Major Histocompatibility Complex Class I-presented Antigenic Peptides Are Degraded in Cytosolic Extracts Primarily by Thimet Oligopeptidase*

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Nearly all peptides generated by proteasomes during protein degradation are digested rapidly to amino acids, but a few proteasomal products escape this fate and are presented to the immune system on cell surface major histocompatibility complex class I molecules. To test whether these antigenic peptides may be inherently resistant to cytosolic peptidases, six different antigenic peptides were incubated with HeLa cell extracts. All six were degraded rapidly by a process involving o-phenanthroline-sensitive metallopeptidases. One antigenic peptide, FAPGNYPAL, was rapidly destroyed in the extracts by a bestatin-sensitive exopeptidase, apparently by the puromycin-sensitive aminopeptidase. The disappearance of the other five was reduced 30–90% by a specific inhibitor of the cytosolic endopeptidase, thimet oligopeptidase (TOP) (EC 3.4.24.15), whose physiological function(s) have been unclear and controversial. All these peptides were sensitive to pure recombinant TOP. Furthermore, upon fractionation of the extracts, the major peptidase peak that degraded the ovalbumin-derived epitope, SIINFEKL, co-purified with TOP. In the extracts, TOP also catalyzed rapid degradation of N-extended variants of SIINFEKL and of other antigenic peptides, which in vivo can serve as precursors of these major histocompatibility complex-presented epitopes. This enzyme (unlike cell proteins that promote production of antigenic peptides) is not regulated by interferon-γ. TOP seems to be primarily responsible for the rapid breakdown of antigenic peptides in cytosolic extracts, and our related studies (A. X. Y. Mo, K. Lemerise, W. Zeng, Y. Shen, C. R. Abraham, A. L. Goldberg, and K. L. Rock, submitted for publication) indicate that TOP by destroying such peptides limits antigen presentation in vivo.

In higher vertebrates, an important function of intracellular protein degradation is to generate the small fragments of cell and foreign proteins that are presented to the immune system on surface MHC class I molecules (1–4). Most of these 8–10 residue peptides are generated by 26S proteasomes during proteolysis by the ubiquitin-proteasome pathway (5–10). Accordingly, inhibitors of the proteasome block MHC class I antigen presentation and suppress T cell responses against various antigens (5–7). Once generated, the antigenic peptides are transported by the TAP complex from the cytosol into the endoplasmic reticulum (ER), where they bind to MHC class I molecules and are then transported to the cell surface (1, 11).

In degrading polypeptides, proteasomes generate peptides ranging from 3 to 25 residues in length (12, 13). Such peptides however, cannot be found in the cytosol (14), because they are rapidly hydrolyzed to amino acids. Efficient breakdown of proteasome products is essential to supply amino acids for the synthesis of new proteins, but rapid breakdown of these peptides is probably also important to prevent the build up of protein fragments that might interfere with critical protein-protein interactions in the cell. The enzymes responsible for this rapid hydrolysis of proteasome products have not been identified, nor is it clear how the MHC-presented peptides can escape this degradative fate and serve in antigen presentation. In fact it is presently unknown whether antigenic peptides, once formed, are inherently stable in the cytosol or are susceptible to the peptidase(s) that degrade completely the great majority of proteasome products.

One theoretical possibility is that these antigenic peptides are inherently resistant to cytosolic peptidases. Alternatively, formation of a complex between antigenic peptides and chaperones might in principle protect them from further degradation and may even help to shuttle them to the TAP complex. It has been proposed that antigenic peptides may be stabilized in the cytosol by binding to the heat shock proteins, Hsp70 and Hsp90 (15). In fact, longer versions of an antigenic peptide have been found to be associated in the cell extracts with an unidentified high molecular weight protein, apparently distinct from Hsp70 (16). It has also been proposed that antigenic peptides may escape destruction in the cytosol, because they may be generated by proteasomes attached to the TAP complex and transported directly to the ER (17); however, many investiga-

1 The abbreviations used are: MHC, major histocompatibility complex; TOP, thimet oligopeptidase; PSA, puromycin-sensitive aminopeptidase; TPP-II, tripetidyl peptidase II; Mcc, 7-methoxycoumarin-3-carboxylyl; Dnp, 2,4-dinitrophenyl; Cpp, C-p-n-carboxy-3-phenylpropyl; pAb, p-aminoanbenzate; Amc, 7-amino-4-methylcoumarin; PMSF, phenylmethylsulfonfluoride; TAP, transporter associated with antigen processing; ER, endoplasmic reticulum; HPLC, high performance liquid chromatography; IDE, insulin-degrading enzyme; Flu NP, influenza virus nucleoprotein; Cbz, benzoxycarbonyl.
tors have tried, but failed to demonstrate such an association of TAP and proteasomes.

There is also clear evidence that antigenic peptides can be modified by cytosolic exopeptidases. It has long been assumed that the proteasome releases the mature epitopes directly (18, 19). However, there is growing evidence that antigenic peptides can be released by 26 S proteasomes as longer precursors that undergo trimming in the cytosol or ER to yield the presented epitopes. If introduced in the cytosol, such N-extended versions can be efficiently trimmed to the presented epitope by cytosolic exopeptidases (16, 20–23). In recent studies, pure proteasomes have been shown to generate from ovalbumin primarily longer versions of the immunodominant epitope SIINFEKL, which contain 1–8 additional residues on its N terminus (24). It is also noteworthy that interferon-γ, which stimulates class I presentation, induces specialized forms of the proteasome (often termed immunoproteasomes), which have been found to produce these N-extended versions of antigenic peptides at increased rates (24), and also have been shown to induce the leucine aminopeptidase, the cytosolic peptidase most active in trimming such longer precursors to the MHC-presented epitopes (21, 25). Therefore, peptide metabolism in the cytosol can be an important factor determining the extent of antigen presentation.

The present studies were undertaken to learn whether antigenic peptides and also N-extended precursors, like other proteasome products, are susceptible to proteolytic digestion in the cytosol. We show here that such peptides are quite labile in cell extracts and that a key enzyme responsible for degradation of most, but not all, peptides studied is the endopeptidase, thimet oligopeptidase (TOP, EC 3.4.24.15) (26). The physiological function(s) of this enzyme have long been unclear. Although primarily cytosolic, this peptidase may also exist in membrane-associated forms in certain cells (27), and a variety of roles for it have been suggested, especially in neuropeptide metabolism (28–30). Surprisingly, TOP has been reported to bind tightly antigenic peptides, but not to degrade them (31). Therefore, it was proposed that the binding of antigenic peptides to TOP may protect them from intracellular destruction (31, 32). On the contrary, the present studies and our related in vivo experiments,2 indicate that this enzyme destroys antigenic peptides in the cytosol and thus is an important new factor that limits the efficiency of antigen presentation in vivo.

EXPERIMENTAL PROCEDURES

Reagents—A variety of known antigenic peptides, whose presentation has been extensively studied in vivo, were examined. Peptides were synthesized by Macromolecular Resources (Colorado State University, Fort Collins, CO) or the Dana Farber Cancer Research Institute core facility (Boston, MA) and were at least 90% pure by HPLC analysis. The following peptides were examined: Bss2, a basic epitope of the influenza virus neuraminidase (Toronto, Canada); LAS, a modified influenza virus neuraminidase (Toronto, Canada); HAA, a basic epitope of the influenza virus neuraminidase (Toronto, Canada); and N218, a basic epitope of the influenza virus neuraminidase (Toronto, Canada). The fluorogenic peptidases was determined with 100 μM Z-GP-Amc at 25°C.

Immunodepletion of TOP and the Insulin-degrading Enzyme (IDE)—The tissue culture cell line HeLa S3 was obtained from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal calf serum and antibiotics. HeLa S3 cells were grown in 100-mm dishes containing 10 ml of culture medium and were treated for different periods of time with 150 units/ml interferon-γ, as detailed in figure legends.

Preparation and Fractionation of Cell Extracts—Cells from confluent cultures were washed twice with ice-cold phosphate-buffered saline, pH 7.4, and were removed into a homogenization buffer by using a cell lifter. Cells were homogenized in a Dounce homogenizer and by vortexing. The cytosolic fraction was obtained by centrifugation of the homogenates for 10 min at 20,000 × g and for 1 h at 100,000 × g. Most of the proteasomes were removed by an additional 6-h centrifugation at 100,000 × g. All extracts were stored at –80 °C until use. The residual proteasomal activity in the extracts was inhibited by pre-incubation with 20 μM MG132 for 15 min at room temperature. Protein concentration in the extracts was determined with the Coomassie Plus protein assay reagent (Pierce).

Fractionation of 0.5 mg of HeLa cell extract (100,000 × g, 6-h supernatant) was performed by ion exchange chromatography on a 1-ml MonoQ 5/5 column (Amersham Pharmacia Biotech, Uppsala, Sweden) in 50 mM Tris-HCl buffer, pH 7.5, 5 mM β-mercaptoethanol, and 0.05% Brij 55. Bound proteins were eluted with a 20-min linear gradient from 0 to 0.5 M sodium chloride and with a flow rate of 1 ml/min. The protein elution profiles were measured at 280 nm, and fractions of 0.5 ml were collected for further analysis.

Peptidase Assays—Hydrolysis of fluorogenic peptide substrates was measured in a continuous assay at 37 °C with 10 μg of HeLa cell extract and the specific fluorogenic substrate in a 500-μl volume of 50 mM Tris-HCl buffer, pH 7.6, 1 mM MgCl2, 0.1 mM ATP, 1 μM dithiothreitol, and 250 mM sucrose. Extracts were prepared by centrifugation of the homogenates for 10 min at 20,000 × g and for 1 h at 100,000 × g. Most of the proteasomes were removed by an additional 6-h centrifugation at 100,000 × g. All extracts were stored at –80 °C until use. The residual proteasomal activity in the extracts was inhibited by pre-incubation with 20 μM MG132 for 15 min at room temperature. Protein concentration in the extracts was determined with the Coomassie Plus protein assay reagent (Pierce).

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HPLC Analysis of Peptide Degradation—Five nmol of the antigenic peptides were incubated at 37 °C for various times with 10 μg of HeLa cell extract and the specific fluorogenic substrate in a 500-μl volume of 50 mM Tris-HCl buffer, pH 7.6, 1 mM MgCl2, 0.1 mM ATP, 1 μM dithiothreitol, and 250 mM sucrose. The tripeptidyl peptide II (TIP-II) was assayed with 100 μM AAF-Amc in the presence of the aminopeptidase inhibitor bestatin. The activity of cytosolic aminopeptidases was determined with 100 μM A-Amc or L-Amc.

Immunodepletion of TOP and the Insulin-degrading Enzyme (IDE)—In order to immobilize antibodies to Protein A-Sepharose, the 25 μl of settled resin was incubated with pre-immune serum or 20 μl of rabbit TOP antiserum for 40 min at room temperature in 300 μl of immunodepletion buffer (50 mM Hepes, pH 7.6, containing 140 mM NaCl, 10 mM KCl). Serum proteins were washed away, and 400 μg of HeLa cytosol (300 μl) was added to each resin. After continuous mixing for 2 h at 4 °C, resins were removed by quick centrifugation at 20,000 × g for 15 s. To ensure complete immunodepletion...
tion of the enzyme, the HeLa cytosol from the first round of immunodepletion was added to a second set of Protein A-Sepharose resins pre-adsorbed with the non-immune or TOP antiserum. These samples were incubated for 1 h at 4 °C. After removing the resin by centrifugation, HeLa cytosol was transferred to new tubes and stored at −80 °C until analysis.

The immunodepletion of the IDE was performed by pre-incubating 400 μg of HeLa cytosol with 5 μg of IDE monoclonal antibodies 9B12 for 2 h in a reaction volume of 300 μl. Protein G-Sepharose was then added to remove antibody-envelope complexes. After a 2 h incubation at 4 °C, resins were removed by centrifugation and the supernatants were transferred to new tubes. IDE antibodies were added for the second round of immunodepletion, which continued as described above. In control reactions, HeLa extract was treated only with Protein G-Sepharose and no antibodies or only with monoclonal antibodies but no Protein G-Sepharose.

The extent of TOP and IDE immunodepletion was assessed by Western blotting with TOP antiserum and IDE 9B12 antibodies. In addition, the depletion of TOP was also monitored in an assay with the fluorogenic TOP substrate, Mcc-PLGPK-Dnp. Two rounds of immunodepletion left only small amounts of TOP and IDE in the HeLa extract, as determined by Western blotting, and reduced the degradation of TOP-substrate Mcc-PLGPK-Dnp by 82%. The levels of these enzymes were not affected in control reactions containing only Protein G- or Protein A-Sepharose or only antibodies. Additionally, these procedures did not affect the activities of other cytosolic enzymes tested (e.g. prolyl oligopeptidase and aminopeptidases).

**Immunoblot Analysis**—The identification and quantification of thimet oligopeptidase in soluble extracts was done by separation of 10 μg of HeLa extract or 40 μl of each sample from fractionated extracts on a 10% SDS-polyacrylamide gel followed by transfer of the proteins to an Immobilon P membrane (Millipore). The membranes were blocked for 30 min at room temperature with 0.5% milk powder in phosphate-buffered saline and incubated overnight at 4 °C with specific primary antibodies or antisera. Bound antibodies on the immunoblots were detected with alkaline phosphatase-conjugated secondary antibodies. Signals were developed by ECL with the alkaline phosphatase substrate, CDP-Star (Tropix, Bedford, MA).

**RESULTS**

**Antigenic Peptides Are Rapidly Digested by Cytosolic Peptidases**—To determine whether antigenic peptides once formed, are stable in the cytosol or can be degraded by cellular peptidases, we established an HPLC assay to follow the fate of a number of antigenic peptides added to soluble extracts of HeLa cells. The six peptides were incubated with high-speed (100,000 × g, 6 h) supernatants, and at different times the remaining peptides were analyzed by reversed-phase HPLC. The following six antigenic peptides were studied: ASNENMETM (influenzavirus nucleoprotein, Flu NP, residues 366–374), SIINFEKL (ovalbumin, residues 257–264), TYQRTRALV (Flu NP, residues 147–154), RGPGRAFVTI (human immunodeficiency virus gp160, residues 318–327), TPHPARGL (β-galactosidase, residues 876–884), and FAPGNYPAL (Sendai virus nucleoprotein, residues 324–332). All these peptides had previously been found associated with murine MHC class I alleles and shown to induce a T cell immune response (33). Consequently, they have often been used in studies of antigen presentation.

At the outset of the incubation, the only peptide peak detectable in the non-dialyzed extracts was the added peptide. The failure to find any endogenous peptides in the cytosol is consistent with earlier observation (14). None of these added peptides were stable in the cell extracts at 37 °C. Peptides were studied at concentrations (50–100 μM), which allowed their degradation to be followed over convenient time periods. Rates of degradation were also dependent on extract concentration, and a concentration was chosen to facilitate the analysis. After 3 h, the amounts of all the peptides studied had decreased by 70–100%, but the rates of disappearance of different peptides varied widely (Fig. 1). Four peptides (ASNENMETM, SIINFEKL, TYQRTRALV, TPHPARGL) were degraded relatively slowly; i.e. ~50% disappeared after 40–100 min under these conditions, while two peptides (RGPGRAFVTI and FAPGNYPAL) were degraded much more rapidly (i.e. at least 50% of each disappeared within 30 min).

**Effect of Protease Inhibitors on Degradation of Antigenic Peptides**—To identify the enzymes responsible for peptide degradation, we initially analyzed the effects of different peptidase inhibitors on the disappearance of the Flu NP-derived antigenic 9-mer, ASNENMETM, and the ovalbumin-derived 8-mer, SIINFEKL. Addition to the extract of the aminopeptidase inhibitor bestatin (100 μM) reduced the degradation of these peptides only slightly (Table I), even though this concentration of bestatin inhibited almost completely the activity of the major cytosolic aminopeptidases, leucine aminopeptidase and the puromycin-sensitive aminopeptidase against fluorogenic substrates (data not shown). High concentrations of PMSF (1 mM) and EDTA (100 μM) caused no inhibition, which argues against the participation of serine and cysteine proteases. By contrast, the metal chelator o-phenanthroline (1 mM) almost completely blocked the degradation of these peptides and also the more rapid digestion of FAPGNYPAL (see below). Thus, metallo-aminopeptidase(s) appear to be responsible for the degradation of all these peptides (Table I).

These data were obtained in soluble extracts from which proteasomes had been removed by extensive ultracentrifugation (100,000 × g, 6 h). However, in additional experiments, a similar breakdown of ASNENMETM, SIINFEKL, and FAPGNYPAL was seen in crude extracts containing active proteasomes. In these preparations, their degradation was also sensitive to the

![Fig. 1. Most antigenic peptides were degraded in HeLa cell extracts by a process sensitive to the thimet oligopeptidase inhibitor, Cpp-AAP-pAb. The degradation of 5 nmol of each of the indicated peptides was analyzed in 10 μg of HeLa extract by reverse phase HPLC. The amount of peptide was determined by integration of the peptide peaks. For inhibitor studies, the extracts were preincubated for 30 min at room temperature with 10 μM Cpp-AAP-pAb prior to addition of the peptide substrate.](http://www.jbc.org/content/doi/10.1074/jbc.M108.986601.full)
Stability of MHC Class I Antigenic Peptides in Cytosol

Peptides (50 nmol/ml) were incubated for 2 h with 100 μg/ml HeLa extract in the presence or absence of these inhibitors as described under “Experimental Procedures.” Reactions were linear and were stopped when ~50–80% of substrate (in the absence of any inhibitor) was degraded, and peptide levels were analyzed by reverse phase HPLC. Percentage of inhibition was calculated from peak areas obtained by integration of reverse phase HPLC peaks of peptides incubated with or without inhibitors. Because the stated ultracentrifugation removed more than 85% of TFP-II, the effect of inhibitor butabindide was assayed in HeLa extract centrifuged only at 20,000 × g for 10 min, and similar results were obtained. At this concentration, butabindide almost completely inhibited TFP-II, as determined by its effect on bestatin-insensitive AAF-Amc degradation in the extracts. These results are representative of at least two independent experiments.

**Table I**

| Inhibitor       | Concentration | Proteases inhibited | Inhibition of Antigenic Peptide |
|-----------------|---------------|---------------------|-------------------------------|
| Bestatin        | 100 μM        | Aminopeptidases     | 6                             |
| PMSF            | 1 mM          | Serine              | 3                             |
| E64             | 100 μM        | Cysteine            | 0                             |
| α-Phenanthroline| 1 mM          | Metalloproteases    | 89                            |
| Butabindide     | 1 μM          | Tripeptidyl peptide II | 0                     |

**Studies with Pure Thimet Oligopeptidase**—The finding that TOP plays a primary role in the breakdown of many class I-presented peptides in cell extracts is quite surprising in light of the recent reports by Portaro et al. (31), who reported that pure TOP binds many antigenic peptides, including ones studied here (e.g. SIINFEKL), but fails to degrade them at all. Because of these apparently contradictory findings, we undertook experiments to determine whether recombinant TOP behaved in a manner consistent with our findings in cell extracts. Two different preparations of recombinant TOP were kindly provided to us, one by Dr. A. C. M. de Camargo and Dr. E. Ferro, and another by Dr. A. Barrett and Dr. P. Dando, whose groups had independently cloned and characterized this enzyme. Both preparations were active against the five antigenic peptides that were degraded by TOP-like activity in HeLa extracts. In fact, the relative sensitivities of these five peptides...
Recent studies have indicated that proteasomes can generate significant amounts of N-extended variants of antigenic peptides (24), and if such peptides are generated in the cytosol, they can be efficiently trimmed and presented on surface MHC molecules (20, 22). Although such longer peptides have been shown to undergo trimming by aminopeptidases in HeLa extracts (21), they may also possibly undergo complete degradation.

Studies were therefore carried out to test whether N-extended variants of antigenic peptides were also rapidly destroyed in cell extracts and whether TOP might be involved. As shown in Table III, ESINFEKL and QLESIINFEKL, which can be processed to the mature epitope in vivo (16, 20) and in cell extracts (21), were also digested in the extracts, although at slower rates than SIINFEKL (Table III). The breakdown of these longer versions was completely blocked by o-phenanthroline (data not shown) and was quite sensitive to the specific inhibitor of TOP, Cpp-AAF-pAb. In fact, the degree of inhibition by this inhibitor was even greater than the inhibition of SIINFEKL breakdown. Moreover, the immunodepletion of TOP, but not of another ubiquitous cytosolic endopeptidase, the insulin-degrading enzyme, also significantly reduced the degradation of these peptides (Table III). Interestingly, the stabilization of these peptides by Cpp-AAF-pAb or immunodepletion was much greater than by bestatin (Table III).

Certain Peptides Are Primarily Degraded by Aminopeptidases—As shown in Fig. 1, the disappearance of one antigenic peptide FAPGNYPAL in the extracts was particularly rapid, and unlike other peptides studied, this process did not involve the endopeptidase TOP. We therefore attempted to define the enzyme(s) responsible for its degradation. The breakdown of FAPGNYPAL involved metallopeptidase(s), since this process was completely inhibited by o-phenanthroline but in contrast to all other peptides studied (e.g., SIINFEKL) its degradation was also very sensitive to bestatin, the general inhibitor of aminopeptidases (Table IV). One major cytosolic peptidase sensitive to bestatin is puromycin-sensitive aminopeptidase (PSA, EC 3.4.11.14) (44). Puromycin markedly stabilized FAPGNYPAL and, as expected, had very little, if any, effect on SIINFEKL breakdown. Although PSA appeared primarily responsible for the breakdown of FAPGNYPAL, its breakdown was also partially inhibited by Z-Pro-t-prolinyl dimethylacetal, a specific inhibitor of the cytosolic endopeptidase, prolyl oligopeptidase (45). Presumably, its sensitivity to this enzyme is due to the presence of two prolines in this antigenic peptide.

Because these results suggested a major role for PSA in degradation of this one peptide, we tested whether the pure recombinant PSA was also particularly active against FAPGNYPAL. This enzyme, at a concentration similar to that in the HeLa extract, digested FAPGNYPAL about 30 times faster than SIINFEKL (Table IV), and this activity (together with some contribution from prolyl oligopeptidase) can easily account for the very rapid, TOP-independent, degradation of FAPGNYPAL in cytosolic extracts. This high rate of the break-
Thimet Oligopeptidase Is Not Regulated by Interferon-γ

The finding that TOP rapidly degrades most antigenic peptides suggests that this enzyme may limit MHC class I antigen presentation in vivo. Because all the peptides tested were degraded almost completely in these cell-free extracts, we found no evidence that such peptides may escape rapid degradation by binding to cytosolic proteins, such as molecular chaperones (14, 16). Another possible mechanism that cells might utilize to reduce degradation of antigenic peptides in the cytosol would be manyfold lower than under our assay conditions. Therefore, antigenic peptides are likely to exist in the cytosol only for seconds (or at most minutes). The present conditions were chosen to facilitate peptide assays and to dissect the roles of different peptidases.

PSA 1 μg/ml 3780 129
TOP 20 μg/ml 270 336

TABLE IV
FAPGYPAL, unlike SIINFEKL, is degraded in HeLa extracts primarily by the exopeptidase, PSA, and is very sensitive to pure PSA

Effects of protease inhibitors were analyzed as described in Table I. Recombinant thimet oligopeptidase (TOP) was a gift from Dr. E. Ferro and the recombinant PSA was from Dr. L. Hersh. Similar results were obtained repeatedly in independent experiments.

| Inhibitor | Concentration | Degradation by pure enzymes | Degradation rate |
|-----------|---------------|----------------------------|-----------------|
|           | PSA | TOP | FAPGYPAL | SIINFEKL |
|           | nmol/mg/h | nmol/mg/h |

| Enzyme | Concentration | Degradation rate |
|--------|---------------|-----------------|
| PSA    | 1 μg/ml       | 3780 129 |
| TOP    | 20 μg/ml      | 270 336 |

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In these studies, we found no evidence for a cytosolic mechanism that may protect a significant fraction of these peptides from further degradation under these conditions. Such a mechanism had been proposed based in part on the observation that MHC class I-presented peptides in extracts bind to the molecular chaperones Hsp70 and Hsp90 (15) or to a distinct cytosolic...
The present findings indicate that, if such a binding site for antigenic peptides do exist, it cannot provide sufficient protection from cytosolic destruction by endopeptidase or trimming by aminopeptidase (21). Moreover, in related studies, we have found that SIINFEKL and other antigenic peptides, if generated by proteolysis in intact cells, are highly susceptible to breakdown by TOP.2

A number of our findings indicate that TOP is responsible for the degradation of most of these antigenic peptides in vitro. (i) The rate of disappearance of five of the six antigenic peptides and of the two N-extended precursors studied was reduced by 30–90% by two highly specific inhibitors of TOP (Fig. 1, Table III). (ii) Chromatography of the extracts revealed that the one major endopeptidase peak active against the antigenic peptide SIINFEKL was also most active against the specific substrate for TOP, Mcc-PLGPK-Dnp (Fig. 2). (iii) The hydrolysis of Mcc-PLGPK-Dnp and that of SIINFEKL by these fractions were sensitive to the specific inhibitor of TOP (Fig. 2). (iv) These activities were eluted together with TOP as shown by immunoblot analysis (Fig. 2). (v) Immunodepletion of TOP with a specific antisera also significantly reduced the degradation of three peptides studied by this method (Table III). (vi) Finally, all antigenic peptides were susceptible to degradation by pure TOP, and their relative rates of hydrolysis by the recombinant enzyme corresponded to their susceptibilities to degradation in the extracts (Table II, Fig. 1). Since no other major enzyme active against these peptides was found, it seems likely that TOP degrades most, but not all, antigenic peptides in vivo. Moreover, since the inhibitors used are competitive agents, the present findings may underestimate the actual contribution of TOP to degradation of certain antigenic peptides in vivo (especially ones to peptides with low $K_m$ values).

The closely related enzyme, neurolysin (EC 3.4.24.16), also can degrade the fluorogenic substrate Mcc-PLGPK-Dnp and is sensitive to the inhibitors used here. Although some neurolysin may also be found in the cytosol (48), it is primarily mitochondrial in location (49) and has a 100-fold lower affinity than TOP for the inhibitor Cpp-AAF-pAb (38, 49). In addition, immunodepletion of TOP with antibodies that do not recognize neurolysin significantly reduced the degradation of three antigenic peptides tested in those extracts (Table III). Therefore, neurolysin is unlikely to account for the effects of Cpp-AAF-pAb on degradation of antigenic peptides described here, although we cannot exclude the possibility for some partial contribution of this enzyme to the remaining, TOP-independent, peptide hydrolysis in these preparations.

Although indicating a major role for TOP, the present findings also indicate additional peptidases must contribute to the breakdown of certain antigenic peptides such as FAPGNYPAL whose degradation was particularly rapid and not affected by TOP inhibitors (Fig. 1) or TOP immunodepletion (data not shown). Because the degradation of this peptide also involved metallopeptidases and was inhibited up to 70% by both puromycin and bestatin, this process seems to involve the major cytosolic aminopeptidase, PSA. Accordingly, FAPGNYPAL was particularly susceptible to degradation by this enzyme at concentrations found in HeLa cells. Recently, the degradation of another class I-presented peptide, RGYVYQGL, and its N-extended precursors have also been reported to be mediated by PSA (23), and in related studies we found that its breakdown in HeLa extracts is not affected by inhibitors of TOP (data not shown). Therefore, PSA seems to hydrolyze rapid elimination of a subset of antigenic peptides, not degraded rapidly by TOP. Although TOP appears to cleave relatively nonspecifically (26, 50), it clearly has strong preferences for certain sequences (29, 38). From the structures of the six peptides studied here, it is quite unclear what features lead to rapid hydrolysis of most by TOP. However, it is likely that the preference of PSA for the N-terminal basic or hydrophobic residues (P1 site) (46) determined the rather high susceptibility of FAPGNYPAL and RGYVYQGL to hydrolysis by this aminopeptidase, although other peptides with basic N-terminal residues were degraded in HeLa extract preferentially by TOP.

The present conclusions differ sharply from the recent proposal that TOP can bind strongly antigenic peptides but is slow or inactive in digesting them (31). These conclusions had led to the suggestion that TOP might function like a chaperone to bind some antigenic peptides and then promote their delivery to the TAP transporter (32). That proposal is inconsistent not only with our findings that TOP plays a major degradative role in cell extracts, but also with our observation that pure recombinant TOP obtained from the laboratories of both A. Camargo and A. Barrett readily degrades these peptides (Table II). Our findings clearly argue that this enzyme may limit the ability of antigenic peptides to be presented in vivo, and related experiments have provided strong confirmation for such a role in intact cells.2

During the breakdown of ovalbumin and presumably other proteins, the proteasome generates the presented 8-residue epitope SIINFEKL, but even larger amounts of N-extended versions (24) that must undergo trimming by leucine aminopeptidase (21) or other aminopeptidases (16, 23) in order to be presented on MHC class I molecules. It is noteworthy, therefore, that TOP not only was very active against the mature antigenic peptides, but also degraded N-terminally extended peptides like the ovalbumin-derived ESIIINFEKL and QLESI-INFEKL (Table III) and in related studies the Flu NP-derived 11-mer QIASNENMETM (data not shown). Thus, it appears likely that the fate of such N-extended versions in the cytosol in vivo is determined by a kinetic competition between degradion by endopeptidases, especially TOP, and trimming to the presented peptide by aminopeptidases. Since interferon $\gamma$ induces the major trimming enzyme, leucine aminopeptidase (21, 25), it presumably can favor processing of N-extended precursors to the mature class I-presented epitopes over their degradation.

In vivo, this degradation of peptides obviously is not so effective as to prevent the development of immune responses.
Sufficient amounts of the peptides must survive attack by TOP (or PSA) and become presented by MHC class I molecules. Peptide digestion prior to antigen presentation would be less likely if there were a close spatial association between the proteasomes and the TAP complex, such that cytosolic peptides would not have access to mature antigenic peptides. However, attempts by many groups to obtain evidence for such a direct association have failed. Another reason that such an association seems unlikely is that the great majority of proteasome products do not serve in antigen presentation and are digested in the cytosol. Therefore, the rate of degradation in the cytosol is likely to be an important factor limiting the extent of MHC class I presentation, as demonstrated elsewhere.2

Interferon γ causes many adaptations that enhance antigen presentation, including induction of MHC class I heavy chain, β2-microglobulin, tapasin, TAP, proteasome β-subunits, and the PA28 complex (47, 51). These latter adaptations appear to enhance the proteasomal production of peptides appropriate for binding to MHC class I molecules (52–54). Moreover, the induction of leucine aminopeptidase by interferon γ can enhance generation of antigenic peptides from larger proteasome products (21). Therefore, we extensively tested whether interferon γ treatment of HeLa cells might lead to a change in the activity or amount of TOP. After several days of interferon γ treatment, no change was seen in expression of TOP (Fig. 3) or PSA (data not shown). Thus, in contrast to those factors that promote epotope generation, the enzymes capable of destroying antigenic peptides, TOP and even PSA, seem to be constitutive. Although suppression of these activities might in principle enhance antigen presentation, decreases in their content could have dire consequences for cell function, since in related studies, we have obtained strong evidence that TOP is playing a major role in the breakdown, not only of antigenic peptides, but of most peptides released by proteasomes.3 On the other hand, overly high levels of TOP result in more efficient destruction of antigenic peptides and a failure of class I presentation, as we shall show elsewhere.4

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Major Histocompatibility Complex Class I-presented Antigenic Peptides Are Degraded in Cytosolic Extracts Primarily by Thimet Oligopeptidase
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