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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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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[1] 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-

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[1] The abbreviations used are: MHC, major histocompatibility complex; TOP, thimet oligopeptidase; PSA, puromycin-sensitive aminopeptidase; TPP-II, tripeptidyl peptidase II; Mcc, 7-methoxycoumarin-3-carboxylyl; Dnp, 2,4-dinitrophenyl; Cpp, *N*-[1(*RS*)-carboxy-3-phenylpropyl]; pAb, *p*-aminobenzoate; Amc, 7-amino-4-methylcoumarin; PMSF, phenylmethylsulfonyl fluoride; TAP, transporter associated with antigen processing; ER, endoplasmic reticulum; HPLC, high performance liquid chromatography; IDE, insulin-degrading enzyme; Flu NP, influenzavirus nucleoprotein; Cbz, benzyloxycarbonyl.

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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 had 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 peptides were dissolved at 10 mg/ml in Me_2SO and stored at 80 °C. The internally quenched fluorogenic substrate Mcc-Pro-Leu-Gly-Pro-D-Lys (Dnp)-OH (Mcc-PLGPK-Dnp) and the inhibitor Cpp-AAF-pAb were obtained from Calbiochem-Novabiochem (San Diego, CA). The fluorogenic substrate for prolyl oligopeptidase (Z-GP-Amc), tripeptidyl peptidase II (AAF-Amc) and aminopeptidases (A-Amc, L-Amc, R-Amc) were obtained from Bachem (Basel, Switzerland). Protein A-Sepharose CL-4B and Protein G-Sepharose 4B Fast Flow, bestatin, *o*-phenanthroline, PMSF, and E64 were purchased from Sigma.

Recombinant human interferon γ was kindly provided by Biogen Inc. (Cambridge, MA). The proteasome inhibitor MG132 (Cbz-Leu-Leu-leucinal) was provided by ProScript Inc. (Cambridge, MA), and phosphinic peptide inhibitor of TOP, Cbz-PheΨ(PO_2CH_2)Ala-Arg-Phe-OH, was a kind gift of Dr. V. Dive (DIEP/DSV, CEA, Gif sur Yvette, France). Recombinant rat thimet oligopeptidase was provided by Dr. P. Dando and Dr. A. Barrett (Babraham Institute, Cambridge, United Kingdom) and also by Dr. A. C. M. Camargo (Instituto Butantan, Sao Paulo, Brazil) and Dr. E. Ferro (Universidade de Sao Paulo, Sao Paulo, Brazil). Recombinant human puromycin-sensitive aminopeptidase was obtained from Dr. L. B. Hersh (University of Kentucky, Lexington, KY). Monoclonal, murine TOP antibody was provided by Dr. C. Abraham (Boston University School of Medicine, Boston, MA), and the polyclonal rat TOP antiserum was a kind gift of Dr. E. Ferro. Rabbit antisera against LMP2 and PA28α were obtained from Dr. K. Tanaka. Rabbit antiserum against purified bovine leucine aminopeptidase was generated in this laboratory. Monoclonal antibody 9B12 against human insulin-degrading enzyme was a gift from Dr. R. Roth (Stanford University, Stanford, CA). Monoclonal antibody against proteasomal α-subunit HC3 was purchased from Affinity Research Products, Ltd. (Mamhead, United Kingdom).

HeLa Cells—The human cervical carcinoma cell line HeLa S3 was obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (Irving 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 with glass beads in 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM ATP, 1 mM dithiothreitol, and 250 mM sucrose. Extracts were prepared by centrifugation of the homogenates for 10 min at 20,000 $\times$ *g* and for 1 h at 100,000 $\times$ *g*. Most of the proteasomes were removed by an additional 6-h centrifugation at 100,000 $\times$ *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 $\times$ *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 7.8, 5 mM β-mercaptoethanol, and 0.05% Brij 35. 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 Hepes-KOH, pH 7.6, 2 mM $MgCl_2$, 0.1 mM dithiothreitol. Thimet oligopeptidase activity was analyzed using the quenched fluorogenic substrate, Mcc-PLGPK-Dnp, at 20 μM, and the fluorescent product was measured at an excitation wavelength of 345 nm and an emission wavelength of 405 nm in a SLM Aminco luminescence spectrometer. The activity of prolyl oligopeptidase was measured with Z-GP-Amc at 100 μM final concentration. The release of Amc was monitored at excitation/emission wavelengths of 380/460 nm. The tripeptidyl peptidase II (TPP-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.

HPLC Analysis of Peptide Degradation—Five nmol of the antigenic peptides were incubated at 37 °C for various time intervals with 10 μg of HeLa extract in 100 μl of 50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, 20 μM MG132. The reaction was terminated by the addition of 100 μl of 20% trichloroacetic acid followed by a 15-min incubation on ice and removal of the precipitated protein by centrifugation for 15 min at 20,000 $\times$ *g*. The peptide-containing supernatant was subjected to reversed-phase HPLC on a 4.6 $\times$ 250-mm Vydac 5-μm C18 column (Vydac, Hesperia, CA) in 0.06% trifluoroacetic acid with a flow rate of 1 ml/min. Elution was performed with a 30-min linear gradient from 5 to 60% acetonitrile. The eluting peptides were calculated by integration of peptide peaks on chromatograms and are given in arbitrary units (21). To study the effects of inhibitors, the extracts were preincubated with the indicated inhibitors for 30 min at room temperature.

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 first incubated with 20 μl of rabbit preimmune 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 $\times$ *g* for 15 s. To ensure complete immunodeple-

[2] A. X. Y. Mo, K. Lemerise, W. Zeng, Y. Shen, C. R. Abraham, A. L. Goldberg, and K. L. Rock, submitted for publication.

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-enzyme 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 antiserum. 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), TPHPARIGL (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, TPHPARIGL) 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).

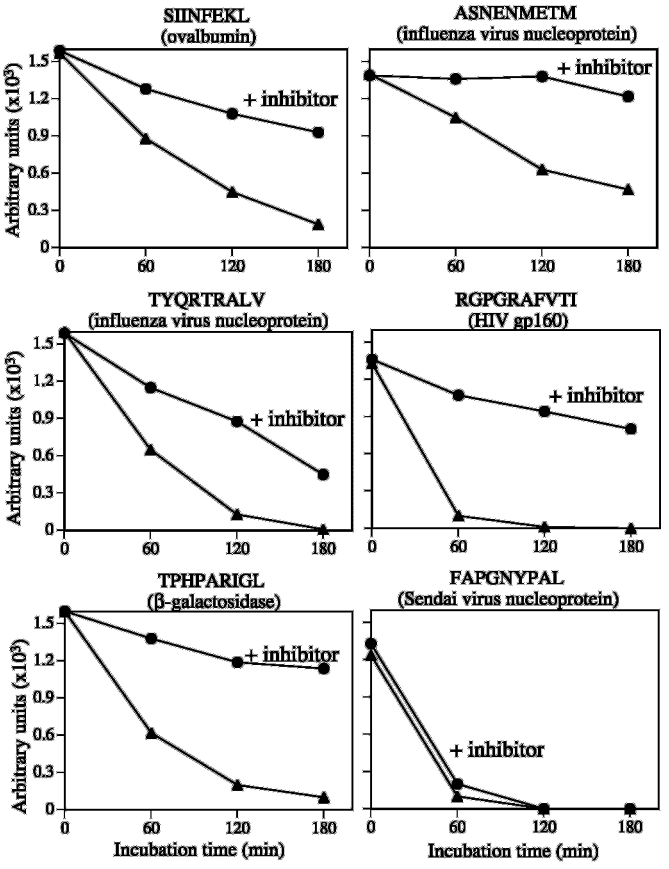

FIG. 1. **Most antigenic peptides were degraded in HeLa cell extracts by a process sensitive to the thimet oligopeptidase inhibitor, Cpp-AAF-pAb.** The degradation of 5 nmol of each of the indicated peptides was analyzed in 10 μg of HeLa extract by reverse phase HPLC. The amount of peptide was determined by integration of the peptide peaks. For inhibitor studies, the extracts were preincubated for 30 min at room temperature with 10 μM Cpp-AAF-pAb prior to addition of the peptide substrate.

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 E64 (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, metalloendopeptidase(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

TABLE I

Effects of protease inhibitors on the degradation of the influenzavirus nucleoprotein-derived antigenic peptide, ASNENMETM, and the ovalbumin-derived peptide, SIINFEKL, in HeLa cell extract

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 ~50–60% 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 TPP-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 TPP-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.

Inhibitor	Concentration	Proteases inhibited	Inhibition	
			ASNENMETM	SIINFEKL
			%	
Bestatin	100 μM	Aminopeptidases	6	14
PMSF	1 mM	Serine	3	3
E64	100 μM	Cysteine	0	12
o-Phenanthroline	1 mM	Metalloproteases	89	100
Butabindide	1 μM	Tripeptidyl peptidase II	0	0

metallopeptidase inhibitor and was not significantly affected by a proteasome inhibitor (data not shown). The ultracentrifugation step did not reduce activity of various soluble peptidases (*e.g.* thimet oligopeptidase, prolyl oligopeptidase, aminopeptidases, or insulin-degrading enzyme) as determined with specific fluorogenic substrates or by immunoblotting. However, this step did remove the very high molecular weight enzyme, TPP-II (34, 35). This enzyme has been proposed to play a role in final steps of intracellular protein degradation by degrading peptides generated by proteasomes (36). Additional experiments were therefore performed with crude extracts (20,000 × *g*, 10 min). Complete inactivation of TPP-II with the specific inhibitor, butabindide, showed that this serine peptidase did not contribute to the hydrolysis of these antigenic peptides (Table I).

Effect of Inhibitors of Thimet Oligopeptidase on Degradation of Antigenic Peptides—These findings suggested the involvement of a soluble metalloendopeptidase in the breakdown of many antigenic peptides. One such cytosolic enzyme, which is sensitive to *o*-phenanthroline and capable of degrading small peptides, is TOP (EC 3.4.24.15) (26, 29, 37). We therefore studied the effects of a specific inhibitor of TOP, the substrate analog Cpp-AAF-pAb (38, 39), on the rates of disappearance of the six mature antigenic peptides in HeLa extracts. This inhibitor (10 μM) reduced the degradation of ASNENMETM, SIINFEKL, TYQRTRALV, RGPGRAFVTI, and TPHPARIGL by ~30–90%, depending on the peptide (Fig. 1). To confirm the participation of TOP in degradation of these peptides, we used another highly specific competitive inhibitor of thimet oligopeptidases, Z-PheΨ(PO_2CH_2)Ala-Arg-Phe-OH (40). A marked inhibition was obtained when the degradation of four of these antigenic peptides (SIINFEKL, ASNENMETM, TYQRTRALV, TPHPARIGL) was monitored in the presence of this slow binding inhibitor (1 μM) (data not shown).

These results indicate that TOP is probably responsible for most of the digestion of these five antigenic peptides. In contrast, the very rapid breakdown of one antigenic peptide, FAPGNYPAL, was not affected by Cpp-AAF-pAb (Fig. 1). Thus, although most antigenic peptides appear to be degraded primarily by TOP, some are rapidly degraded by a distinct enzyme (see below).

Further Evidence Implicating Thimet Oligopeptidase in Degradation of Antigenic Peptides—To obtain more definitive evidence about the role of TOP in hydrolysis of antigenic peptides, soluble HeLa extracts were fractionated on an anion exchange column (Fig. 2). The fractions were initially assayed for TOP activity using a specific substrate for this enzyme, the quenched fluorogenic peptide Mcc-PLGPK-Dnp (41). Incubation of the unfractionated extracts with MG132, PMSF, E64, Z-Pro-L-prolinal dimethylacetal (specific inhibitor of prolyl oligopeptidase), or phosphoramidon (inhibitor of neprilysin) reduced hydrolysis of this substrate by less than 10%. By contrast, the specific inhibitor of TOP, Cpp-AAF-pAb, reduced breakdown of this substrate by 85–90%, as did immunodepletion of TOP with the polyclonal antiserum that specifically recognizes TOP, but not the related enzyme neurolysin (EC 3.4.24.16) (42). When each fraction from the anion exchange column was analyzed for Mcc-PLGPK-Dnp hydrolysis, one major peak of activity was found, which was eluted at 0.15 M NaCl. This activity was inhibited by 97% with Cpp-AAF-pAb (Fig. 2). In addition, all fractions were tested by HPLC analysis for their ability to degrade the antigenic peptide SIINFEKL. The maximum SIINFEKL degradation was found exactly in those fractions, which contained the major activity against Mcc-PLGPK-Dnp (Fig. 2).

To confirm that this activity was due to thimet oligopeptidase, each fraction was also analyzed in an immunoblot with the monoclonal antibody (IVD6), which reacts with this enzyme, but not with neurolysin (43). This antigen was detected specifically in those fractions that exhibited the peak of activity against the fluorogenic substrate and against SIINFEKL (Fig. 2). These various observations, together with immunodepletion studies (see below), indicate that TOP must be responsible for most of the degradation of SIINFEKL and other antigenic peptides in the HeLa extract.

Although no other fraction of the gradient contained TOP, there were additional small peaks of activity degrading SIINFEKL, one of which was also able to hydrolyze Mcc-PLGPK-Dnp (Fig. 2). These minor peaks were also sensitive to *o*-phenanthroline (date not shown) and presumably may also contribute to the degradation of SIINFEKL and other antigenic peptides, since the TOP inhibitor Cpp-AAF-pAb did not completely stabilize these peptides in the extracts (Fig. 1).

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

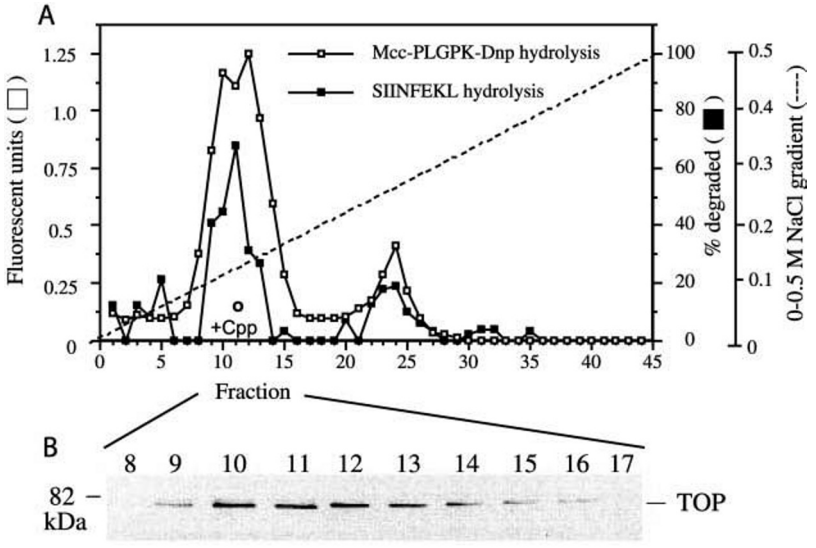

FIG. 2. **Thimet oligopeptidase in HeLa extract is responsible for most of the Mcc-PLGPK-Dnp hydrolysis and degradation of antigenic peptide SIINFEKL.** *A*, the HeLa extract (0.5 mg) was fractionated on a Mono-Q column as described under "Experimental Procedures." Fifty-μl aliquots were analyzed for their ability to hydrolyze Mcc-PLGPK-Dnp. The SIINFEKL-degrading activity of each fraction was tested by incubating 5 nmol of the peptide with 50 μl of each fraction for 3 h at 37 °C, followed by reverse phase HPLC. *B*, 40 μl of each fraction was subjected to SDS-polyacrylamide gel electrophoresis, and an immunoblot was performed with a monoclonal antibody against thimet oligopeptidase. No immunoreaction was seen in the minor peak (fractions 21–26). *Cpp*, Cpp-AAF-pAb.

TABLE II
Relative susceptibilities of antigenic peptides to degradation by recombinant TOP correlates with their relative rates of degradation in HeLa extracts

Peptides are listed according to their relative rates of degradation in Fig. 1. Degradation rates were calculated from linear curves obtained in reactions containing 50 nmol/ml each peptide and 20 μg/ml pure TOP. Reactions with pure TOP were incubated at 37 °C for 90 min until 18–58% of substrate was degraded. These data utilized recombinant TOP provided by Dr. E. Ferro, but similar results were also obtained with recombinant enzyme from Dr. A. Barret.

Peptide	Degradation rate
	nmol/mg/h
ASNENMETM	237
SIINFEKL	336
TYQRTRALV	368
TPHPARIGL	792
RGPGRAFVTI	840

to pure TOP correlated with their relative sensitivities to degradation in the HeLa extracts (Table II). The peptide, FAPGNYPAL, which appeared to be degraded rapidly by some other enzyme in the extracts, was also digested by the pure TOP, but was much more sensitive to a cellular exopeptidase (see below).

Similar degradation of these various antigenic peptides by pure TOP was seen under several different assay conditions. Although we have no explanation for the failure of Portaro *et al.* (31) to observe peptide degradation with an identical enzyme preparation, this failure may be due to much shorter incubation time (12 min) and much lower enzyme concentration (2.4 μg/ml) used in their experiments (compare with our conditions in Table II). The findings presented here and our related *in vivo* studies[2] provide strong evidence against the proposal that binding of antigenic peptides to TOP may protect them from degradation and promote antigen presentation *in vivo* (31, 32). On the contrary, these and related studies[2] indicate that TOP degrades such peptides and can limit antigen presentation *in vivo*.

Degradation of Longer Precursors of Antigenic 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, ESIINFEKL 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-L-prolinal 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-

TABLE III

N-terminally extended versions of SIINFEKL, ESIINFEKL, and QLESIINFEKL, are also degraded primarily by thimet oligopeptidase in HeLa extract

Total degradation rates in HeLa extract were calculated from linear portions of degradation curves as described in Table II, and percentage of inhibition by 100 μM bestatin, 10 μM Cpp-AAF-pAb (Cpp) or by TOP immunodepletion (TOP-IP) were calculated as in Table I. Rates of peptide degradation by aminopeptidases and by TOP were calculated by multiplying the total degradation rate by the fraction assignable to aminopeptidases (as determined by bestatin inhibition) and to TOP (as measured by Cpp-AAF-pAb inhibition). Reactions were performed with 10 μg of HeLa extract and 5 nmol of each peptide in 100 μl of 50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$ containing 20 μM MG132. Reactions were stopped when 50% SIINFEKL, 43% of ESIINFEKL, and 32% QLESIINFEKL were degraded. The inhibitor Cpp-AAF-pAb, at the concentration of 10 μM, reduced the hydrolysis of TOP substrate Mcc-PLGPK-Dnp by 86%, while the immunodepletion of TOP decreased its degradation by 82%. The immunodepletion of insulin-degrading enzyme had no effect on degradation of SIINFEKL or ESIINFEKL (data not shown). Similar results were obtained repeatedly in at least two independent experiments.

Peptide	Total degradation rate	Inhibition by			Rates of degradation by	
		Bestatin	Cpp	TOP-IP	Aminopeptidases	TOP
	nmol/mg/h		%		*nmol/mg/h*	
SINNFEKL	220	14	45	37	31	99
ESIINFEKL	152	24	89	88	36	135
QLESIINFEKL	79	43	46	42	34	35

TABLE IV

FAPGNYPAL, 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	Degradation by HeLa extract		
	Concentration	Inhibition	
		FAPGNYPAL	SIINFEKL
		%	
o-Phenanthroline	1 mM	100	100
Bestatin	100 μM	72	14
Puromycin	100 μM	65	5
Z-Pro-L-Prolinal	50 μM	19	0

Enzyme	Degradation by pure enzymes		
	Concentration	Degradation rate	
		FAPGNYPAL	SIINFEKL
		nmol/mg/h	
PSA	1 μg/ml	3780	129
TOP	20 μg/ml	270	336

down of FAPGNYPAL, but not of SIINFEKL, by the pure PSA is in agreement with the preference of this enzyme for the bulky hydrophobic or basic residues in P1 and P1′ sites (46), and with the fact that Phe-Amc is also one of the best fluorogenic substrates for PSA.[3]

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 (15, 16). Another possible mechanism that cells might utilize to reduce degradation of antigenic peptides in the cytosol would occur if the activity of TOP were reduced under conditions when antigen presentation is stimulated by interferon-γ. This cytokine induces many components of the MHC class I pathway, including MHC molecules, the proteasome activator PA28, three alternative catalytic subunits of the proteasome (LMP2, LMP7, and MECL-1), and the trimming enzyme leucine aminopeptidase (47). To test if TOP activity might be down-regulated under these conditions, HeLa cells were treated with interferon γ for 1–5 days. In some initial experiments (11), the TOP activity appeared to decrease after interferon-γ-treatment. However, these experiments proved to be complicated due to cell death with prolonged interferon γ treatment, and in more controlled studies, no consistent decrease was seen in TOP activity or in its levels as assayed with the fluorogenic substrate Mcc-PLGPK-Dnp or by Western blot analysis, respectively (Fig. 3). In these same extracts, interferon γ treatment did cause a dramatic induction of LMP subunits, a 4–6-fold increase in leucine aminopeptidase, and a 2-fold increase in PA28. By contrast, the levels of a constitutive α-subunit of the proteasome (HC3) were not affected (Fig. 3). It is also noteworthy that interferon γ treatment did not influence other cytosolic peptidases that possibly could play a role in this pathway, including the activities of puromycin-sensitive aminopeptidase and prolyl oligopeptidase or the levels of the insulin-degrading enzyme assayed by immunoblotting (data not shown). Thus, the enzymes that may degrade antigenic peptides, such as TOP and PSA, are not regulated by interferon γ, in contrast to cellular components that generate antigenic peptides.

DISCUSSION

Despite the continuous breakdown of cell proteins and release of peptides by the proteasome, such peptides are not detectable in the cytosol (14, 16) due to their rapid breakdown to amino acids. Very little is known about this degradative process, and it remains unclear whether the peptides that serve in antigen presentation are metabolized in a similar or different manner than the bulk of proteasome products. The present findings suggest that the antigenic peptides and longer precursors, once generated by proteasomes *in vivo*, are not quantitatively transported into the ER, but that a significant fraction of these peptides may be hydrolyzed to amino acids, like most peptides released by proteasomes. All six antigenic peptides studied here were hydrolyzed almost completely in extracts from HeLa cells, even though they were added in rather high amounts. Presumably, peptide degradation is much faster *in vivo* than these highly diluted extracts, and also the cytosolic concentration of the proteasome products should 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.

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

[3] T. Saric, unpublished observation.

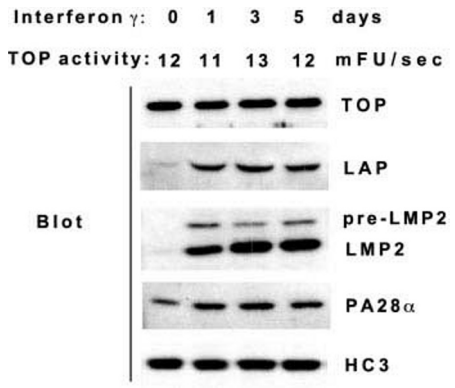

FIG. 3. **Treatment of HeLa cells with interferon γ does not affect levels or the activity of thimet oligopeptidase.** Proteins (10 μg) from control and interferon γ-treated HeLa cells were fractionated by SDS-polyacrylamide gel electrophoresis and analyzed with polyclonal sera against thimet oligopeptidase, leucine aminopeptidase, proteasome activator PA28α, and immunoproteasomal subunit LMP2, and a monoclonal antibody against proteasomal α-subunit HC3. The specific signals were developed using the ECL method. The TOP activity was measured in control and treated cell extracts with a fluorogenic substrate Mcc-PLGPK-Dnp as detailed under "Experimental Procedures." These data were the same in low and high speed supernatants and were repeated in several independent experiments.

protein (16). The present findings indicate that, if such a binding sites for antigenic peptides do exist, it cannot provide strong 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 antiserum 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 degraded 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 ESIINFEKL and QLESIINFEKL (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 destruction by endopeptidases, especially TOP, and trimming to the presented peptide by aminopeptidases. Since interferon γ 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

(33). 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 peptidases 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, β-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 epitope 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 major role in the breakdown, not only of antigenic peptides, but of most peptides released by proteasomes.[4] 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.[2]

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