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Interferon-γ Can Stimulate Post-proteasomal Trimming of the N Terminus of an Antigenic Peptide by Inducing Leucine Aminopeptidase*

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Most antigenic peptides presented on major histocompatibility complex class I molecules are generated during protein breakdown by proteasomes, whose specificity is altered by interferon-γ (IFN-γ). When extended versions of the ovalbumin-derived epitope SIINFEKL are expressed *in vivo*, the correct C terminus is generated by proteasomal cleavage, but distinct cytosolic protease(s) generate its N terminus. To identify the other protease(s) involved in antigen processing, we incubated soluble extracts of HeLa cells with the 11-mer QLESIINFEKL, which *in vivo* is processed to the antigenic 8-mer (SIINFEKL) by a proteasome-independent pathway. This 11-mer was converted to the 9-mer by sequential removal of the N-terminal residues, but surprisingly the extract showed little or no endopeptidase or carboxypeptidase activity against this precursor. After treatment of cells with IFN-γ, this N-terminal trimming was severalfold faster and proceeded to the antigenic 8-mer. The IFN-treated cells also showed greater aminopeptidase activity against many model fluorogenic substrates. Upon extract fractionation, three bestatin-sensitive aminopeptidase peaks were detected. One was induced by IFN-γ and was identified immunologically as leucine aminopeptidase (LAP). Purified LAP, like the extracts of IFN-γ-treated cells, processed the 11-mer peptide to SIINFEKL. Thus, IFN-γ not only promotes proteasomal cleavages that determine the C termini of antigenic peptides, but also can stimulate formation of their N termini by inducing LAP. This enzyme appears to catalyze the trimming of the N terminus of this and presumably other proteasome-derived precursors. Thus, susceptibility to LAP may be an important influence on the generation on immunodominant epitopes.

Mammalian cells present to the immune system small fragments of intracellular proteins in the form of 8–10-residue peptides bound to surface major histocompatibility complex (MHC)[1] class I molecules. This process allows cytotoxic T cells to screen for intracellular pathogens (*e.g.* viruses) and for transformed cells. The great majority of these antigenic peptides is generated during the course of protein degradation by 20 S or 26 S proteasomes (1), which catalyze the breakdown of most cell proteins. Nearly all of the peptide products of the proteasomes undergo rapid proteolytic destruction to amino acids. However, some oligopeptides, perhaps after further proteolytic processing, are transported from the cytosol into the endoplasmic reticulum (ER), where they bind to MHC class I molecules, and this complex is transported to the cell surface (2–4). Many lines of evidence have indicated that the proteasome is essential for the generation of most antigenic peptides. 1) Proteasome inhibitors, such as peptide aldehydes (5, 6) and lactacystin β-lactone (7, 8), prevent the generation of most class I-presented peptides and the cytotoxic T-cell response. 2) Antigen presentation from certain proteins requires their conjugation to ubiquitin, which leads to rapid breakdown by the 26 S proteasome (9, 10). 3) The cytokine IFN-γ, which stimulates many steps in antigen presentation, induces the expression of three special β-subunits (LMP2, LMP7, and MECL1). Their incorporation into the 20 S proteasome modifies its peptidase activities and thus appears to increase the generation of peptides with hydrophobic and basic C termini (11–14). Such peptides are selectively transported into the ER (15) and bind preferentially to MHC class I molecules (16). These adaptations are clearly important *in vivo,* since deletion of LMP2 (17) or LMP7 (18) genes in mice leads to defects in their ability to generate cytotoxic T-cell responses. 4) IFN-γ also induces an 11 S complex, PA28, which stimulates the proteasome's peptidase activity (19, 20) and thus may also promote antigen presentation (21).

One important aspect of this process that is poorly understood concerns the exact fate of peptides released by the proteasome. The peptide-binding cleft of the MHC class I molecule binds strongly only peptides of 8–10 residues in length (22, 23). The sizes of the peptides containing antigenic epitopes that are released by the mammalian proteasomes are unknown. Recently, proteasomes from the achaeon, *Thermoplasma acidophilum,* have been shown to cleave proteins to peptides ranging in length from 3 to 25 amino acids. Even though the eukaryotic proteasome has many fewer active sites, and these sites differ in cleavage specificity (1), it generates peptides during protein breakdown whose size distribution resembles that of the achaeal proteasome (25).[2] With both types of proteasomes, 10–15% of the peptide products are of the correct length for MHC class I binding (24). It thus remains unclear whether most presented peptides are produced directly by the proteasome, as suggested by some workers (27, 28), or whether additional proteolytic steps are necessary to generate the final

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[1] The abbreviations used are: MHC, major histocompatibility complex; ER, endoplasmic reticulum; LAP, leucine aminopeptidase; IFN, interferon; HPLC, high performance liquid chromatography; AMC, aminomethylcoumarin; pkLAP, porcine kidney LAP.

[2] A. F. Kisselev, T. Akopian, K. M. Woo, and A. L. Goldberg, submitted for publication.

8–10-mers (29) (see below). Also, the enzymatic steps in epitope production may differ for different proteins. One of the epitopes studied more extensively is the ovalbumin-derived, H2-K^b-presented epitope, SIINFEKL. When SDS-activated 20 S proteasomes were incubated with ovalbumin (25) or with fragments of ovalbumin (27) for prolonged periods, SIINFEKL was generated, but also some N-terminal-extended versions of this peptide were produced. However, the physiological relevance of such experiments is unclear; for example, it remains questionable whether the products released by these 20 S particles under these artificial conditions are the same as those generated from ubiquitinated proteins by the 26 S proteasomes *in vivo*.[2]

Craiu and co-workers (29) showed that if longer peptides that contain this antigenic sequence are injected into cells or expressed from minigenes, these peptides could be proteolytically trimmed to SIINFEKL and be presented on surface MHC class I molecules. Interestingly, generation of this antigenic peptide from ovalbumin with C-terminal extensions of SIINFEKL of 1–15 amino acids was completely blocked by treatment of the cells with the proteasome inhibitor, β-lactone (29). Although the proteasome thus seems to play a critical role in the generation of the C terminus of this antigenic peptide, this particle was not necessary for the cleavages that define their N termini. When peptides containing 2–25 additional residues on the N terminus of SIINFEKL were expressed or injected into cells, SIINFEKL was presented by MHC class I molecules, and this process was not affected by a proteasome inhibitor. The 20 S proteasome, although it contains several endopeptidase activities, lacks aminopeptidase activity. Thus, some other proteolytic enzyme(s) must be generating the correct N terminus of this (and presumably of other) antigenic peptides. Several findings indicate that the major peptidase(s) active in this N-terminal trimming are located in the cytosol (29). For example, a SIINFEKL-containing precursor with 25 additional N-terminal residues was efficiently trimmed to the presented octapeptide by a non-proteasomal mechanism. However, peptides longer than 16 residues are quite poor substrates for the TAP transporter on the ER (30). Therefore, most of the additional N-terminal residues must have been cleaved off in the cytosol before uptake into the ER for MHC class I binding, although some exopeptidase(s) clearly capable of trimming antigenic peptides are also found in the ER (31, 32).

A number of cytosolic proteases degrade preferentially oligopeptides and therefore may function in the trimming or further degradation of proteasome products, including the heterogenous group of aminopeptidases (33–35). Unidentified bestatin-sensitive aminopeptidases have been shown to catalyze the final steps in the ATP-dependent proteolytic pathway in the conversion of small peptides to amino acids (33, 36). The present experiments were undertaken to investigate which cytosolic proteases may be involved in the post-proteasomal processing of class I-presented peptides, and specifically in the cytosolic trimming of N-terminal-extended peptides to SIINFEKL. In addition, we have tested whether IFN-γ treatment, which promotes many important steps in antigen presentation, also stimulates the processing of such peptides.

EXPERIMENTAL PROCEDURES

Peptides and Reagents—Peptides with the sequences SIINFEKL, ESIINFEKL, LESIINFEKL, and QLESIINFEKL were synthesized by Macromolecular Resources (Colorado State University, Fort Collins, CO) and were over 90% pure (by HPLC analysis). The peptides were dissolved at 10 mg/ml in dimethyl sulfoxide and stored at −80 °C. Purified porcine kidney leucine aminopeptidase and tissue carboxypeptidase A were obtained from Sigma. Bestatin was purchased from Sigma and the proteasome inhibitor, MG132 (Cbz-LLLal), was kindly provided by ProScript Inc. (Cambridge, MA). The 10-mer QLESIINFEK was prepared by incubation of the 11-mer with carboxypeptidase A, and the product was isolated by HPLC.

Cell Lines—The human cervical carcinoma cell line, HeLa S3, and the human macrophage cell line, U937, were 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 and U937 cells were treated for 5 days with 1500 and 3000 units/ml of human recombinant IFN-γ (a kind gift from Biogen, Cambridge, MA).

Preparation of Soluble Extracts—Cells were homogenized in a Dounce homogenizer and by vortexing with glass beads in 50 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM ATP, 1 mM dithiothreitol, and 250 mM sucrose, pH 7.4. Cytosolic extracts were prepared by centrifugation of the homogenates for 20 min at 10,000 × *g* and 1 h at 100,000 × *g*, and proteasomes were removed by an additional 6 h centrifugation at 100,000 × *g*. All extracts were stored at −80 °C until use. For some experiments, the residual proteasomes in the extracts were inactivated by incubation with 100 μM MG132 for 15 min at room temperature.

Peptidase Assay—Aminopeptidase activity was analyzed using fluorogenic substrates of the amino acid-AMC type (Bachem, King of Prussia, PA). Substrates containing 19 different N-terminal amino acids (except tryptophan) were used at a concentration of 200 μM of each in a 1-ml volume of 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 8.5. Substrate hydrolysis was determined using 10 μg of cytosolic proteins from HeLa or U937 cells, as measured using the Coomassie kit from Pierce (Rockford, IL). Samples were incubated for 75 min at 37 °C, and the reaction was stopped by adding 1 μl of 10% SDS, and then fluorescence was measured at an excitation wavelength of 380 nm and an emission wavelength of 440 nm in a SLM-AMINCO spectrometer (Rochester, NY). Analysis of fractionated extracts was carried out with 50 μl of each fraction in 500 μl of 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 8.5, stopped after 75 min incubation at 37 °C with 500 μl of 2% SDS, and analyzed as described above.

High Performance Liquid Chromatography (HPLC) Analysis of Peptides—5 nmol of the synthetic peptide QLESIINFEKL was incubated with 10 μg of extract from HeLa cells or with 25 μg of extract from U937 cells in 100 μl of 50 mM Tris-HCl and 5 mM $MgCl_2$, pH 8.5, for various times at 37 °C. The reaction was terminated by addition of 100 μl of 20% trichloroacetic acid followed by 15 min incubation on ice, and the precipitated protein was removed by centrifugation for 15 min at 14,000 rpm. The peptide-containing supernatant was subjected to reverse-phase HPLC on a 4.6 × 250-mm Macrosphere 300A C8-column (Alltech, Deerfield, IL) at 40 °C in 10 mM phosphate buffer, pH 6.8, with a flow rate of 0.75 ml/min. Elution was performed with a 25-min linear gradient from 15 to 40% acetonitrile. The eluted peptides were detected by measuring their absorbance at 214 nm. The relative concentrations of each eluted peptide were calculated by integration of the areas under the peaks and are given in arbitrary units.

Fractionation of Soluble Extract by Ion Exchange Chromatography—Fractionation of 2 mg of U937 and 0.5 mg of HeLa cell extracts was performed by ion-exchange chromatography in 50 mM Tris-HCl buffer, pH 8.5, on a 1-ml MonoQ 5/5 column (Pharmacia, Upsala, Sweden). Bound proteins were eluted with a 20-min linear salt gradient from 0 to 0.5 M sodium chloride and with a flow rate of 1 ml/min. The eluted peptides were measured at 280 nm, and fractions of 0.5 ml were collected for further analysis.

Electrophoretic Methods and Immunoblot Analysis—The identification and measurements of LAP in soluble extracts were done by immunoblot analysis. 30 μg of crude extract or 20 μl of each sample from fractionated extracts were separated on a 12.5% SDS-polyacrylamide gel, and the proteins were transferred onto an Immobilon P membrane (Millipore). The filters were blocked for 1 h at room temperature with 0.5% milk powder in phosphate-buffered saline and incubated overnight at 4 °C with a rabbit antiserum against bovine lens LAP (kindly provided by A. Taylor, Tufts University, Boston, MA). Bound antibodies were detected with ^{125}iodinated-protein A (NEN Life Science Products) and visualized and quantified with a PhosphorImager (Molecular Dynamics).

RESULTS

The Precursor QLESIINFEKL Is Processed to SIINFEKL in Cell Extracts—Because *in vivo* studies (29) indicated that cytosolic protease(s) distinct from the proteasome generate the N terminus of the MHC class I-presented SIINFEKL from longer precursors, such as QLESIINFEKL, we tested whether a similar processing of this precursor occurs in soluble extracts of HeLa cells. The processing reaction was followed until the

antigenic 8-mer was generated (on the assumption that once the 8-mer is generated *in vivo*, it would be efficiently transported into the ER and would bind to MHC class I molecules where it would be protected from further proteolysis). In principle, conversion of the ovalbumin-derived 11-mer to SIINFEKL can occur by a single endoproteolytic cleavage, by sequential removal of the N-terminal residues (generating the 10-mer and then the 9-mer), or by some mixture of endo- and exopeptidase reactions. To follow these reactions, an assay using reverse-phase HPLC was developed, which allowed resolution of the 11-mer (QLESIINFEKL), 10-mer (LESIINFEKL), 10-mer (QLESIINFEK), 9-mer (ESIINFEKL), and 8-mer (SIINFEKL) peptides. The 11-mer peptide QLESIINFEKL was incubated with the 100,000 $\times$ *g* supernatant from HeLa cells at 37 °C for various periods of time. To prevent proteasomal activity, the extracts were depleted of proteasomes by prolonged ultracentrifugation, and any residual activity was blocked by the addition of the inhibitor, MG132 (37), which blocks proteasome function, but does not inhibit aminopeptidases or carboxypeptidases. The reaction was stopped by addition of trichloroacetic acid, and the peptide-containing supernatant was fractionated by HPLC. Determination of the retention times of standard peptides allowed us to test whether the same peptides were generated in the extracts, and their relative concentrations were determined by integration of peak areas.

Initially, the 11-mer was the only peptide peak detected at 214 nm in the HeLa extract (Fig. 1). The lack of endogenous peptides in these undialyzed extracts confirms prior observations that the concentration of free peptides in the mammalian cytosol is very low (38). Extract concentrations (100 μg/ml) were studied that allowed easy measurement of the disappearance of the added 11-mer in a few hours. In the extracts, the amount of the added 11-mer decreased at a linear rate, and by 3 h, nearly 50% had disappeared (Fig. 2). After 30 min, additional peaks could be detected, which correspond to the 10-mer and 9-mer. These two peptides increased with time and reached similar maximal levels at 2 and 3 h. Only a very small peak corresponding to the C-terminal truncated 10-mer (QLESIINFEK) was detected after addition of the 11-mer to the HeLa extracts (data not shown). In these extracts, the amount of this C-terminal truncated peptide was always much less than the amounts of the N-terminal truncated peptides. These findings and inhibitor studies (see below) all indicated that in these preparations peptide processing occurred primarily by aminopeptidase(s), with little or no carboxypeptidase activity or endoproteolytic generation of the 9- or 8-mer.

Since IFN-γ is known to alter the peptidase activity of the proteasome (11–13, 17, 18) and to promote expression of other components of the antigen presentation pathway, we tested whether IFN-γ also promotes processing of the 11-mer. In extracts from IFN-treated HeLa cells, the 11-mer disappeared 2 to 3 times faster than in the control extract (Fig. 2). At the same time, IFN treatment stimulated the generation of the 10- and 9-mer by 2.2–2.4-fold. After 90 min, the antigenic 8-mer SIINFEKL was present in the extract from the IFN-treated cells. The amount of SIINFEKL increased with time, although its level was always much less than that of the 10- or 9-mer. By contrast, SIINFEKL was not detected at any time in the control extract. These findings indicate sequential trimming of the precursor by an IFN-induced aminopeptidase(s). Since the sum of the amounts of 10-, 9-, and 8-mer peptides recovered at every time point nearly equaled the amount of the 11-mer that had disappeared, no other protease contributes significantly to the destruction of the 11-mer or to the generation of the 8-mer.

We tested whether the enzyme that processes QLESIINFEKL was sensitive to bestatin, a competitive inhibitor of many aminopeptidases. When the extracts of HeLa cells were preincubated with bestatin at a concentration (100 μM) that inhibited the aminopeptidase activity against Leu-, Gln-, and Lys-AMC by 80–99% (data not shown), the rates of disappearance of the 11-mer and of the appearance of the 10-mer in control and IFN-treated HeLa cell extracts decreased by 50% (Fig. 3). Furthermore, in the bestatin-treated extracts, neither the 9-mer nor the antigenic 8-mer could be detected. These data further argue that aminopeptidases are responsible for the generation of SIINFEKL. Presumably, this inhibition by bestatin is only partial, because the 11-mer has a much higher affinity than the amino acid-AMC substrates. Alternatively, there may well be some bestatin-resistant aminopeptidase that converts it to the 10-mer. In fact, in a similar experiment with extracts from U937 cells (data not shown), the disappearance of

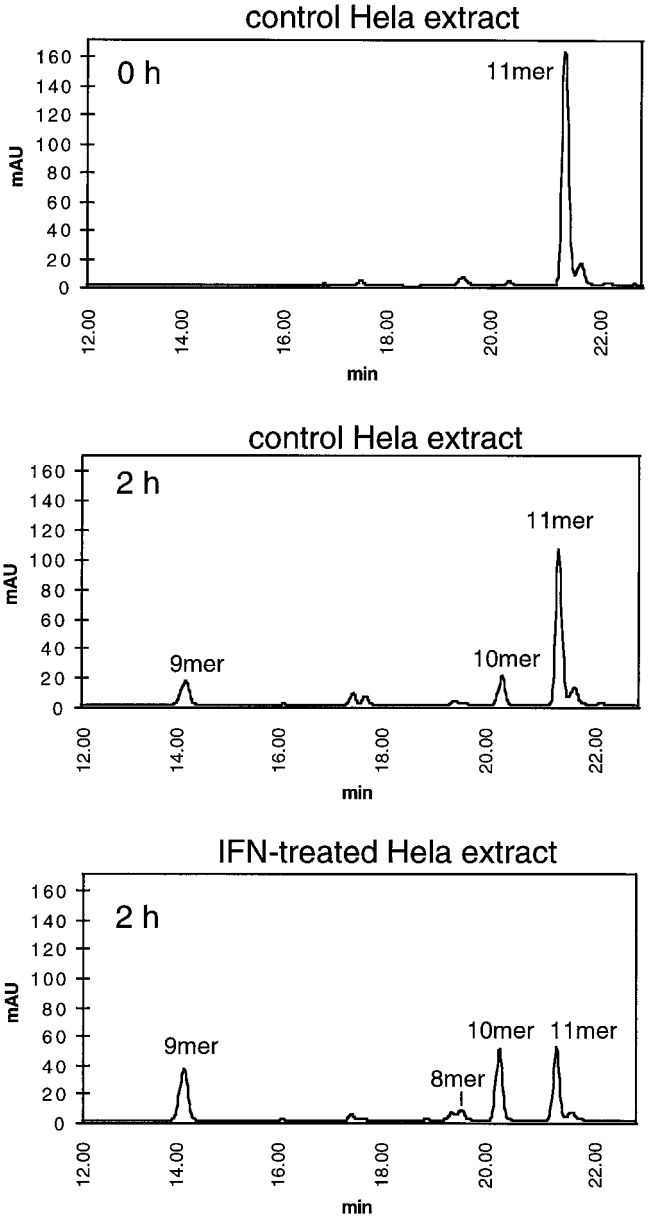

FIG. 1. **HPLC analysis of the processing of QLESIINFEKL in extracts from control and IFN-treated HeLa cells.** The processing of the 11-mer peptide was analyzed by reverse-phase HPLC after 2 h incubation at 37 °C with 10 μg of each extract. The generated 10-mer (LESIINFEKL), 9-mer (ESIINFEKL), and 8-mer (SIINFEKL) were identified by their elution times using peptide standards.

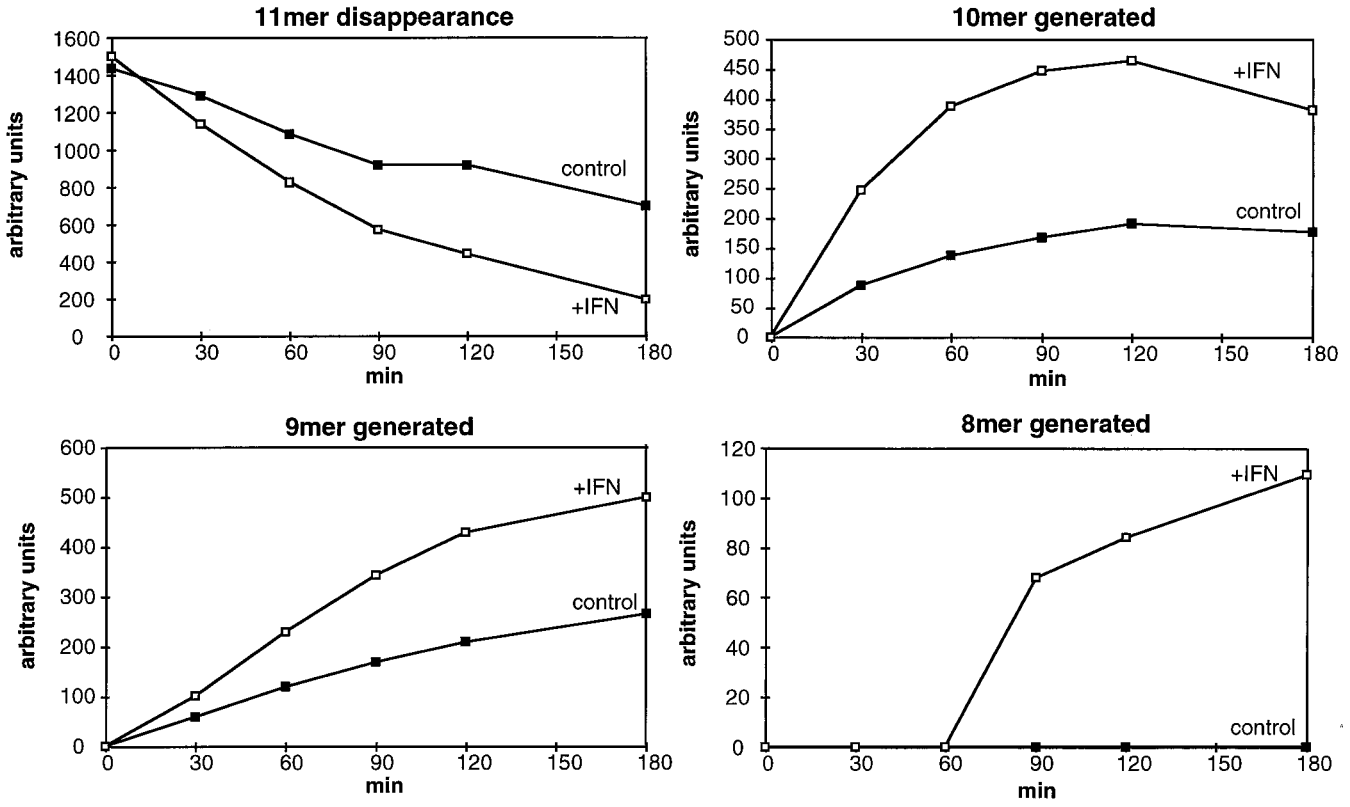

FIG. 2. **The kinetics of the disappearance of QLESIINFEKL and the generation of smaller peptides in extracts from control and IFN-treated HeLa cells.** The levels of the 11-mer and related products were analyzed in the two extracts at 30-min intervals, as described in Fig. 1. The relative amounts of all peptides were calculated by integration of the peptide peaks on the chromatogram.

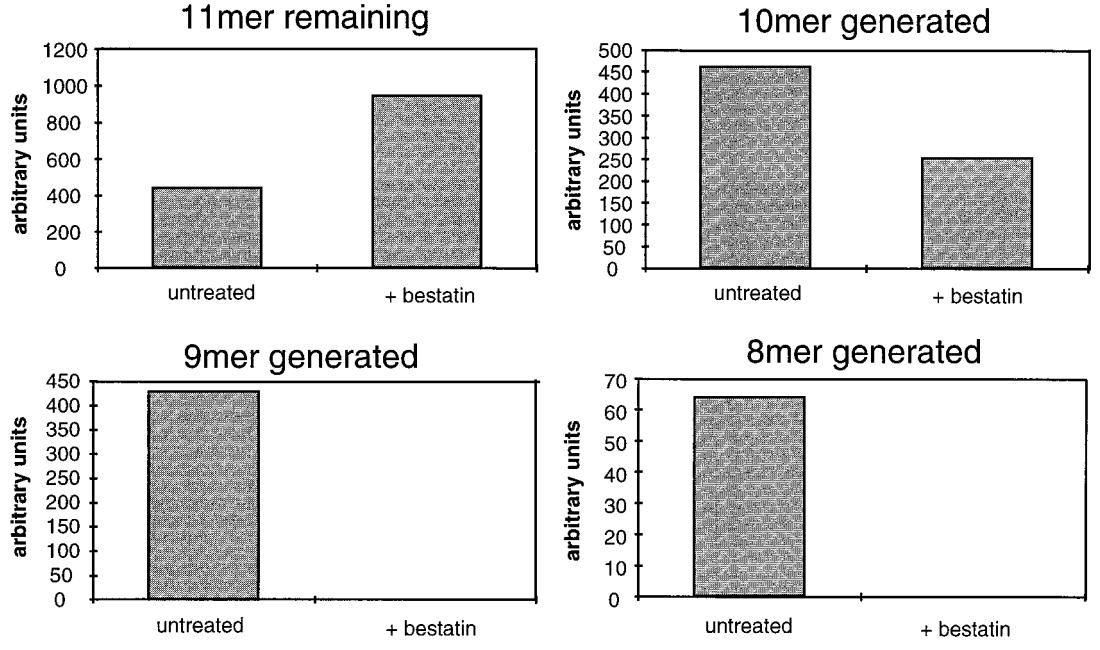

FIG. 3. **Proteolytic processing of the 11-mer QLESIINFEKL is blocked by bestatin.** 10 μg of soluble extract from IFN-treated HeLa cells were preincubated for 30 min with 100 μM bestatin. Then 5 nmol of the 11-mer peptide was added and incubated at 37 °C. After 2 h, the amounts of the 11-mer remaining and the generated 10-, 9-, and 8-mer were determined as described in the legend to Fig. 2.

the 11-mer was not affected by bestatin in the control extract, but was inhibited by 50% in the extract from IFN-treated cells. Thus, in U937 cells, multiple peptidases can degrade the 11-mer, but the IFN-induced N-terminal removal also involves a bestatin-sensitive aminopeptidase.

IFN-γ Increases Cellular Aminopeptidase Activities—To further characterize the aminopeptidase(s) induced by IFN-γ, we used a variety of fluorogenic aminopeptidase substrates. Initial experiments with control and IFN-treated HeLa and U937 extracts using Leu-AMC as substrate indicated that its hydrolysis occurred at a linear rate for up to 75 min. The rate of this reaction was directly proportional to the substrate concentration (which ranged from 50 to 600 μM) and to the amounts of extract protein (which ranged from 10 to 50 μg) (data not

TABLE I

Hydrolysis rates for 19 amino acids in soluble extracts from control and IFN-treated HeLa and U937 cells and pkLAP

Substrate hydrolysis was determined in a peptidase assay with 10 μg of extract or 0.05 μg of pure pkLAP and 200 μM of each amino acid-AMC. Results are given as the mean values from two experiments for HeLa and three experiments from U937 cells, each with two independent samples. X represents each of the amino acids indicated below with the C-terminal coupled fluorophor, amidomethylcoumarin (-AMC). Any stimulation of 40% over control is highly reproducible.

Substrate, X-AMC	HeLa cells				U937 cells				pkLAP
	Control	IFN-treated			Control	IFN-treated			
		nmol/mg/h	Difference	% Stimulation		nmol/mg/h	Difference	% Stimulation	
									nmol/mg/h
Good substrates									
L	74	116	42	57	52	104	52	100	53
K	87	104	17	20	67	71	4	6	5.7
M	44	80	36	82	37	57	20	54	29
C	27	41	14	52	21	45	24	114	26
F	35	58	23	66	22	26	4	18	9.7
R	33	37	5	12	36	39	3	8	5.5
A	34	46	12	35	23	25	2	9	7.6
P	19	18	−0.1	0	19	21	2	10	2.7
Y	10	16	6	60	9.1	10	0.9	10	2.1
Poor substrates									
T	3.1	4.9	1.8	58	0.7	3.4	2.7	386	2.3
Q	3.6	6.9	3.3	92	1.7	5.7	4	235	4.0
E	2.5	3.2	0.7	28	0.4	1	0.6	150	0.7
N	2.9	1.7	−1.2	0	0.9	1.3	0.4	44	3
H	0.9	2.1	1.2	133	0.3	1.5	1.2	400	1.4
V	1.6	2.4	0.8	50	0.9	1.3	0.4	44	0.7
S	1.6	2.4	0.8	50	0.4	0.6	0.2	50	0.3
I	1.1	1.9	0.8	73	0.8	1.5	0.2	88	0.7
D	0.9	2.7	1.8	200	1.7	1.6	−0.1	0	0.9
G	1.7	2	0.3	18	0.2	0.4	0.2	100	ND[a]

[a] ND, not determined.

shown). In extracts from IFN-treated cells, the rate of Leu-AMC hydrolysis was consistently 2–3-fold higher than in extracts from controls. We then compared the activity of the control and IFN-treated cell extracts against 18 other amino acid-AMC substrates (Table I). The substrates fell into two groups, according to their rates of hydrolysis. In control extract, the amino acid-AMC substrates containing Leu, Lys, Met, Cys, Phe, Arg, Ala, Pro, and Tyr were hydrolyzed at least 2–20-fold faster than those containing Thr, Gln, Glu, Asn, His, Val, Ser, Ile, Asp, and Gly.

Interestingly, IFN-γ treatment stimulated the hydrolysis of only certain amino acid-AMC substrates. In HeLa cells, IFN-γ enhanced the hydrolysis of the Lys, Met, Cys, Phe, Tyr, Thr, Gln, His, Val, Ser, Ile, and Asp containing substrates between 50 and 200%. In U937 cells, IFN treatment also promoted hydrolysis of nearly all these substrates (except for Phe, Tyr, and Asp), but it also enhanced activity against Gly-AMC and Glu-AMC. The ability of IFN-γ to stimulate the hydrolysis of only certain substrates indicates that in these extracts, IFN-inducible and non-inducible aminopeptidases are probably present.

Like the N-terminal processing of the 11-mer to SIINFEKL, the cleavage of these fluorogenic substrates was also very sensitive to bestatin. For control extracts from U937 cells, pretreatment with bestatin (100 μM) reduced hydrolysis of Leu-AMC by 86% and of Gln-AMC by 80% (data not shown). In extract from IFN-treated cells, bestatin treatment reduced the hydrolysis of Leu-AMC and of Gln-AMC by 95%, which was 2–3-fold greater than in controls. In addition, the hydrolysis of Lys-AMC, which was not enhanced by IFN-γ, was also inhibited by 99% in controls and 88% after IFN treatment. Thus, the nature of the N-terminal residue of a peptide can have a major effect on its stability in the cytosol.

The IFN-γ-inducible Aminopeptidase(s)—To identify the aminopeptidase(s) which are induced in the cytosolic extracts by IFN-γ, extracts from control and IFN-treated HeLa cells were fractionated by ion exchange chromatography, and the fractions were analyzed for aminopeptidase activity against Leu-, Cys-, Lys-, and Gln-AMC. These substrates represent ones that were hydrolyzed rapidly (Leu-, Cys-, and Lys-AMC) or slowly (Gln-AMC) and ones whose hydrolysis was unaffected (Lys-AMC) or stimulated by IFN-γ (Leu-, Cys-, and Gln-AMC). In both control and IFN-treated extracts, the cleavage of Leu-AMC was found in two distinct peaks, which were eluted at 0.2 M (*peak A*) and at 0.35 M (*peak B*) NaCl (Fig. 4). The peptidase activity in peak A was 5-fold greater after IFN treatment than in the controls. In contrast, IFN-γ treatment had no stimulatory effect on the activity in peak B. The hydrolysis of Cys-AMC was observed in the same two peaks, and again this activity in peak A increased 2.4-fold after IFN treatment, while peak B did not change significantly.

After prolonged incubation of these fractions with the more slowly degraded substrate Gln-AMC, only one peak of activity was detected, and it corresponded to peak A. Gln-AMC hydrolysis by peak A was stimulated 10-fold by IFN-γ treatment. Lys-AMC was hydrolyzed by peak B and by a distinct aminopeptidase activity which was eluted at 0.24 M NaCl (peak C). These activities were not stimulated by IFN treatment. In similar experiments, nearly identical results were obtained with control and IFN-treated U937 cells. Leu-AMC and Cys-AMC were hydrolyzed in two peaks (A and B), and only the activity in peak A was stimulated by IFN treatment. Gln-AMC hydrolysis was observed only in peak A, and its cleavage was greatly stimulated by IFN-γ, while the activity against Lys-AMC in peaks B and C were not affected by IFN-γ. Accordingly, the degree of stimulation by IFN-γ in peak A of Leu- and Cys-AMC hydrolysis was significantly greater than in crude extracts. Because these three peaks had different substrate specificities, they presumably are distinct enzymes. For example, only peak A was induced by IFN-γ and was able to hydrolyze Gln-AMC, while peak C only cleaved Lys-AMC.

In all cell extracts, these aminopeptidase peaks were very

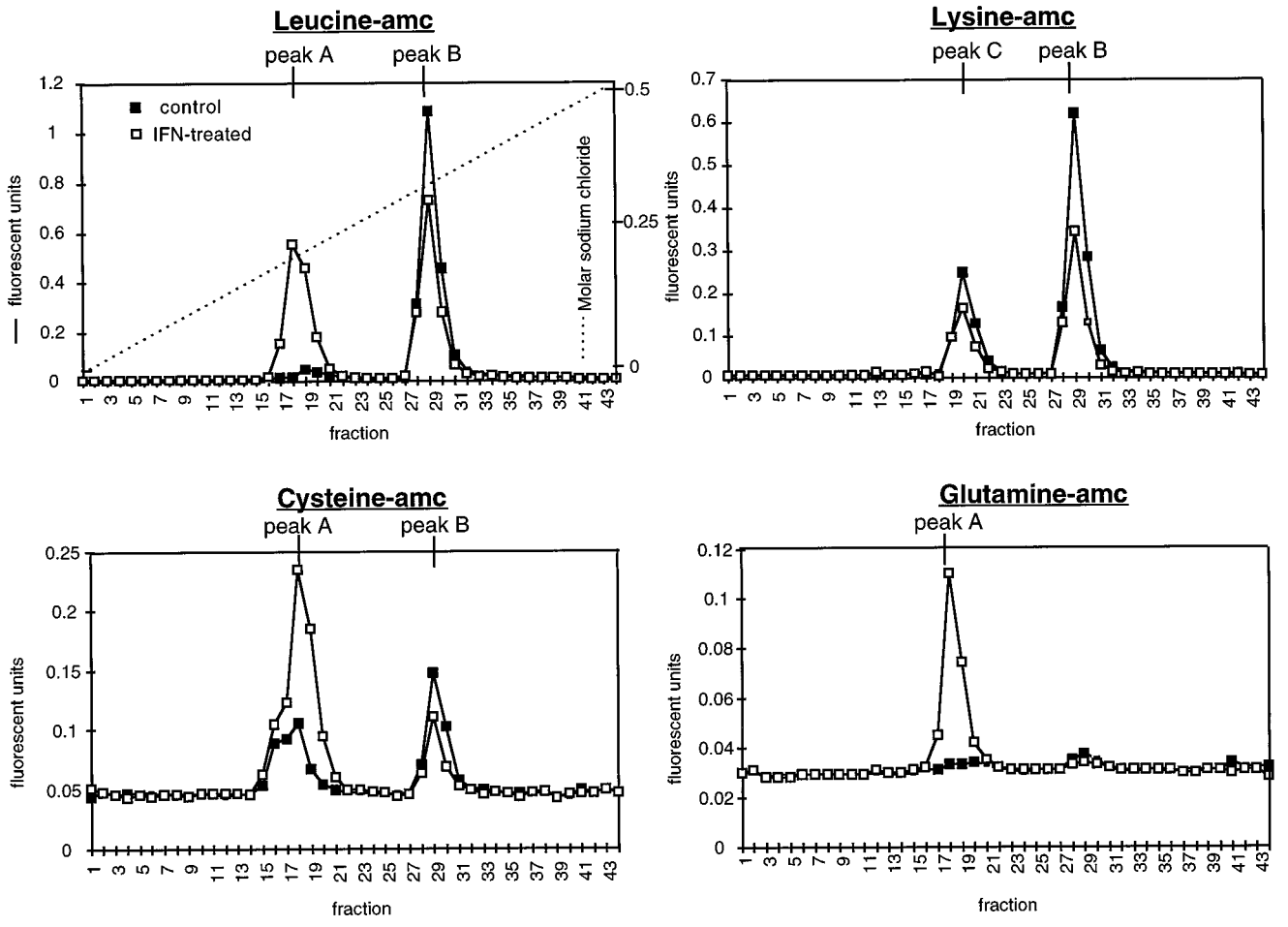

FIG. 4. **Three different aminopeptidase activities are present in the extracts of HeLa cells, one of which is induced by IFN-γ.** 0.5 mg of the extracts from control and IFN-treated HeLa cells were fractionated by ion-exchange chromatography with a 0–0.5 M salt gradient. Fractions of 0.5 ml were collected, and 1/10 volume of each fraction was incubated with 200 μM of the indicated substrate for 75 min (Leu-, Lys-, and Cys-AMC) or 225 min (Gln-AMC) at 37 °C.

sensitive to bestatin (100 μM), which reduced Leu-AMC hydrolysis in peaks A and B, and Lys-AMC hydrolysis in peak C by 80 to 99% (not shown). When each peak was incubated with QLESIINFEKL, no degradation of the 11-mer was seen with any peak from the control extracts (not shown), probably because of the large dilution of the activity during column chromatography. Also, peak B from the IFN-treated extract lacked trimming activity. However, the IFN-induced peptidase in peak A was able to process QLESIINFEKL and to generate the 10- and 9-mer, despite the appreciable dilution. Processing was also seen with peak C of the IFN-treated extracts, but this activity can be accounted for by its contamination by the IFN-induced peptidase in peak A. Thus, only the IFN-induced aminopeptidase peak was able by itself to mimic the processing of the 11-mer seen in crude extracts.

The IFN-inducible Protease Is Leucine Aminopeptidase (LAP)—Because the IFN-induced aminopeptidase peak preferentially hydrolyzed Leu-AMC and was sensitive to bestatin, it seemed possible that this enzyme corresponded to LAP. In fact, Harris *et al.* (39) had found that one of the genes induced by IFN-γ in human fibroblasts and certain carcinoma and melanoma lines codes for LAP. To test whether peak A corresponded to LAP, we used a polyclonal rabbit antiserum against bovine lens LAP (kindly provided by A. Taylor, Tufts University School of Medicine, Boston, MA) in an immunoblot analysis on control and IFN-treated extracts from HeLa and U937 cells. With this serum, we were able to detect cross-reacting bands in the extracts from IFN-treated HeLa and U937 cells that had a similar molecular weight as porcine kidney LAP (*pkLAP*) (Fig. 5*A*). Only a faint band could be detected in the control extracts from these cells. Quantification of the Western blot using ^{125}I-protein A showed a 6-fold induction of LAP in HeLa cells and a 14-fold induction in U937 cells after treatment with IFN-γ.

Furthermore, when the individual fractions were analyzed by immunoblotting, bands cross-reacting with LAP and showing the same molecular weight as pkLAP were detected in both control and IFN-treated U937 cells (Fig. 5*C*). The much higher signal in IFN-treated extracts was found in those fractions in peak A containing the IFN-induced aminopeptidase activity (Fig. 5*B*). Furthermore, with the HeLa extracts, where lower amounts of the extracts were fractionated, no LAP-specific band was observed in the controls, but bands that corresponded to the activity in peak A were clearly detected in the IFN-treated extracts (data not shown). This serum also reacted with other nonspecific bands, which did not correlate with any peak of aminopeptidase activity.

To test whether LAP could account for the IFN-induced processing of QLESIINFEKL, the 11-mer was incubated with purified pkLAP. After 15 min, peptide peaks corresponding to the 10- and 9-mer were detected, and after 30 min, the antigenic 8-mer SIINFEKL appeared (Fig. 6*A*). The amounts of 10-, 9-, and 8-mer thus increased with time in a similar fashion, as they did in the crude extracts from IFN-treated HeLa cells (Fig. 6*B*). Furthermore, pretreatment of pkLAP with bestatin (100 μM) reduced the rate of disappearance of the 11-mer and the appearance of the 10-mer by approximately 50%, and neither

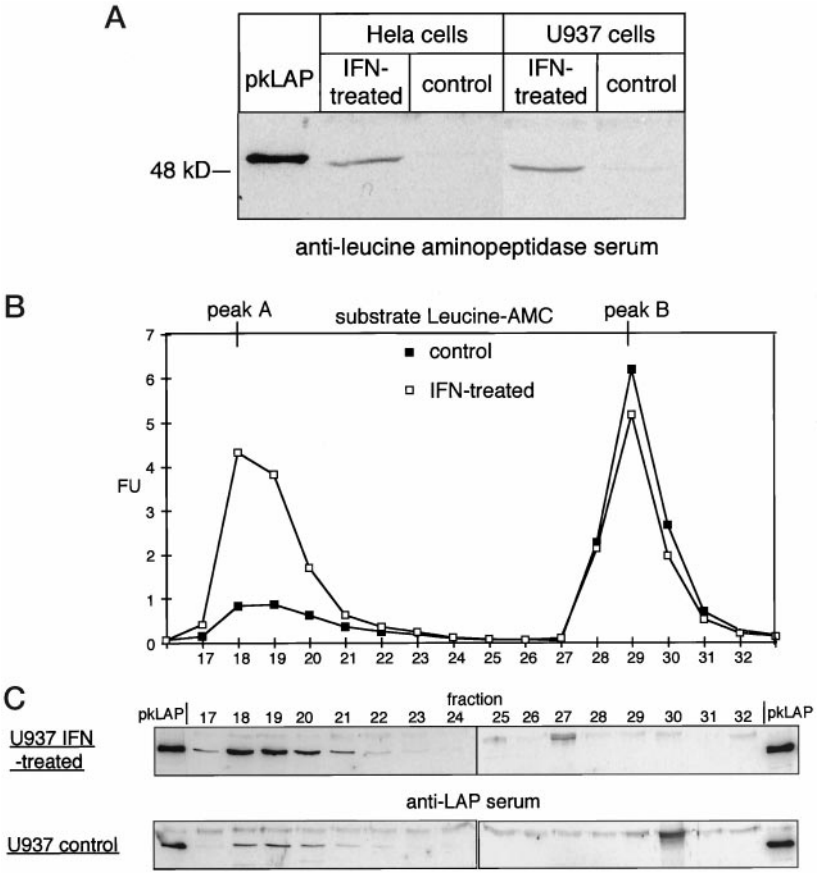

FIG. 5. **LAP is the IFN-γ-inducible protease in extracts from HeLa and U937 cells.** *A*, 0.5 μg of pure pkLAP and 30 μg of extract from control and IFN-treated HeLa and U937 cells were separated by SDS-polyacrylamide gel electrophoresis, transferred onto an Immobilon P membrane, and incubated with rabbit serum against bovine lens LAP. The bound antibodies were detected with ^{125}I-protein A by phosphor imaging. *B,* the fractionated extracts from U937 cells were tested for aminopeptidase activity in a peptidase assay, as described in the legend to Fig. 4. *C,* 20 μl of each fraction were separated by SDS-polyacrylamide gel electrophoresis and analyzed with the serum against LAP in an immunoblot analysis, as described above.

the 9-mer nor the antigenic 8-mer could be detected at 2 h (data not shown). The degree of pkLAP inhibition by bestatin resembled that found in the cell extracts; thus, the failure of bestatin to inhibit completely is probably because this enzyme has a higher affinity for the peptide precursor than for the fluorogenic substrates.

In addition, we systematically compared the substrate preference of the pure pkLAP with that of the enzyme(s) induced by IFN-γ in HeLa and U937 cells. The relative rates of hydrolysis of the 19 different fluorogenic amino acid-AMC substrates by pkLAP resembled closely the absolute increases in their rates of hydrolysis in HeLa and U937 cell extracts following IFN-γ treatment (Table I and data not shown). Together, these results strongly suggest that induction of LAP by IFN-γ can by itself account for the enhanced capacity of the cells to trim the 11-mer to SIINFEKL and for the increased aminopeptidase activity against model substrates.

These data also raised the question whether the IFN-induced trimming process stops once the SIINFEKL is generated and whether this peptide is stable in these extracts. Since the amount generated under our standard conditions was low or negligible, we incubated HeLa cell extracts with the 8-mer in the same molar concentration as the 11-mer. In both extracts, SIINFEKL was hydrolyzed, and in 3 h, nearly 50% disappeared. However, this process was not stimulated by IFN-γ (data not shown). These observations and related ones[3] indicate that SIINFEKL degradation does not involve LAP, which catalyzes its production from the 11-mer. Moreover, these findings indicate that in these cytosolic extracts, there is no mechanism or molecular chaperone that protects antigenic peptides from proteolytic attack, as has been suggested (40).

[3] J. Beninga, K. L. Rock, and A. L. Goldberg, submitted for publication.

DISCUSSION

Although the great majority of the peptides released by proteasomes during protein breakdown are rapidly degraded to amino acids, a fraction is used for antigen presentation (6, 7). Depending on the nature of the products produced, these peptides may either be presented without further modification (25, 27) or may have to be further trimmed by other proteases before presentation (29). In the present study, we found that cytosolic extracts of HeLa cells, like intact cells, can convert extended forms of the immunodominant ovalbumin epitope, SIINFEKL, to the presented peptide, and that this trimming process is stimulated by IFN-γ. Interestingly, these proteasome-free cytosolic extracts did not trim the various SIINFEKL-related peptides on their C termini to any significant degree. In fact, N-terminal processing accounted for over 90% of the metabolism of the 11-mer peptide in these extracts. Therefore, there appears to be very little carboxypeptidase activity in these cells. It is noteworthy that no carboxypeptidase has been described within the cytosol of mammalian cells (41), although some such activity has been reported in the ER (42, 43).

This lack of C-terminal processing provides biochemical support for our prior conclusion that the proteasome generates the correct C terminus of SIINFEKL and presumably also of most other presented peptides. Accordingly, when the C terminus of the SIINFEKL was extended by 1–15 residues, its presentation was markedly inhibited by blockers of proteasome function (29). This conclusion also is in accord with the finding that IFN-γ, by altering the proteasome's peptidase activities should enhance the production of peptides with hydrophobic and basic C termini, exactly the types of peptides that bind preferentially to the TAP transporter (15) and MHC class 1 molecules (16). By contrast, when the N terminus of SIINFEKL was extended by up to 25 residues, the cells could generate the antigenic peptide

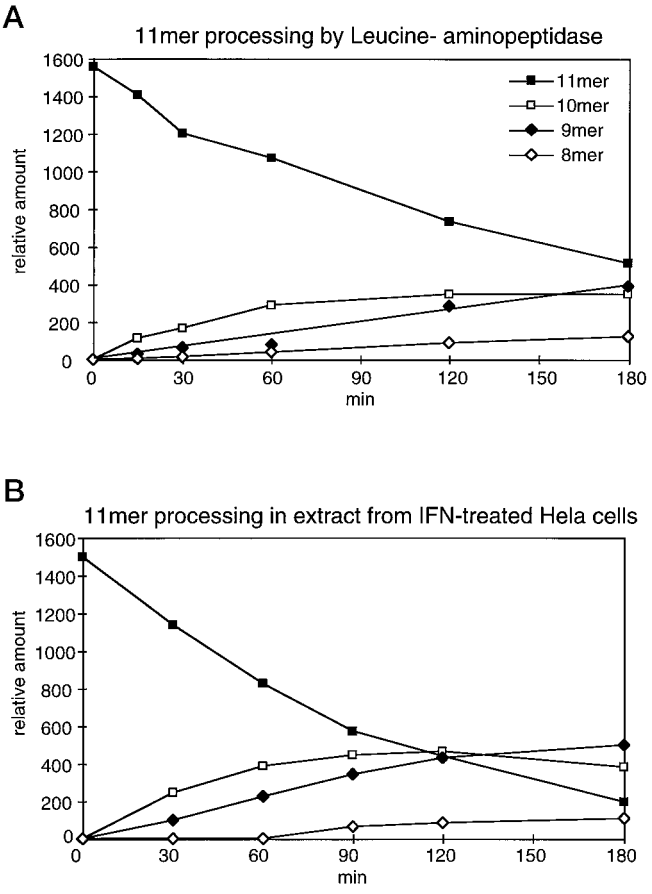

FIG. 6. **LAP can process the 11-mer QLE*SIINFEKL* to SIINFEKL.** *A,* 5 nmol of the 11-mer was incubated with 0.5 μg of pure pkLAP or with 10 μg of extract from IFN-treated HeLa cells at 37 °C for 3 h. The disappearance of the 11-mer and generation of the 10-, 9-, and 8-mer peptides were analyzed by HPLC, as described in the legend to Fig. 1. *B,* 11-mer processing in IFN-treated HeLa extract (summarized data from Fig. 2).

by a non-proteasomal mechanism; and in HeLa or U937 extracts, we found that the 11-residue precursor was shortened by progressive removal of single residues, especially in IFN-γ-treated cells. Several findings indicate that LAP is the critical enzyme in this generation of the N terminus of SIINFEKL. (i) The purified enzyme trimmed the 11-mer to peptides of 8–10 residues in a very similar manner as the extracts of HeLa cells treated with IFN-γ. (ii) This release of the N-terminal residues was inhibited by bestatin, an inhibitor of LAP, and the extent of inhibition was similar with the extract and the pure enzyme. (iii) This trimming is stimulated by IFN treatment; in fact, in the absence of IFN-γ, the antigenic peptide was not generated under the conditions used here. (iv) Although three major aminopeptidase peaks were detected in these extracts, only one, LAP, was induced by IFN-γ, and it alone was active in the processing of the 11-mer. This enzyme was identified by immunoblot analysis and sensitivity to bestatin. (v) Moreover, the substrate preference of pure porcine LAP against 19 different amino acid-AMC substrates correlated well with that of the IFN-induced enzyme in the human cell extracts. Thus, although IFN-γ may induce other peptidases, LAP induction by itself seems to account for all of the changes in peptidase activity and SIINFEKL trimming in these extracts. These findings, however, do not exclude the possibility that the non-induced aminopeptidases or endopeptidases may also be involved in processing of other antigenic peptides or in the complete digestion of most proteasomal products to amino acids.[3]

IFN-γ thus stimulates multiple steps in the MHC class I pathway, which together should have additive or synergistic effects in promoting antigen processing. In addition to inducing LAP, IFN-γ enhances the proteasome's capacity to generate peptides with the appropriate C termini and induces the PA28 proteasome activator, which seems to favor the generation of 8–10-mer peptides (28). By trimming longer epitope-containing peptides, LAP should enhance the yield of peptides of proper length for tight binding to MHC class I molecules. These findings clearly suggest a collaboration between the proteasomes and this cytosolic peptidase to enhance the efficiency of antigen presentation. The coordinated induction of the TAP transporter and MHC class I molecules by IFN-γ should further increase the number of presented peptides.

It appears likely that peptides released from the proteasome that are too long for MHC class I binding undergo N-terminal trimming in the cytosol by LAP prior to transport into the ER. The TAP complex is able to efficiently translocate peptides containing 5–16 residues (30). Once the peptides are transported, their N termini may be further trimmed in the ER (29, 32, 44), which contains aminopeptidases that can also process the N-terminal-extended SIINFEKL peptides. Alternatively, the peptides once in the ER, may be rapidly transported back into the cytosol by the ER retrograde-transport system (43, 45, 46). Unoccupied MHC class I molecules are associated with the TAP transporter, and if transported peptides are too long to bind tightly to the MHC molecule, they can be readily transported back into the cytoplasm for further trimming by LAP. Repeated aminopeptidase cycles should thus eventually generate peptides of appropriate length for association with MHC class I molecules. Once tight binding is achieved, it should prevent further transport back into the cytosol and further proteolysis.

These models apply if the antigenic peptides released by the proteasome are too large for optimal TAP transport or MHC class I binding. It remains uncertain to what extent 26 S proteasomes do in fact generate 8-mer or N-terminal-extended versions of SIINFEKL (or other antigenic peptides). Recently, Kisselev *et al.* (24)[2] found that the 20 S and 26 S proteasomes degrade proteins to oligopeptides ranging from 3 to 25 amino acids long, in clear contrast to the proposal that proteasomes generate uniformly octapeptides according to a "molecular ruler" mechanism (27, 47). Less than 15% of the products were 8 residues long, while up to 15% are 10 amino acids or longer (24),[2] and these peptides would clearly be potential substrates for processing by LAP for antigen presentation (if they have the appropriate C termini).

Such N-extended, longer versions of antigenic peptides have in fact been detected *in vivo*. For example, efforts to purify the transplantation antigen recognized by the alloreactive T-cell clone, C-2, identified both the antigenic 8-mer and a 16-mer precursor (48, 49). Similarly, Uenaka *et al.* (50) found not only the 8-mer peptide for the immunogenic BALB/c radiation-induced leukemia RLo1, but also found a 10-mer. Purified proteasomes also were found to generate N-terminal-extended peptides; *e.g.* among the peptides released during the hydrolysis of ovalbumin (29) and of 22- and 41-residue fragments of ovalbumin (27) were the N-terminal-extended 9-mer and the 11-mer studied here (29). Although these reactions were run under rather nonphysiological conditions, proteasomes clearly can generate N-extended versions of antigenic peptides. The induction of LAP by IFN-γ strongly suggests that the trimming of such peptides is a rate-limiting step in the presentation of certain antigenic peptides.

Implications for the Immunodominance of Antigenic Peptides—Antigenic proteins must contain a large number of se-

quences that can potentially be presented on MHC class I molecules, and it is unclear why the immune system mounts responses to a very limited number of these sequences (26). The present findings imply that there are 4 or 5 proteolytic processes which influence whether or not an antigenic peptide appears on the surface: (i) whether the proteasome cleaves precisely at the correct C terminus of the epitope; (ii) whether these particles release a peptide of sufficient length to bind to MHC class I molecules; (iii) whether the proteasome by chance also cleaves before the appropriate N terminus; if not, (iv) whether the N-terminal residues flanking the epitope can be removed efficiently by aminopeptidases. As shown in Table I, the presence of certain amino acids in the N terminus of a peptide can make it a very good or very poor substrate for cytosolic aminopeptidases. In fact, the N-terminal residues differ up to 20-fold in their susceptibilities to exopeptidases and to IFN-stimulated hydrolysis. Therefore, the presence of residues which are released faster by LAP in the N-terminal-extended region would lead to faster trimming, while residues which are poorly released should slow down its trimming to a size that binds to MHC class I molecules; (v) whether cytosolic endopeptidases or exopeptidases destroy the correct epitope once formed.[3] As noted here, SIINFEKL is susceptible to cytosolic proteases, and it is noteworthy that this degradation, unlike SIINFEKL generation, is not stimulated by IFN-γ. Thus, this proteolytic step which may limit antigen presentation seems to involve distinct protease(s) from those involved in epitope generation.[3] Identity of the enzyme(s) that degrade antigenic peptides will be important to establish. It is also noteworthy that we found no evidence for a peptide-binding chaperone (40) that might bind and protect the mature peptide from further digestion.

The finding that LAP may be important in the generation of antigenic peptides leads to the novel idea that the susceptibility of the N-terminal flanking residues to trimming by this enzyme could have an impact on its immunodominance under basal conditions and also influence whether presentation is stimulated by IFN-γ. These predictions should be testable by mutagenesis of upstream regions of antigenic peptides or by genetic alterations in LAP level. These arguments also raise the possibility that amino acids, which are poorly released, may be found in disproportionate amounts at the N terminus of antigenic peptides to retard further cleavages. Rigorous examination of these possibilities, *i.e.* of the importance of LAP in processing antigenic peptides generally and in determining immunodominance of different peptides, must await cell-free reconstitution of the entire process for generation of antigenic peptidase.

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