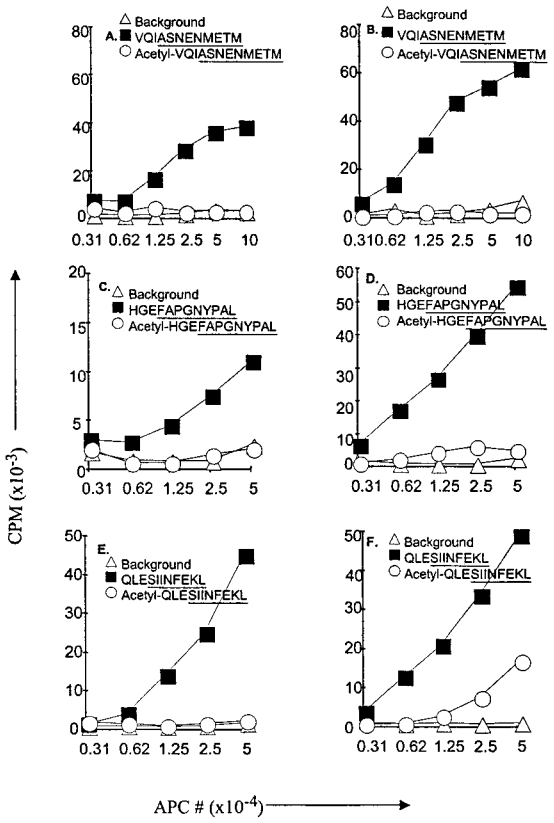

FIGURE 5. Ag presentation from oligopeptides with blocked N termini was impaired. E36/D^b APCs (*A* and *B*) and LB27.4 APCs (*C*, *D*, *E*, and *F*) were electroporated with 3 μg (*A*) or 10 μg (*B*) of VQIASNENMETM or acetylated-VQIASNENMETM peptides; 5 μg (*C*) or 20 μg (*D*) of HGEFAPGNYPAL or acetylated-HGEFAPGNYPAL peptides; and 0.2 μg (*E*) or 1 μg (*F*) of QLESIINFEKL or acetylated-QLESIINFEKL peptides, as described previously (53). Cells were then either fixed immediately (background) or after a 2-h incubation at 37°C. The specific responses were assayed with 12.33 hybridoma (for ASNENMETM) or with B3.4D8 hybridoma (for FAPGNYPAL) and RF33.70 T hybridoma (for SIINFEKL). The data for each different Ag (Flu, Sendai, and OVA) come from independent experiments. However, the data for each pair of acetylated and its corresponding unmodified peptide come from the same experiment. All experiments were repeated at least three times.

Discussion

The present studies provide further strong evidence that most MHC-presented peptides are derived from peptides produced during protein degradation within the proteasome. Using specific proteasome inhibitors, we demonstrated here that this particle is essential for the generation of the D^b-restricted peptide, ASNENMETM, from full-length influenza NP and of the K^b-restricted peptide, FAPGNYPAL, from full-length Sendai NP. Analogous studies have also indicated that proteasomes are essential for the generation of a number of antigenic epitopes including, OVA-derived SIINFEKL (8, 10), β-galactosidase-derived TPHPARIGL (39), influenza NP-derived ASNENMETM, nonstructural protein 1-derived EEGAIVGEI, hemagglutinin-derived IEGGWTGWI epitopes (12), and vesicular stomatitis virus NP-derived RGYVYQGL (40), as well as the endogenous antigenic peptides, whose binding allows MHC class I assembly to occur (8, 10). It is noteworthy that the presentation of the ASNENMETM and FAPGNYPAL from their full-length proteins was markedly inhibited by lactacystin, although the presentation of these antigenic peptides when they were directly expressed from minigenes (encoding the 9-mers) was not reduced by this agent. Therefore, proteasomal function is required for the generation of these peptides or larger precursors, and these inhibitors do not affect any subsequent steps in the presentation pathway.

More specifically, the proteasome was shown to be necessary for the generation of the C termini of ASNENMETM and FAPGNYPAL. Because proteasome inhibitors block completely the presentation of these peptides from the C-terminal extended versions, it appears that mammalian cells lack other proteases, such as carboxypeptidases, which can make the correct C-terminal cleavage necessary to generate presented peptides. Accordingly, in HeLa extracts, we failed to demonstrate any carboxypeptidase activity capable of attacking the OVA-derived peptide, SIINFEKL, or model fluorogenic peptides (32). Presumably, it is advantageous to mammalian cells because appreciable carboxypeptidase activity against such oligopeptides might remove the hydrophobic or basic C-terminal residues that are essential for peptide binding to MHC class I molecules and for transport into the ER by TAP.

In sharp contrast to these findings with the C-terminal extended peptides, the trimming of N-terminal flanking residues was completely resistant to proteasome inhibitors. Mammalian cells do contain multiple aminopeptidases (41), such as leucine aminopeptidase (42), which can sequentially remove the N-terminal residues from N-extended precursors (32). Therefore, leucine aminopeptidase or some other aminopeptidase(s) is likely to catalyze the trimming of these extended precursor peptides to generate the presented epitopes in vivo. This conclusion is also supported by the finding that IFN-γ, which promotes Ag presentation, induces leucine aminopeptidase (32), as well as those novel proteasomal subunits (LMP-2, LMP-7, etc.) that alter the particle's peptidase activities so as to potentially favor the production of peptides with C termini appropriate for MHC class I binding (19, 20). Thus, two distinct proteolytic processes can generate the C and N termini of the antigenic peptides of ASNENMETM and FAPGNYPAL, as was found previously for SIINFEKL (30) and for the vesicular stomatitis virus NP-derived peptide RGYVYQGL ($NP_{52–59}$) (40). Because similar results were obtained with four unrelated antigenic peptides, it seems very likely that the C termini of presented peptides are generally determined by proteasomal cleavages, while the N terminus can be generated by leucine aminopeptidase or some other cytosolic aminopeptidase.

Several studies have reported that the presentation of certain Ags is not blocked or is only partially reduced by proteasome inhibitors (23–25), including influenza $NP_{55–57}$ and $NP_{147–155}$ peptides (43), K^d-restricted epitopes of NP, hemagglutinin, or PB1 proteins of influenza PR8 virus (24), and a human class I-associated peptide of influenza M1 protein (38). While these findings may indicate that other cellular proteases can generate class I-presented peptides, very high concentrations of peptide aldehydes (8) or lactacystin-β-lactone (10) are needed to completely inhibit intracellular protein breakdown. In this report, we have found examples where high concentrations of the β-lactone are needed to block the presentation of some extended peptides. Therefore, some caution is needed in interpreting experiments, in which a proteasome inhibitor failed to block Ag presentation, especially when only low or moderate doses of the inhibitors were used, and where only certain of the proteasome's active sites may be inhibited (see below). In such studies, it is important that the actual extent of inhibition of protein breakdown or of proteasomal activity be measured in the cells.

The concentrations of the proteasome inhibitors needed to block Ag presentation differed widely with these different antigenic constructs. A much lower concentration of the β-lactone was needed to inhibit the presentation from full-length NPs (Fig. 1) than from the corresponding C-terminally extended oligopeptide constructs (Fig. 2). For example, $\leq$5 μM of lactacystin (Fig. 1) or clasto-lactacystin-β-lactone (data not shown) was sufficient to inhibit markedly the presentation of ASNENMETM and FAPGNYPAL from full-length proteins, whereas $\geq$20 μM of inhibitor was needed for the short constructs. These differences are not surprising, because the degradation of proteins is highly processive (44), involving many peptide-bond cleavages, and partial inhibition of these individual proteolytic cleavage steps should have additive effects in reducing the breakdown of the protein. In contrast, to generate the MHC-presented peptide from these short constructs, the proteasome needs to make only one or at most a couple of cleavages. It is also noteworthy that the concentration of clasto-lactacystin-β-lactone needed to block the C-terminal cleavage of ASNENMETM- or FAPGNYPAL-extended peptides was much higher (Figs. 2 and 4) than for SIINFEKL-extended peptides (2–5 μM) (30). Most likely, these differences may be due to these constructs being cleaved by different active sites in the proteasome, which differ appreciably in sensitivity to these inhibitors. For example, the "chymotryptic site" is particularly sensitive to lactacystin and β-lactone, while the "BrAAP" activity and the peptidylglutamyl peptide hydrolyzing activity are quite resistant to these inhibitors (6, 45). Thus, a relative resistance to lactacystin or the β-lactone may well indicate the involvement of different active sites of the proteasome. The earlier findings that high concentrations of lactacystin ($>$20 μM) are required to maximally inhibit protein degradation in intact cells are consistent with this interpretation (10).

In those examples where the proteasome is required for Ag presentation, an important issue is whether the proteasome generates peptides of the correct size or extended precursors that must be trimmed by other enzymes. Mammalian proteasomes degrade polypeptides to oligopeptides that range in length from 4 to 24 residues, although about 70% of these peptides are shorter than 8 residues and cannot function in Ag presentation (29). Approximately 10% of these peptides contain 8–10 residues, the precise length required to bind with high affinity to class I molecules. Although purified proteasomes can make the precise cuts in the full-length OVA or β-galactosidase proteins (39, 46) or in extended synthetic oligopeptides (47) to generate the correct presented peptides, these studies have used highly unphysiological conditions (e.g., incubation for 24 h and activated 20S proteasome), and therefore it is uncertain whether these results can be extrapolated to in vivo situations. By contrast, our approach, using minigene constructs and proteasome inhibitors, has allowed us to characterize where proteasomes cleave substrates in intact cells and to establish that in vivo proteasomes are essential to generate the correct C termini of many (and presumably most) antigenic peptides, but not their N termini.

The specificity of cleavage by proteasomes has been assumed to be determined by sequence preceding the scissile bond (2). One unexpected finding with these extended constructs was the marked influence of certain P′ residues on Ag presentation. Although SIINFEKL + 1 (30) and FAPGNYPAL + 1 (Fig. 4) constructs were presented readily, ASNENMETM with one or two additional C-terminal residues failed to be presented, while ASNENMETM with longer C-terminal extensions could be presented. A similar observation has been reported for another influenza NP epitope ($NP_{147–155}$), whose presentation from extended minigene constructs also required three residues of carboxyl-terminal flanking residues (surprisingly, the presentation of that peptide was not found to be inhibited by proteasome inhibitors, although it is possible that the inhibitor was not used at high-enough concentrations to block proteasome function). This surprising influence of the P3′ position on presentation may indicate that one of the proteasome's active sites must bind at least three downstream residues for activity. Alternatively, it is possible the constructs with additional residues at only the P2′ and/or P1′ positions may be much more susceptible to destruction by other cytosolic peptidases.

In all cases studied, Ag presentation from the N-terminally extended constructs was not affected by the proteasome inhibitors. These results with N-terminally extended ASNENMETM or FAPGNYPAL were almost identical with earlier ones with N-extended SIINFEKL (30) and RGYVYQGL (40). In all cases, a proteolytic activity that is resistant to β-lactone seems to trim the N termini of extended peptides. It is unlikely that this trimming process is mediated by the proteasome's peptidylglutamyl peptide hydrolyzing activity site, whose inhibition requires high concentrations of β-lactone (6). This latter site tends to prefer acidic residues in the P1 position of model peptides, which is not present in the N-extended ASNENMETM and RGYVYQGL (40). Moreover, our biochemical studies have identified bestatin-sensitive cytosolic

aminopeptidases in cells that can remove these N-terminal flanking residues (32). However, the most definitive evidence was the finding that acetylation of the N-terminal residues of three different extended peptides (Fig. 5), which blocks trimming by aminopeptidases, also inhibits Ag presentation.

The N-terminal trimming process by aminopeptidases probably can occur both in the cytosol and the ER. The first evidence for trimming in the cytoplasm came from the analysis of antigenic constructs that appear almost too long (25 + SIINFEKL, 33 residues) to be transported into the ER without removal of the N-terminal flanking region. Yet the presentation of this constructs was not blocked by proteasome inhibitors (30), suggesting a nonproteasomal activity in the cytosol. More direct evidence comes from the demonstration that the cytosolic extracts contain aminopeptidases that can remove these N-terminal flanking residues. However, this trimming process can also occur in the ER, because N-terminally extended constructs that are targeted into the ER via a signal sequence can still be trimmed and presented (30, 48, 49). Because TAP is unable to efficiently transport peptides with blocked N termini (5, 50), it is possible that the impaired presentation of our acetylated constructs (Fig. 5) was due in part to their failure to be transported into the ER. However, even if some trimming occurred in the ER, it would not alter our conclusion that the N-terminally extended sequence is removed by a nonproteasomal activity.

Our data certainly do not exclude the possibility that the proteasome also sometimes makes the cleavages that generate the proper N termini of presented peptides. When this happens, proteasome inhibitors might not block presentation because trimming is not rate-limiting (i.e., aminopeptidases are sufficient to catalyze this process) or the proteasome generates relatively few of the correct peptides. In vitro experiments with pure 20S proteasomes (47), although highly artificial, suggest that the proteasome can make the correct cleavages, and a "2-cut" model has been proposed to account for such behavior (51, 52). In addition, the isolated proteasomes also generate peptides that were too long for presentation and would require trimming for presentation (28). Presumably, the ability of the proteasome to generate either directly the presented peptide or N-extended versions depends on the nature of the N-terminal flanking sequence and their susceptibility to active sites of the proteasomes or the "immunoproteasome" variants. Therefore, it remains to be established with different proteins to what extent 26S proteasomes under in vivo conditions actually do generate N-extended precursors, which are then trimmed by cellular aminopeptidases to the presented epitopes.

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