Pathway for Degradation of Peptides Generated by Proteasomes

A KEY ROLE FOR THIMET OLIGOPEPTIDASE AND OTHER METALLOPEPTIDASES

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Tomo Saric‡, Claudia I. Graef§, and Alfred L. Goldberg¶
From the Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115

The degradation of cellular proteins by proteasomes generates peptides 2–24 residues long, which are hydrolyzed rapidly to amino acids. To define the final steps in this pathway and the responsible peptidases, we fractionated by size the peptides generated by proteasomes from β-[14C]-casein and studied in HeLa cell extracts the degradation of the 9–17 residue fraction and also of synthetic deca- and dodecapeptide libraries, because peptides of this size serve as precursors to MHC class I antigenic peptides. Their hydrolysis was followed by measuring the generation of smaller peptides or of new antigenic peptides. Their hydrolysis was followed by measuring the generation of smaller peptides or of new amino groups using fluorescamine. The 14C-labeled peptides released by 20 S proteasomes could not be degraded further by proteasomes. However, their degradation in the extracts and that of the peptide libraries was completely blocked by o-phenanthroline and thus required metallopeptidases. One such endopeptidase, thimet oligopeptidase (TOP), which was recently shown to degrade many antigenic precursors in the cytosol, was found to play a major role in degrading proteasome products. Inhibition or immunodepletion of TOP decreased their degradation and that of the peptide libraries by 30–50%. Pure TOP failed to degrade proteasome products 18–24 residues long but degraded the 9–17 residue fraction to peptides of 6–9 residues. When aminopeptidases in the cell extract were inhibited with bestatin, the 9–17 residue proteasome products were also converted to peptides of 6–9 residues, instead of smaller products. Accordingly, the cytosolic aminopeptidase, leucine aminopeptidase, could not degrade the 9–17 residue fraction but hydrolyzed the peptides generated by TOP to smaller products, recapitulating the process in cell extracts. Inactivation of both TOP and aminopeptidases blocked the degradation of proteasome products and peptide libraries nearly completely. Thus, degradation of most 9–17 residue proteasome products is initiated by endoproteolytic cleavages, primarily by TOP, and the resulting 6–9 residue fragments are further digested to amino acids by aminopeptidases.

Most intracellular proteins, both short and long lived, are degraded by the ATP-dependent proteolytic complex known as the 26 S proteasome (1). This structure generates diverse peptides, most of which range in length from 2 to 24 residues, with approximately two-thirds less than 8 residues long (2–5). Nearly all of these peptides are then rapidly degraded to amino acids in the cytosol or nucleus. However, in higher vertebrates, a small fraction of proteasome products escapes this complete destruction and is translocated from the cytosol into the endoplasmic reticulum, where they bind to MHC1 class I molecules (either directly or after N-terminal trimming) and are transported to the cell surface to serve in antigen presentation (6).

Although much attention has focused on the initial steps in the pathway for protein breakdown, the ubiquitination of polypeptides and their degradation in proteasomes, very few studies have concerned the subsequent degradation of the proteasome products to amino acids. Oligopeptides generated by proteasomes derived from cell proteins are not found in cell extracts (7, 8), because they are degraded very rapidly in vivo (9, 10). This rapid clearance of peptides released by proteasomes appears to be essential for cell viability, because this process serves several key functions. 1) The hydrolysis of these proteasome products provides amino acids for use in the synthesis of new proteins. The efficient digestion of cell proteins to amino acids must be of particular importance under conditions where the supply of exogenous amino acids is limiting. 2) An accumulation of undegraded peptide fragments of cell proteins in the cytosol could interfere with important protein-protein interactions, and such peptides could by themselves aggregate or be toxic (11, 12). 3) In mammals, the peptidases that degrade the bulk of proteasome products, by degrading potential precursors of MHC class I-presented antigenic peptides, limit antigen presentation on the cell surface and thus can affect the immune response against virally infected or transformed cells. In fact, recent studies indicate that antigenic peptides and longer precursors are quite susceptible to rapid degradation in cytosolic extracts (13, 14) and in the cytosol in vivo (9, 10, 15, 16).

Free peptides in the cytosol are short lived and are rapidly broken down to amino acids (8–10, 17). However, the responsible peptidases are largely unknown. In eukaryotes, aminopeptidases have been implicated in the final steps of intracellular proteolysis. Botbol and Scornik (18–22) have shown that treatment of various cells with the aminopeptidase inhibitor bestatin causes accumulation of short peptides of 2–5 residues, which are generated during cytosolic protein degradation. These observations
suggest that the final steps in ubiquitin-proteasome pathway involve unidentified aminopeptidases, which very rapidly digest the small proteasome products (<5 residues). Presumably, the longer peptides released by proteasomes must be first broken down to smaller fragments by other enzymes, specifically by cystolic endopeptidases or by exopeptidases insensitive to bestatin (23). One such peptidase, tripeptidyl peptidase II (TPP-II), has been shown by Reits et al. (10) to be important in the degradation of peptides longer than 15 residues and in the generation of many of the 8–10 residue antigenic peptides presented on MHC class I molecules (24). These investigators reported that in vivo peptides smaller than 15 residues are digested exclusively by aminopeptidases and not by endopeptidases (9, 10). However, this observation is not consistent with the findings of Botbol and Scornik (18–22, 25, 26) that inhibition of aminopeptidase results in accumulation only of peptides shorter than 5 residues, the latter observation suggesting that peptides 6–15 residues long are initially broken down by endopeptidases to smaller fragments that are further digested by aminopeptidases.

In addition to TPP-II, three other cystolic endopeptidases have been proposed to function in this process: the insulin-degrading enzyme, prolyl oligopeptidase, and neurolysin (23, 27, 28). However, no detailed studies of their roles have been undertaken. While studying whether potential antigenic peptides are degraded in the cytosol, we found that in the HeLa cytosol, the seven antigenic peptides studied were all rapidly degraded by a process involving two specific metallopeptidases sensitive to o-phenanthroline (14). Three of these 8–10 residue peptides were degraded rapidly by a bestatin-sensitive exopeptidase, the puromycin-sensitive aminopeptidase. However, the destruction of the five others was mediated by a single endopeptidase, thimet oligopeptidase (TOP, EC 3.4.24.15). This monomeric and highly conserved 78 kDa metallopeptidase is present in all cells and preferentially degrades peptides 6–17 residues long (28–30). In these extracts, TOP also catalyzed the degradation of N-extended variants of antigenic peptides (14), which in vivo appear to be the primary precursors of the MHC class I-presented epitopes (13, 25). These findings suggested that TOP, by destroying antigenic peptides or precursors in the cytosol, might inhibit their presentation on MHC molecules. Recent in vivo studies, in which TOP was overexpressed or eliminated using siRNA, have proven that the activity of TOP limits the extent of antigen presentation on the cell surface (15, 16).

These findings raised the possibility that TOP also catalyzes the breakdown of the bulk of proteasome products, especially those 6–17 residues long. The present studies were undertaken to test this possibility, to determine whether other peptidases also contribute to this process, and to clarify the likely sequence of actions of different peptidases in hydrolysis of these proteasome products. We demonstrate here that their degradation in the cytosol is a two-step process in which many (perhaps most) are cleaved initially by the endopeptidase TOP yielding smaller peptides, which are further digested to single amino acids by cystolic aminopeptidases.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine milk β-casein, o-phenanthroline, bestatin, and fluorescamine were purchased from Sigma. The inhibitor N-[1(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-p-aminobenzoate (Cpp-AAP-pAb) and all fluorogenic substrates used for assaying peptidase activities were purchased from Bachem (Basel, Switzerland). [14C]Formaldehyde was obtained from PerkinElmer Life Sciences. The N-terminally biotinylated decapetide library (bionit-X$_n$-COOH) was a generous gift from Dr. Z. Songyang (Baylor College of Medicine, Houston), and the random dodecapeptide library with unsubstituted termini (NH$_2$-X$_n$-COOH) was kindly provided by Dr. B. Turk and Dr. L. Cantley (Harvard Institutes of Medicine, Boston). Porcine kidney leucine aminopeptidase (LAP) was purchased from Sigma and was additionally purified by hydrophilic interaction chromatography using Butyl-Sepharose 4 Fast Flow (Amersham Biosciences). Recombinant rat thimet oligopeptidase was a kind gift from Dr. A. Barrett (Cambridge, UK), Dr. E. Ferro (Universidade de Sao Paulo, Brazil) and Dr. A. C. M. Cavargo (Instituto Butantan, Sao Paulo, Brazil), who also provided the rabbit polyclonal antiserum against TOP. Affinity-purified polyclonal anti-tripeptidyl peptidase II (TPP-II) antibody was a generous gift from Dr. B. Tomkinson (Swedish University of Agricultural Sciences, Uppsala, Sweden), and the monoclonal antibody BB12 against the insulin-degrading enzyme (IDE) was kindly supplied by Dr. R. A. Roth (Stanford University School of Medicine, Stanford, CA). Butyrolactone was a kind gift from Dr. J.-C. Schwartz (Unité de Neurobiologie et Pharmacologie de l’INSERM, Paris, France).

**Protease and TOP-II Purification**—The 20 S proteasomes and TOP-II were simultaneously purified from rabbit psoas muscle by a modification of the procedure of Kisselev et al. (3). Briefly, frozen muscle was homogenized in a buffer in four volumes of buffer A (50 mM Trias-HCl, pH 7.5, 2 mM ATP, 5 mM MgCl$_2$, 0.5 mM EDTA, 1 mM DTT, 250 mM sucrose). The homogenate was centrifuged for 15 min at 10,000 × g, and the supernatant was spun further for 60 min at 100,000 × g. The proteasomes and TOP-II in the supernatant were pelleted by centrifugation for 2.5 h at 240,000 × g in a Ti45 rotor (Beckman Coulter, Fullerton, CA) over 1 mL of cushion of 20% glycerol. Pellets were resuspended in buffer B (20 mM Tris-HCl, pH 7.5, 5 mM MgCl$_2$, 0.5 mM EDTA, 1 mM DTT, 10% glycerol), loaded onto a DEAE AffiBlue column (Bio-Rad), and the proteasomes and TOP-II were eluted with an isotonic step of 150 mM NaCl. This fraction was directly loaded on an Uno Q-12 anion exchange column (Bio-Rad Laboratories) and fractionated as described previously (3). Proteasomes and TOP-II were completely separated by this chromatographic step. The 20 S proteasomes were eluted at 240 mM NaCl, and the TOP-II at 260 mM NaCl. 20 S proteasomes were dialyzed against buffer C (50 mM Na-Hepes, pH 7.5, 1 mM DTT, 10% glycerol) and further purified to homogeneity on a heparin column (EconoPac Heparin cartridge, 5 ml, Bio-Rad) as described previously (3).

Protein fractions were concentrated by ultrafiltration, and aliquots were kept frozen at −70 °C. 20 S proteasomes eluted from the Uno Q-12 column were further purified by further gel permeation chromatography on a Superdex 75 10/300 GL column (Pharmacia). Fractions were combined and concentrated by ultrafiltration. The fluorogenic activities were measured by 15-18-fold and had more than 90% purity as determined by SDS-PAGE and Coomassie Blue staining. The proteasomes and TOP-II were stored at −20 °C. The TPP-II fractions from the Uno Q-12 column were concentrated by ultrafiltration and 0.2 mg were loaded on top of a 10-ml glycerol gradient density gradient (5%–20% glycerol in 50 mM K$_2$HPO$_4$, pH 7.6, 100 mM K$_2$HPO$_4$, pH 7.6, 1 mM DTT, and 10% glycerol), centrifuged for 20 h at 100,000 × g in a Ti45 rotor at 4 °C. Peak fractions containing 26 S proteasomes were dialyzed against buffer C (50 mM Na-Hepes, pH 7.5, 1 mM DTT, 10% glycerol) and then treated with 0.2 mg/ml TOP activity was measured at an excitation wavelength of 345 nm and an emission wavelength of 460 nm.

**Peptidase Assays with Fluorogenic Substrates**—Peptidase activities were assayed at 37 °C in a continuous assay containing 1–10 μl of each chromatographic fraction or 10–20 μg of HeLa cell extract and the specific fluorogenic substrate in a reaction volume of 500 μl. The 20 S proteasome activity was determined with 100 μM Suc-L-LYV-Amc in 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.02% SDS. The fluorescence of liberated Amc was measured in a SLM Aminco luminescence spectrometer at an excitation wavelength at 380 nm and emission wavelength at 460 nm. The activity of TPP-II was measured with the substrate AAF-Amc at 100 μM in 50 mM potassium phosphate buffer, pH 7.6, containing 1 mM DTT, 10% glycerol, 100 μM bestatin, and 10 mM MgCl$_2$. Thimet oligopeptidase activity was analyzed in buffer containing 50 mM Tris-HCl, pH 7.6, 5 mM MgCl$_2$, 0.1 mM DTT, 0.05% Brij 35, and 20 μM fluorogenic peptide substrate Mec-Pro-Leu-Gly-Pro-$\beta$-Lys(Dnp)-OH-Dnp (Mcc-PLGPK-Dnp). The fluorescent product was measured at an excitation wavelength of 345 nm and an emission wavelength of 405 nm. The activity of prolyl oligopeptidase (POP) was measured with Z-GP-Amc at a final concentration of 100 μM in buffer containing 50 mM Tris-HCl, pH 7.6, 0.4 mM NaCl, and 1 mM DTT. The activity of cytosolic aminopeptidases was determined routinely in 50 mM Hepes-KOH, pH 7.6, 2 mM MgCl$_2$, 1 mM DTT with 100 μM A-Amc or L-Amc. The levels of the insulin-degrading enzyme were measured by immunoblotting.

**Purification of Peptides Generated by Proteosomal Degradation of Casin**—Peptides were first generated by digestion of β-[14C]casein by proteasomes as described by Kisselev et al. (3), and then they were
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purified from undigested substrate and proteasomes by successive size exclusion HPLC (S.E.-HPLC) and RP-HPLC, as described below. The $\beta^\text{14C}$Casein was prepared by reductive methylation as described by Detwiler-Martin and Moore (31) and had a specific activity of $3 \times 10^7$ cpm/mg. This procedure radiolabeled the casein exclusively on lysine residues; the 95% of other amino acids (as calculated from the frequency of lysine residues in bovine $\beta$-casein) remained unmodified. Rabbit muscle 20 S proteasomes (25 pmol) were incubated with 2.15 mol (2.1 $\times$ 10$^8$ cpm) of $\beta^\text{14C}$Casein in 50 $\mu$l of 50 mM Na-Hepes, pH 7.5, 0.02% SDS, 5% glycerol. The reaction mixtures were incubated at 37 °C for different periods of time. The reactions were then stopped by cooling on ice, and the amount of substrate hydrolyzed was determined by the trichloroacetic acid precipitation assay or by SDS-PAGE and autoradiography. An aliquot of each reaction was directly analyzed by size exclusion (S.E.-)HPLC or the complete reaction mixtures were frozen and lyophilized for later analysis.

S.E. Chromatography of Degradation Products—An aliquot (5 $\mu$l) of each reaction or lyophilized samples were dissolved in 50 $\mu$l of S.E. buffer (200 mM sodium sulfate and 25% acetonitrile, pH 3.0 adjusted with phosphoric acid). 50 $\mu$l of the peptide mixture were loaded on a polyhydroxethyl aspartamide S.E.-HPLC column (200 $\times$ 4.6 mm, 200 A, PolyLC, Inc., Columbia, MD) at a flow rate of 0.125 ml/min, and fractions were collected at 0.5-min intervals. Fractions corresponding to absorbance at 280 nm were pooled. After lyophilization, the samples were dissolved in a 0.06% trifluoroacetic acid. Peptides were eluted in a single peak that contained more than 90% of the loaded radioactivity. Peptides were pooled, lyophilized, and redissolved in a 0.06% trifluoroacetic acid and was desalted on a C18 reverse phase column (20 $\times$ 2 mm, Vydac, Hesperia, CA), which was equilibrated in 0.06% trifluoroacetic acid. Peptides were eluted with a 2 min, followed by a linear gradient from 38 to 48% for the next 1.2 min, and again with an isocratic step with 48% acetonitrile for the last 2 min at a flow rate of 1.5 ml/min.

Peptides of each pool were eluted in a single peak that contained more than 90% of the loaded radioactivity. Peptides were pooled, lyophilized, and redissolved in sterile water to a final concentration of 100–200 pmol. Aliquots were subjected to RP-HPLC analysis (S.E.-HPLC, as described above). Identical elution profiles of control reactions (peptides and extracts at time 0) were obtained in a number of independent runs. The buffer components used in the degradation reactions and the presence of cell proteins did not affect the elution profile of radiolabeled peptides.

Degradation of Peptide Libraries by Cell Extracts—The HeLa extracts (10–20 μg) were incubated with 10–20 nmol of the N-terminally biotinylated decapeptide library or 5–10 nmol of the unsubstituted dodecapeptide library in 50 μl of 50 mM Hepes-KOH, pH 7.5, containing 0.1 mM DTT, and 20 μl of MCMG132. In reactions with inhibitors, the extracts were preincubated with inhibitors for 15–30 min before the addition of substrates. After incubation at 37 °C, newly formed amino groups were measured using fluorescamine (32). 5 μl of each sample were mixed with 50 μl of 0.2 M phosphate buffer (pH 6.8) and 25 μl of 0.3 mg/ml acetonitrile solution of fluorescamine. After vortexing for 5 min, 200 μl of each sample was transferred to the 96-well plate. The fluorescence was measured in the FLUOstar Galaxy plate reader (BMG Labtechnologies, Inc., Durham, NC) at the excitation of 370 nm and emission of 480 nm. The reactions with fluorescamine were performed at pH 6.8 in order to assess the contributions of endopeptidases to the degradation of peptide libraries. At this pH, the new amino groups in peptides, but not free amino acids, react with fluorescamine (32).

RESULTS

Products of Proteasome Degradation of $\beta^\text{14C}$Casein—in order to identify the intracellular peptidases that hydrolyze the large variety of peptides released by proteasomes, we first analyzed in HeLa cell extracts the effect of various protease inhibitors on the degradation of peptides generated during the digestion of $\beta^\text{14C}$Casein by 20 S proteasomes. $\beta$-Casein has been widely used as a model proteasome substrate and in studies of its products (3). Radioactive $\beta$-casein was used here to maximize the sensitivity of the assays and to follow specifically the fate of the added proteasome products independently...
of other peptides that might be generated or destroyed in the extracts. During incubation of SDS-activated 20 S proteasomes with this protein, aliquots were collected at different times and analyzed by SDS-PAGE and size exclusion HPLC (S.E.-HPLC). The column yielded a highly reproducible separation of standard peptides by size (Fig. 1A). By 10 h of incubation, most of the β-[14C]casein was digested, and this process reached completion by about 20 h as demonstrated by SDS-PAGE and autoradiography (data not shown).

In accord with prior results (3), 95% of the [14C]-labeled peptides ranged in size between 3 and 25 residues with a median size slightly longer than 8 residues (Fig. 1B). The size distribution of these products was very similar after different periods of digestion (Fig. 1B). Interestingly, the peptides released by proteasomes during this incubation were not cleaved further, even after prolonged incubations (>20 h) when all of the protein substrate was consumed (Fig. 1B). Similar results were obtained when two other substrates, reductively labeled [14C]lactalbumin and metabolically labeled [35S]calmodulin, were degraded by 20 S proteasomes (not shown). The impression that peptides, once released by proteasomes, are not further cleaved by proteasomes to any significant extent was tested by isolating the peptides generated from casein and incubating them again with pure 20 S proteasomes. As suggested by the S.E.-HPLC data, no further cleavages of these peptides could be detected using fluorescamine to measure the formation of new amino groups (not shown). These in vitro results are in good agreement with recent in vivo observations indicating that cellular peptidases must act after proteasomes to complete the process of protein degradation (9, 10).

To prepare the [14C]-peptides for use as substrates, the β-[14C]casein was incubated with proteasomes until 45% of the casein was consumed. After fractionation of the products by S.E.-HPLC, the peptides were pooled into three groups: peptides from 6–8 residues (28% of the total), ones from 9 to 17 residues (22% of the total), and ones from 18 to 26 residues (about 6%) (Fig. 1B). When rechromatographed on the same column, these size fractions were eluted at their expected positions (Fig. 1C). This grouping was based on prior reports (29, 33) that TOP preferentially degrades peptides of 6–17 residues in length, and that MHC-class I-associated antigenic peptides are 8–10 residues in length but may frequently arise from longer precursors (34–40). This grouping also was essential because it enabled the detection by S.E.-HPLC of the conversion of the peptides of 9–17 and 18–26 residues to smaller fragments by cell extracts and/or pure peptidases. However, the 6–8 residue pool and peptides smaller than 6 residues were too small for analysis of their degradation by this approach and were not used further.

Degradation of 9–17 Residue Peptides in HeLa Extracts Requires Metallopeptidases—In HeLa extracts many antigenic peptides and longer precursors were degraded by metallopeptidases sensitive to the heavy metal chelator, o-phenanthroline (14), which inhibits many aminopeptidases and several en-
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**Thimet Oligopeptidase Contributes to Degradation of Proteasome Products**—The cytosolic metallopeptidase found to be responsible for breakdown of most antigenic peptides was the endopeptidase, TOP (14). To determine if TOP is also involved in degradation of proteasome products generally, the 9–17 residue peptides from β-[14C]casein were incubated in HeLa extracts in the presence of the selective TOP inhibitor, Cpp-AAF-pAb. In control experiments, this agent at 10 μM blocked TOP activity in extracts against the specific fluorogenic substrate, Mcc-PLGP-Dnp by 84%. The remaining activity was presumably due to action of the closely related metallopeptidase, neurolysin, which also cleaves this substrate (see “Experimental Procedures”) (14). At this concentration, Cpp-AAF-pAb blocked the breakdown of the 9–17 residue fraction to smaller peptides by about 50% (Fig. 2A). Because many peptides normally degraded by TOP are hydrolyzed by some alternative route under these conditions, this decrease in peptide hydrolysis (up to 50%) must underestimate the actual contribution of TOP to hydrolysis of proteasome function. Thus, TOP appears to be important for the initial cleavage of a large fraction, probably most but not all, of 9–17 residue peptides. These findings also indicate that the smaller peptides degraded in the absence of TOP are hydrolyzed by other peptidases in the cytosol. At the concentration used, Cpp-AAF-pAB can also partially inhibit neurolysin (28). However, neurolysin has a 100-fold lower affinity than TOP for this inhibitor, and only small amounts of this enzyme could be demonstrated in HeLa extracts, as assessed by immunoblotting and immunodepletion (see “Experimental Procedures”).

**Pure TOP Cleaves 9–17 Residue Proteasome Products**—These findings strongly suggest that in the cytosol, TOP converts the longer fraction of proteasome products to smaller peptides. To further test this possibility, we determined the susceptibility of the casein-derived peptides (9–17 and 18–26 residues) to pure recombinant TOP. The 9–17 residue peptides were readily degraded by TOP. TOP generated fragments of mainly 6–9 residues long (Fig. 3B), and the inhibitor Cpp-AAF-pAb at 10 μM blocked this shift in peptide size by the pure enzyme (supplemental Fig. 1B). Higher concentrations of pure TOP caused no further degradation of the 9–17 residue peptides (Supplemental Fig. 1A). Therefore, this endopeptidase appears to perform only limited endoproteolytic cleavages, and other peptidases are necessary to complete the degradation of these endoproteolytic products to amino acids. In analogous experiments pure TOP degraded only a small fraction (<10%) of peptides in the 18–26 residue pool, and the peptides generated...
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| Inhibitor            | Peptidase-targeted | Concentration | Fluorogenic substrate | 10-mer library | 12-mer library |
|----------------------|--------------------|---------------|-----------------------|----------------|----------------|
| o-Phenanthroline     | Metallopeptidases  | 2 mM          | 70                    | 100            |                |
| PMSF                 | Serine peptidases  | 100 μM        | 12                    | 0              |                |
| E64                  | Cysteine peptidases| 100 μM        | 3                     | 4              |                |
| Pepstatin A          | Aspartic peptidases| 5 μM          | 0                     | 2              |                |
| Cpp-AAF-pAb          | Thimet oligopeptidase| 10 μM        | 86                    | 32             | 38             |
| Z-Pro-L-Proinal      | Prolyl oligopeptidase| 50 μM        | 91                    | 0              | 0              |
| Butabindinea         | Tripeptidyl peptidase II | 1 μM        | 97                    | NDb            | 0              |
| Ultracentrifugationc| Tripeptidyl peptidase II | 85          | ND                    | 0              | 0              |

a The effect of TPP-II inhibitor, butabindine, on degradation of peptide libraries was assayed in crude HeLa extract (20,000 × g, 5 min supernatant), which was not depleted of TPP-II.

b ND, not determined.
c The centrifugation at 250,000 × g for 20 min depleted the 85% of TPP-II from HeLa extract, but did not affect the degradation rates of peptide libraries.

Degradation of synthetic peptide libraries in HeLa cytosol is mediated largely by thimet oligopeptidase

HeLa cytosol (250,000 × g, 20 min) was preincubated for 15–30 min in 50-μl reactions with inhibitors and with 10 μM MG132 to eliminate contribution of proteasomes. The N-biotinylated decapetide library (bixin-X₈Q₄-COOH) or the unsubstituted dodecapeptide library (NH₂-X₁₂-COOH) was added, and the reactions were incubated at 37°C for 2 h. The reactions were stopped by chilling on ice, and the amount of new amino groups generated was determined with fluorescamine. The effect of inhibitors on specific peptidases was determined in a continuous assay with the corresponding fluorogenic substrates. The values for percent inhibition are representative of results obtained in several independent experiments.

Degradation of proteasome products above.

Degradation of 9–17 residue proteasome products, this TOP-independent hydrolysis could be caused by the slower breakdown of these peptides by aminopeptidases (see Fig. 3A). Therefore, we analyzed the effects of simultaneous inhibition of TOP by Cpp-AAF-pAb and aminopeptidases by bestatin on their degradation in HeLa extract. As shown in Fig. 3D, simultaneous inactivation of TOP and aminopeptidases had additive effects and profoundly inhibited the degradation of 9–17 residue peptides. Thus, much of the TOP-independent hydrolysis is probably due to the action of cytosolic aminopeptidases (such as puromycin-sensitive aminopeptidase and leucine aminopeptidase). However, this combination of inhibitors did not completely abolish their degradation as occurred upon addition of o-phenanthroline (see Fig. 2). Thus, although TOP plays a major role, an additional metallopeptidase(s) (presumably an endopeptidase or bestatin-insensitive exopeptidase) also contributes to this degradative process. This conclusion was corroborated by data obtained with peptide libraries (see Table I).

Degradation of Deca- and Dodecapeptide Libraries in HeLa Extract Is Mediated by TOP and Not by TPP-II—In order to characterize the other peptidases that may hydrolyze proteasome products, we used a more sensitive and more quantitative assay based on the ability of fluorescamine to react with newly formed amino groups generated by peptide bond cleavage to yield a highly fluorescent adduct. In these assays, we used as substrates a synthetic decapetide library with N termini biotinylated and a dodecapeptide library with unblocked N termini. These highly heterogeneous libraries are easier to generate in high amounts than the proteasome products.

Our experiments with the [β-1⁴C]casein-derived peptides and with antigenic peptides (14) showed that metallopeptidases, both endo- and exopeptidases, catalyze the degradation of these substrates. To test if the synthetic peptide libraries are hydrolyzed by the same enzymes in the extract, we assayed their degradation using fluorescamine (32). Under conditions where the generation of new amino groups from these peptides occurred at linear rates, the o-phenanthroline blocked the cleavage of the biotinylated decapetides by 70% and the unmodified dodecapeptides by 100% (Table I). In contrast, the inhibitors of serine (phenylmethylsulfonyl fluoride), cysteine (E64), and aspartic proteases (pepstatin A) caused little or no reduction in hydrolysis of these peptides (Table I). Therefore, metallopeptidases appear to be primarily responsible for the breakdown of these model substrates, as was found with proteasome products above.

Other Enzymes Probably Contribute to Degradation of Proteasome Products—Although our data suggest that some other endopeptidase(s) besides TOP can also contribute to degradation...
To determine if TOP is responsible for the degradation of the deca- and dodecapeptide libraries, as it is of antigenic- (14) and casein-derived proteasomal products, these libraries were incubated with HeLa extracts in the presence of the specific inhibitor, Cpp-AAF-pAb. This agent at 10 μM decreased the cleavages of both biotinylated and unsubstituted peptide libraries by 33–39% (Table I). This incomplete inhibition implies that TOP is important in this process, but that other metalloendopeptidase(s) can also play a role in the cleavages of these model substrates, in agreement with our observations made when proteasome products or antigenic peptides were the substrates. The specific inhibition of the serine peptidase, POP, had no effect on degradation of the peptide libraries (Table I). Similarly, the inhibition in crude HeLa extract of another serine peptidase, TPP-II, by butabindide (1 μM) or the depletion of TPP-II by prolonged ultracentrifugation did not affect the degradation of the dodecapeptide library with free N termini, a requirement for cleavage by TPP-II (Table I). Thus, TOP, but not POP or TPP-II, plays a major role in degradation of decas- and dodecamers. This conclusion was corroborated by our observation that pure TOP degraded the decapeptide library and that the TOP inhibitor, Cpp-AAF-pAb, and α-phenanthroline blocked this process nearly completely (data not shown) and is supported by recent in vivo data suggesting that TPP-II is mostly responsible for degrading peptides larger than 15 residues but not for the shorter ones (10).

Further Evidence for the Involvement of TOP in Degradation of Peptide Libraries—To define the responsible peptidases, we prepared extracts immunodepleted of TOP or of another cytosolic metalloendopeptidase of broad substrate specificity, the insulin-degrading enzyme, which has been proposed to play a role in this degradative pathway (27). Immunodepletion of TOP with a polyclonal antiserum that specifically recognizes TOP, but not the related enzyme neurolysin (EC 3.4.24.16) (41), completely depleted TOP from the cytosol (Fig. 4A). This antibody did not affect the activities or levels of aminopeptidases, neurolysin, prolyl oligopeptidase or TPP II, as determined with specific fluorogenic substrates or Western blotting (not shown). Immunodepletion of TOP reduced the degradation of the biotinylated decapeptide library by 33% (Fig. 4B) and the unmodified dodecapeptide library by 57% (not shown). By contrast, complete immunodepletion of insulin-degrading enzyme had no effect on the degradation of the dodecapeptide library in HeLa extract (not shown). Thus, both inhibitor and immunodepletion studies indicate that TOP is a major enzyme responsible for degradation of these substrates, although another α-phenanthroline-sensitive peptidase distinct from the insulin-degrading enzyme also appears to contribute to this degradative process (as also shown with 9–17 residue proteasome products).

DISCUSSION

During protein degradation in eukaryotic cells proteasomes continually generate oligopeptides ranging from 3 to 24 residues. However, due to their size, these peptides are not likely to be captured by cells unless hydrolyzed to amino acids. In agreement with these observations, in the present experiments the peptides released by 20 S proteasomes were not cleaved further by 20 S proteasomes at measurable rates (Fig. 1B), apparently because the great majority of the bonds in the protein most susceptible to the proteasome’s active sites were already hydrolyzed. Even if the peptides once released by proteasomes could reenter these particles, they are unlikely to compete efficiently with proteins, which are highly preferred as substrates. It is also noteworthy that many types of peptides are inherently resistant to the proteasome’s active sites (42). Finally, as shown here, the intracellular exo- and endopeptidases degrade proteasomal products at much higher rates than proteasomes, since inhibition or removal of the proteasomes from the extracts did not slow significantly the breakdown of these oligopeptides. Together, these observations make it very unlikely that proteasome products reenter these particles for further processing to antigenic peptides (43) or that proteasomes in vivo digest proteins mainly into fragments larger than 15 amino acids (10), as has been proposed by some investigators.

To identify the peptidases responsible for degradation of proteasome products, we focused on the fate of peptides 9 residues or longer, because they may serve as precursors for MHC class I antigen presentation. Prior in vivo studies had suggested that the proteasome products shorter than 6 residues are degraded by aminopeptidases (18–20, 25, 26) and that ones over 15 residues in length require TPP-II for their degradation (10). We showed here that degradation of 9–17 residue peptides in cell extracts requires metalloendopeptidases (Figs. 2 and 3 and Table I), which is in agreement with our earlier findings that such enzymes catalyze destruction of antigenic peptides and their N-extended precursors (14). Thus, degradation of 9–17 residue peptides consists of at least two steps. First, these peptides are cleaved by endopeptidases to intermediate-sized products (Fig. 3, A and B), which are then degraded by bestatin-sensitive aminopeptidases to amino acids (Fig. 3, B–D).

The zinc-containing enzyme, thimet oligopeptidase, appears to be the metalloendopeptidase responsible for many, probably most, of these initial endopeptidolytic cleavages in HeLa cells (Figs. 3, A and B and 4 and Table I). The biological function(s) of this enzyme have long been unclear. TOP was initially discovered by Camargo (44, 45) and Orlowski (46) and their co-
workers by its ability to degrade a variety of circulating peptides and has therefore been assumed to function primarily in the catabolism of neuropeptides. However, it is very unlikely that its primary role is in metabolism of extracellular peptides, since TOP is localized in most cells primarily in the cytosol and nucleus, and at most only about 5% of cellular TOP is found on the surface of neuroendocrine cells (47). Instead, as shown here, the TOP major function is most probably in the hydrolysis of proteasome products. Like the proteasome, TOP is ubiquitously distributed in tissues and has broad sequence specificity. TOP cleaves almost exclusively peptides 6–17 residues long (29, 30, 33). Recently, the crystal structure of human TOP has been solved; its active site is located at a base of a deep channel that probably excludes long peptides from degradation (48). The 6–17 residue long substrates of TOP correspond to the sizes of about 50% of proteasome products (3), and the fraction found here to be degraded primarily by TOP (Fig. 3, A and B). TOP is a highly conserved enzyme and close homologs are present in bacteria (49) and in yeast (50). The yeast enzyme, oligopeptidase yscD (EC 3.4.24.37), and TOP have 35% amino acid sequence identity, similar inhibitor susceptibilities, and similar preferences for peptide bonds in several substrates (50). Interestingly, yeast mutants lacking yscD accumulate soluble peptides in their cytosol (50), which suggests a role in degrading proteasome products.

Together, these various findings indicate a key role of TOP in protein degradation in the nucleus and cytosol after the proteasome. This broad specificity means that it can hydrolyze the highly diverse peptides generated by 20 S proteasomes (Fig. 3, A and B), the highly degenerate peptide libraries (Fig. 4 and Table I), as well as many MHC class I presented antigenic peptides and their N-extended precursors (14). Moreover, related studies by our group (15) and others (16) have shown that in cultured cells TOP destroys antigenic peptides in the cytosol, limiting their presentation to the immune system. These findings are not consistent with the proposal that cytosolic TOP binds antigenic peptides but is unable to digest them and can even protect them from cytosolic degradation (53, 54).

The present observations indicate that the enzymes that degrade proteasome products in eukaryotic cells are not part of a very large complex. In extracts of the Archaea Thermoplasma acidophilum, the breakdown of proteasome products is mediated by the tricorn protease, a giant endopeptidase, which is associated with several aminopeptidases (55). Despite appreciable effort, we were unable to find any evidence for the existence of a similar large complex in extracts of HeLa cells or rabbit muscles. Instead, the analysis of these extracts revealed that the enzyme most active in degrading the decapetide library and the antigenic peptide SIINFEKL had an apparent molecular mass of 70–80 kDa similar to TOP. Although very important, TOP is not the only endopeptidase cleaving peptides released by the proteasome. Additional metalloendopeptidases must contribute, since o-phenanthroline almost completely blocked the degradation of the 9–17 residue peptides and the peptide libraries but other types of protease inhibitors were ineffective (Fig. 2 and Table I). These findings exclude a significant role for cytosolic serine proteases (e.g. TPP-II) or cysteine peptidases (e.g. bleomycin hydrolase) in this process. Accordingly, inhibition of TPP-II and of prolyl oligopeptidase did not slow the degradation of unmodified dodecapeptide library by the extracts (Table I). Accordingly, the degradation of peptides smaller than 15 residues was not affected by TPP-II in intact cells (10). However, TPP-II appears to be important for the breakdown of longer proteasome products (>15 residues) (10), a size range that is not susceptible to degradation by TOP. Also, we have found that pure TPP-II degraded the 18–26 residue proteasome products better than 9–17 residue peptides.2

Only a few metalloendopeptidases in eukaryotic cells have been characterized that can act on peptides. Neurolysin (EC 3.4.24.16) is a ubiquitous enzyme very similar to TOP in structure, tissue distribution, specificity, and inhibitory profile (48, 56–58), and is present in the cytosol, nucleus, and mitochondria (59). However, neurolysin has about a 100-fold lower affinity than TOP for the inhibitor Cpp-AAF-pAb (28), and the concentration that reduced degradation of proteasome products by 50% (10 μM) does not markedly inhibit neurolysin. In addition, DTT (0.5 mM) did not affect the degradation of proteasome products in HeLa extracts,2 though it inhibits neurolysin but not TOP (60). Moreover, selective immunodepletion of TOP with specific antibodies had similar effects on peptide degradation as Cpp-AAF-pAb. Thus, neurolysin is unlikely to contribute significantly to the peptide hydrolysis studied here, although it may account for the TOP-independent activity in these extracts. Possibly, in certain tissues that express low levels of TOP, neurolysin could play a more important role in degrading proteasome products. In addition, since neurolysin is also present in mitochondria, and since proteases in the mitochondrial matrix degrade proteins to amino acids (61), neurolysin might function to hydrolyze peptides generated by mitochondrial ATP-dependent proteases in an analogous way to TOP in the cytosol.

The insulin-degrading enzyme (EC 3.4.24.56) is another highly conserved metalloendopeptidase (62). All known IDE substrates are peptides longer than 20 residues, which comprise only a very small fraction of the total proteasomal products (3). In the present studies, IDE was not found to play a role in the degradation of dodeca- or decapetide libraries (Table I) or antigenic peptides (14) in HeLa extracts. A related cytosolic peptidase, nardilysin (EC 3.4.24.61), has restricted cleavage specificity for doublets of basic amino acids, but like IDE, it prefers substrates larger than most proteasome products (63). A newly described enzyme, termed endo oligopeptidase A, has broad substrate specificity but is expressed predominantly in the brain (64). These enzymes may also degrade a fraction of proteasome products that cannot be hydrolyzed by TOP or TPP-II (Fig. 3C), but it is likely that some unidentified endopeptidase functions with TOP in the initial degradation of 9–17 residue proteasome products.

It is unfortunately impossible to quantify precisely the relative contributions of TOP or any unidentified enzyme in the breakdown of proteasome products. Proteasome-generated peptides from a single protein must vary widely in sequence and properties, and all cells must have evolved enzymes capable of insuring rapid digestion of each. Degradation of some relatively rare peptide sequences may require a specific peptidase (e.g. prolyl endopeptidase), which would appear unimportant in the present studies of most proteasome products. Also, more typical peptides can probably be degraded by multiple routes. So, even if a peptide is hydrolyzed primarily by TOP in the extracts, when this enzyme is inhibited or removed, another endopeptidase or exoproteolytic degradation by aminopeptidases may assume this function. Another inherent barrier to quantifying the relative contributions is that these different enzymes can function in a synergistic fashion by generating substrates for each other. Furthermore, proteasome products are present in cells at low concentrations and the relative contributions of different peptidases to their degradation must depend on their concentrations and on the enzyme affinities for these peptides. To mimic in vivo conditions, radiolabeled proteins were used as the source of 20 S proteasome substrates in order to achieve high sensitivity in

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assays. Using this approach, we could not follow the degradation of these peptide substrates all the way to amino acids, because these substrates were radioactively labeled only on lysines and only 5% of peptides in bovine β-casein would contain lysines. Although further degradation of some peptides might have been prevented by lysine methylation and though the detection of free lysine was not possible by gel filtration, these issues do not prevent breakdown of microinjected synthetic peptides modified with fluorescent and quenching group, which may make them relatively resistant to cleavage by TOP or other endopeptidases, Botbol and Scornik bas their conclusions on analysis of the breakdown of endogenous peptides generated in the process of natural protein degradation.

In our studies, TOP was required for hydrolysis of at least 30–50% of 9–17 residue peptides, and the simultaneous inhibition of TOP and aminopeptidases had additive effects, and blocked degradation of these peptides by 80–90%. Thus, it is likely that both endo- and aminopeptidases contribute to peptide bond hydrolysis in this size fraction. Most cytosolic exopeptidases are very sensitive to bestatin and are ubiquitously distributed. Some have restricted substrate specificities (e.g. aminopeptidase B), while others (e.g. leucine aminopeptidase) can catalyze the degradation of diverse short peptides. It is also noteworthy that cellular extracts lack carboxypeptidase activity (8–10). Therefore, these various aminopeptidases together account for the rapid elimination of short proteasome products (2–5 residues and also some longer peptides), while most longer protein fragments must first be cleaved by endopeptidases, such as TOP (peptides 8–17 residues) or TPP-II (peptides over 15 residues in length), before digestion by aminopeptidases. Fig. 5 summarizes our present understanding of the different pathways for metabolism of proteasome products based on the current findings.

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**Fig. 5. Summary of pathways for degradation of proteasome products in mammals.** Intracellular proteins are degraded by proteasomes to peptides ranging from 3–24 amino acids (aa), nearly all of which are degraded in the cytosol to single amino acids. The smallest products (2–6 residues) and some larger peptides are directly degraded by aminopeptidases. Larger peptides are primarily cleaved by endopeptidases to shorter pieces, which are then further hydrolyzed by aminopeptidases. TOP appears of particular importance for peptides of 9–17 residues in length. The enzyme primarily responsible for degrading the longest products (>15 residues) appears to be TPP-II (10) but other peptidases (e.g., the insulin-degrading enzyme) may also be involved. A very small fraction of proteasome products escapes destruction in the cytosol and is utilized for MHC class I antigen presentation or without additional processing by aminopeptidases in the cytosol, such as LAP or TPP-II (10, 24), and/or in the endoplasmic reticulum by the recently identified aminopeptidase ERAP1 (34, 35, 65).
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Pathway for Degradation of Peptides Generated by Proteasomes: A KEY ROLE FOR THIMET OLIGOPEPTIDASE AND OTHER METALLOPEPTIDASES
Tomo Saric, Claudia I. Graef and Alfred L. Goldberg

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