Specific Proteolytic Cleavages Limit the Diversity of the Pool of Peptides Available to MHC Class I Molecules in Living Cells

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Specific Proteolytic Cleavages Limit the Diversity of the Pool of Peptides Available to MHC Class I Molecules in Living Cells[1]

Thomas Serwold and Nilabh Shastri[2]

MHC class I molecules display peptides selected from a poorly characterized pool of peptides available in the endoplasmic reticulum. We analyzed the diversity of peptides available to MHC class I molecules by monitoring the generation of an OVA-derived octapeptide, OVA$_{257-264}$ (SL8), and its C-terminally extended analog, SL8-I. The poorly antigenic SL8-I could be detected in cell extracts only after its conversion to the readily detectable SL8 with carboxypeptidase Y. Analysis of extracts from cells expressing the minimal precursor Met-SL8-I by this method revealed the presence of SL8/K^b and the extended SL8-I/K^b complexes, indicating that the peptide pool contained both peptides. In contrast, cells expressing full length OVA generated only the SL8/K^b complex, demonstrating that the peptide pool generated from the full length precursor contained only a subset of potential MHC-binding peptides. Deletion analysis revealed that SL8-I was generated only from precursors lacking additional C-terminal flanking residues, suggesting that the generation of the C terminus of the SL8 peptide involves a specific endopeptidase cleavage. To investigate the protease responsible for this cleavage, we tested the effect of different protease inhibitors on the generation of the SL8 and SL8-I peptides. Only the proteasome inhibitors blocked generation of SL8, but not SL8-I. These findings demonstrate that the specificities of the proteases in the Ag-processing pathway, which include but are not limited to the proteasome, limit the diversity of peptides available for binding by MHC class I molecules in the endoplasmic reticulum. *The Journal of Immunology,* **1999, 162: 4712–4719.**

C D8$^+$ T cells recognize and kill virally and bacterially infected cells as well as tumors and tissue transplants that display foreign peptides bound to MHC class I molecules on their cell surface. The peptides presented by MHC class I molecules are generated by proteolytic processing of intracellular precursors, and represent a very complex mixture (1–3). Yet, analysis of processed peptides in cell extracts, by direct microsequencing or by functional T cell assays, has revealed that individual peptides presented by MHC class I molecules are cleaved to specific lengths and have conserved features (4–7). For example, the K^b MHC molecule, in cells expressing the OVA precursor, presents the octapeptide SIINFEKL (SL8)[3] (8, 9). The sequence of this SL8 peptide conforms exactly to the octapeptide consensus motif, xxxx(F,Y)xx(I,L,M), which is shared by most peptides presented by K^b (10). How cells generate a diverse set of peptides to satisfy the varying preferences of length and consensus sequence motifs for the polymorphic MHC class I molecules is not understood.

Successful presentation of a given peptide/MHC complex on the cell surface from an endogenously synthesized precursor depends upon three key events (reviewed in Refs. 1 and 11–14). First, the precursor proteins are fragmented in the cytosol by proteases including the multicatalytic proteasome (15). Second, these peptide fragments are transported into the endoplasmic reticulum (ER) via the TAP transporter (16, 17). Third, the peptides bind to MHC class I molecules in ER, a process that involves transient associations with the calreticulin/calnexin chaperones, the thiol oxidoreductase, ERp57, and with the TAP and tapasin molecules (18–25). The resulting peptide/MHC class I complexes are then transported to the cell surface via the constitutive secretory pathway (26).

Many naturally processed peptides generated by the Ag-processing pathway and presented by MHC class I molecules have been identified, but the mechanisms that produce them are poorly understood. It is possible that the cleavage of a given precursor by the multicatalytic proteasome and other cytosolic proteases generates a large set of random 8- to 10-mer peptides which via TAP transport are made available for binding to the MHC molecules in the ER. It is also possible that the number and diversity of peptides available to MHC molecules is severely limited by the selectivity of proteases, TAP transport, or the MHC-loading mechanisms (27, 28). Consistent with the latter model, in vitro assays have indicated preferences in proteasomal cleavage after basic and hydrophobic amino acids (29, 30). These are the same C-terminal residues of the majority of MHC class I-bound peptides (10). TAP may further restrict the peptide pool by preferentially transporting peptides between 8 and 12 amino acids in length (31). While human TAP is relatively nonselective in the sequences of the peptides it transports, murine TAP, as well as the product of one rat TAP allele, preferentially transports peptides containing hydrophobic C termini (32). These preferences are reflected in the pool of peptides presented by MHC class I of cells expressing these TAP alleles (10, 33). Together, the preferences of the proteasome and TAP correlate well with the observed characteristics of the MHC-bound

peptide pool, and provide a potential explanation for why certain peptides are immunodominant (34, 35). Yet, in vitro assays also show that cleavages can occur at multiple sites within the precursors, and that suboptimal peptides can be transported into the ER (34, 36, 37), implying that a large set of peptide fragments may be available for MHC binding. These two models have not been resolved, largely because of the difficulties associated with analyzing nonantigenic peptides and peptides present at a low abundance present in living cells.

In this study, we address the diversity of the peptide pool by analyzing the processing of OVA to the SL8 peptide and its extended analogs. We compared the generation of two possible peptide products from OVA: SL8 and its C-terminally extended analogue, SL8-I. To enable detection of the poorly antigenic SL8-I peptide, we treated peptides extracted from cells with carboxypeptidase Y (CPY), which cleaves off the C-terminal isoleucine residue, liberating the readily detectable SL8 peptide. Use of this method revealed that SL8-I was generated in cells, but only from precursors with SL8-I at the C terminus with no additional flanking residues. Notably, generation of SL8 from these precursors was blocked by proteasome inhibitors while generation of SL8-I was proteasome independent. Our results provide evidence for multiple proteases in the Ag-processing pathway, and indicate that the diversity of peptides presented by MHC class I molecules is limited by the specificities of these proteases.

Materials and Methods

Cell lines, plasmid DNAs, and synthetic peptides

β-Galactosidase (LacZ)-inducible, SL8/K^b-specific B3Z T cell hybrid, K^b-L, and COS-7 (COS) cell lines have been described (38). Cell lines were maintained in RPMI 1640 with 10% FCS, 2 mM glutamine, 1 mM pyruvate, 50 μM β-mercaptoethanol, 200 U/ml penicillin, and 200 μg/ml streptomycin. Plasmid DNA constructs encoding the minigene precursors methionine (Met)-SL8-X were either previously described (39, 40), or were prepared by inserting complementary synthetic oligonucleotides into the *Bst*XI and *Xba*I sites of the pcDNAI vector (Invitrogen, San Diego, CA). All plasmids encoding mutated or truncated OVA constructs were derived from the full length OVA cDNA subcloned into the *Eco*RI site of the pCDNAI vector. The OVA(T265I) mutant was made by inserting complementary oligonucleotides into the *Stu*I and *Ava*I sites of full length OVA, which introduced an AUC (isoleucine) codon at codon 265, and a silent *Hin*dIII site at amino acids 263–264. OVA$_{1-265}$(T265I), lacking all C-terminal residues flanking SL8-I was made by cutting the pCDNAI/OVA(T265I) with *Hin*dIII and *Xba*I and inserting complementary oligonucleotides into the site encoding 263–265 followed by a stop codon (TAG). OVA$_{257-386}$(T265I), lacking all N-terminal residues flanking SL8-I was constructed by digesting pCDNAI/OVA(T265I) with *Bst*XI and *Hin*dIII and inserting complementary oligonucleotides encoding Met and residues 257–263. Synthetic peptides were prepared using standard f-moc chemistry on an Applied Biosystems (Foster City, CA) automated peptide synthesizer. All peptides were purified by HPLC, their concentrations determined by UV absorbance, and their identity confirmed by mass spectrometry. Peptide sequences in their single letter code are SIINFEKL (SL8), MSIINFEKL (M-SL8), MSIINFEKLI (M-SL8-I), MSIINFEKLV (M-SL8-V), MSIINFEKLA (M-SL8-A), MSIINFEKLT (M-SL8-T), and SIINFEKL-I, -A, -T, OR -V.

Peptide/MHC binding assays

Relative peptide-binding ability of K^b MHC was measured in a competitive peptide-binding assay described previously (9). Briefly, T2-K^b cells were incubated with varying concentrations of competitor peptides in PBS in 2% FCS for 20 min on ice. FITC-conjugated SL8 was then added at concentration of approximately 1 nM for an additional 20 min. Cells were washed and analyzed for their FITC fluorescence by flow cytometry.

Protease inhibitors

COS cells electroporated with expression vectors encoding K^b and OVA$_{138-265}$(T265I) (5 μg of each) were treated 4 h after transfection with the indicated inhibitors. Leu-chloromethylketone (LCMK) and Ala-Ala-Phe-chloromethylketone (AAF-CMK) (Sigma-Aldrich, St. Louis, MO) and lactacystin (Calbiochem-Novabiochem, San Diego, CA) were used at 10

μM. E64 (Sigma-Aldrich) was used at 50 μM. Cells were harvested at 12 h after addition of the inhibitors and peptides were extracted by boiling for 10 min in 10% acetic acid + 10 μM nonspecific peptide to minimize nonspecific loss of Ag during the procedure. Extracts were run through a 10-kDa cutoff filter and separated by HPLC. Fractions were dried and treated with CPY as described below and assayed for B3Z-stimulating activity.

Transient DNA transfections and peptide extraction

Cesium chloride-purified plasmid DNAs were used in all transfection experiments. For peptide extraction experiments, 1×10^7 COS cells were electroporated at 650–700 V with 5 μg of each plasmid DNA in 270 mM sucrose, 7 mM sodium phosphate, and 1 mM MgCl$_2$, using the BTX Transfector 800 (Biotechnologies and Experimental Research, San Diego, CA). Cells were harvested 2 days after electroporation. Total cellular peptide extracts were prepared by lysing cells in 500 μl 0.1% trifluoroacetic acid (TFA) in water as described (9), or by lysing cells in 500 μl 10% acetic acid and boiling for 10 min. The samples were then spun down in a microfuge and the supernatants were passed through a 10-kDa cutoff filter (Millipore, Bedford, MA). The filtrate was either dried in a vacuum centrifuge (Savant Instruments, Holbrook, NY) and then resuspended in 0.1% TFA or injected directly into the HPLC.

T cell activation assays

The Ag-specific T cell response was measured as the β-galactosidase activity induced in the T cell hybrids following Ag-specific activation (38, 41). T cell hybrids were cocultured with K^b-L cells in wells with synthetic peptides or the resuspended HPLC fractions for 12–16 h. The β-galactosidase activity induced in the T cells was detected by the addition of substrate chlorophenol red-β-D-galactopyranoside (CPRG). After 1–5 h, the absorbance of the cleaved chlorophenol red in the wells was measured at 595 nm with 635 nm as the reference wavelength. Data shown are representative of at least three independent experiments.

HPLC analysis

Peptides and cellular peptide extracts were fractionated on a Hewlett Packard 1050 quaternary pump HPLC using a 4.6 × 250-mm C18 column with 5-μm particle size and 300 Å pores (Vydac, Hesperia, CA). Samples were separated using an acetonitrile:H$_2$O gradient with TFA as the ion-pairing agent. Buffers used were 0.1% TFA in H$_2$O (buffer A) and 0.1% TFA in acetonitrile (buffer B). The following protocol was used to separate the peptides: 0–5 min, 23% B; 5–35 min, 23–38% B; and 35–40 min, 38–77% B. One or 0.5 ml fractions were collected during the indicated times, dried in a vacuum centrifuge, resuspended in 30 μl PBS, and used in the T cell activation assay. For carboxypeptidase treatment, the fractions were resuspended in 30 μl PBS and 2% CPY-conjugated beads (Pierce Chemical, Rockford, IL). Samples were incubated at room temperature for 2 h and were then centrifuged to remove the CPY beads. The supernatants were tested for B3Z-stimulating activity using K^b-L cells as APC as described above. Mock injections of 300 μl 0.1% TFA/H$_2$O were performed before each sample run and assayed in parallel to ensure absence of any cross-contamination from previous runs. Retention times were reproducible within 0.5 min between runs.

Results

Analysis of naturally processed SL8 peptide and its analogs

To determine if extended products are generated in the Ag-processing pathway, we first tested whether our T cell-based assay was capable of detecting these longer peptides. In K^b-expressing cells synthesizing the Met-SIINFEKL-I (M-SL8-I) precursor, a single amino acid is cleaved from the N and C termini to yield the octapeptide product SL8 bound to K^b (Fig. 1*A*) (40). In the T cell activation assay using K^b-L cells to present exogenous antigenic peptides to the B3Z T-cell hybridoma that specifically recognizes SL8 bound to K^b, the SL8 peptide is readily detected by the T cell response at a concentration of <10 pM (Fig. 1*B*). In contrast, extended SL8 analogs are far less active. The N-terminally extended M-SL8, the C-terminally extended SL8-I, and both N- and C-terminally extended M-SL8-I peptides were, respectively, about 30-, 1000-, and 30,000-fold less active than SL8 in this assay.

To distinguish whether the higher detection limit of the extended SL8 analogs was due to their failure to bind K^b or to the
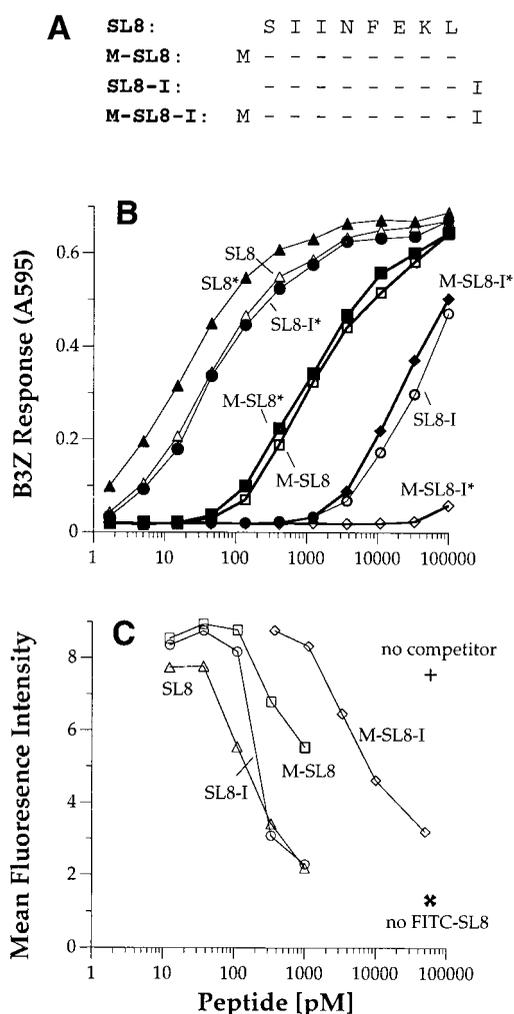
FIGURE 1. T cell-stimulating and K[b] MHC-binding capacity of SL8 and its N- and/or C-terminally extended analogs. *A* Amino acid sequences, in single letter code, of SL8 and its extended analogs abbreviated as M-SL8, SL8-I and M-SL8-I. *B*, Stimulation of SL8/K[b]-specific LacZ-inducible B3Z T cells by varying concentrations of the indicated synthetic SL8 analogs. The peptides were either left untreated (open symbols) or were treated with CPY prior to the assay (asterisk superscript, closed symbols). The peptide dilutions were incubated with K[b]-L cells (3×10^4) and B3Z cells (1×10^5) overnight. Accumulation of LacZ in activated B3Z cells was detected by incubating culture lysates with the LacZ substrate, CPRG, and measuring the absorbance of the product at 595 nm. *C*, Relative K[b]-binding capacity of synthetic SL8 analogs in a competitive K[b]-binding assay. Varying concentrations of the indicated peptides, and a constant concentration of FITC-labeled SL8 were incubated with T2-K[b]. The cells were washed and their FITC fluorescence was measured by flow cytometry. Data are plotted as the mean fluorescence intensity vs the concentration of competitor peptides.

FIGURE 2. CPY treatment converts SL8-I to SL8 and enables its detection in complex cell extracts. K[b]-HeLa cells (5×10^6) were spiked with 0.17 pmol of synthetic SL8-I peptide and immediately extracted by TFA as described in Materials and Methods and split into three parts. Two-thirds of the TFA extract was fractionated by reverse phase HPLC. The fractions were dried and resuspended in PBS alone (*A*) or PBS + CPY (*B*) before assaying for B3Z T cell-stimulating activity with K[b]-L cells as APC. *C*, The final third of the TFA extract was treated with CPY before HPLC fractionation, and the fractions were assayed for the presence of B3Z-stimulating activity. Mock injections of sample buffer alone were also HPLC fractionated under identical conditions before each run and assayed in parallel to demonstrate the absence of any contaminating activity from previous runs (open symbols). The elution times of SL8 and SL8-I peptides, indicated by vertical arrows, were determined by HPLC fractionation of synthetic peptides run and assayed under identical conditions.

lack of recognition of the K[b]-bound peptides by the B3Z hybridoma, we tested the ability of each peptide to bind K[b] in a competitive-binding assay. The K[b]-specific binding of FITC-conjugated SL8 on the surface of TAP-negative T2-K[b] cells can be specifically inhibited by unlabeled peptides according to their relative K[b]-binding ability and measured by flow cytometry (9). Surprisingly, the C-terminally extended SL8-I peptide bound K[b] as well as SL8 despite being 1000-fold worse in stimulating the T cell response (Fig. 1 *B* and *C*). The binding activity of M-SL8 was intermediate while the decapeptide, M-SL8-I, was about a hundred-fold less efficient in binding K[b] than SL8 or SL8-I. These

results show that the low T cell-stimulating activity of the longer SL8 analogs, especially SL8-I, is due to poor T cell recognition of the peptide/K[b] complexes, rather than a failure of the peptides to bind K[b].

The poor T cell stimulatory capacity of the C-terminally extended SL8 analogs would normally preclude their detection in the B3Z T cell-based assay in the complex peptide mixtures extracted from cells. To facilitate the detection of these C-terminally extended peptides, we treated them with CPY, a yeast carboxypeptidase that cleaves single amino acids from the C termini of its substrates (42, 43). Treatment of SL8-I or MSL8-I with CPY markedly improved the ability to detect these peptides with the SL8/K[b]-specific B3Z hybridoma (Fig. 1*A*). It is possible that CPY also destroys a fraction of the SL8 peptide during digestion because of its broad specificity, but even if CPY cleaved one carboxyl-terminal residue from 50% of both the SL8 and SL8-I epitopes, it would lead to only twofold reduction in the amount of detectable SL8, but a 500-fold increase in detection limit of SL8-I. Thus treatment with CPY dramatically improved the ability to detect the C-terminally extended analogs of the SL8 peptide.

We next tested whether CPY treatment would enable detection of the SL8-I peptide when it was present in a typical cell extract containing a large diversity of peptides. K[b] HeLa cells were spiked with the SL8-I peptide and were immediately extracted with TFA. The low m.w. material of the TFA extract was fractionated by

FIGURE 3. SL8-X is present in cells expressing M-SL8-X precursors and K^b. COS cells were transfected with constructs encoding M-SL8-I with *A* or without *B* a cDNA encoding K^b. Two days later the total peptide pool of the transfected cells was extracted with TFA, fractionated by HPLC, and each fraction was tested for B3Z-stimulating activity with (closed symbols) or without (open symbols) CPY treatment as described in Materials and Methods and the legend to Fig. 2. *C* to *E*, HPLC fractionation and B3Z stimulation of extracts from cells transfected with K^b and M-SL8-V, M-SL8-A, or M-SL8-T DNA constructs. All fractions were treated with CPY. The elution times of synthetic peptides are indicated by vertical arrows. Fractions collected from one of the mock runs are shown in *E*. The small activity peak indicated by the asterisk is a contaminating activity from a previous run.
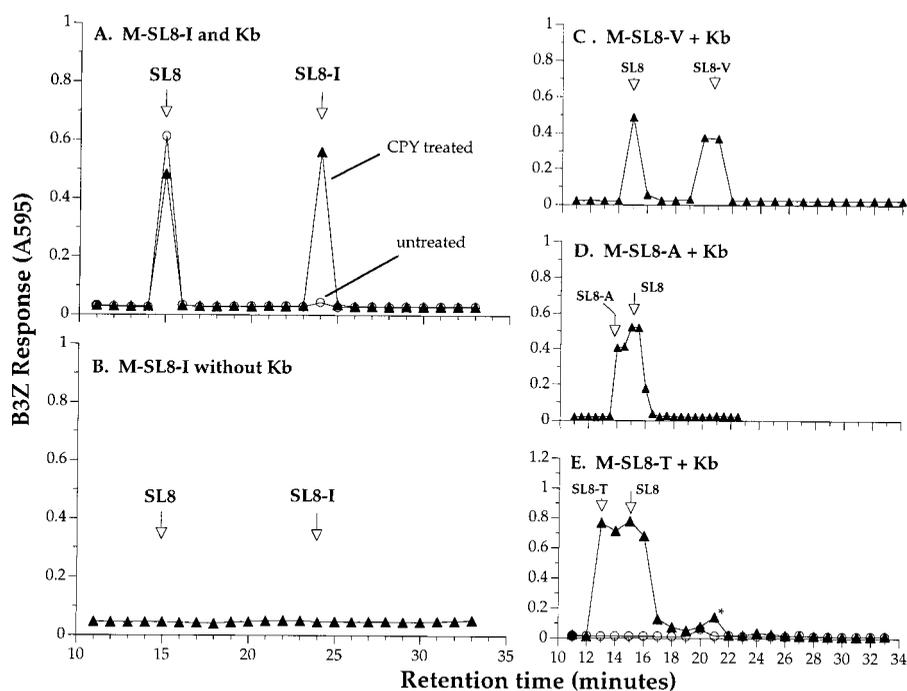
reverse phase HPLC and each fraction was assayed for T cell-stimulating activity with or without CPY treatment. As expected from the poor stimulatory capacity of SL8-I, no B3Z-stimulating activity was detected in the untreated fractions (Fig. 2*A*). However, when each fraction was treated with CPY prior to the assay, a single peak of activity was detected that comigrated with the synthetic SL8-I peptide (Fig. 2*B*). When the SL8-I-spiked extract was treated with CPY prior to the HPLC run, the single-activity peak now comigrated with SL8 rather than with SL8-I peptide, confirming that the CPY treatment converted the SL8-I nonapeptide to SL8 (Fig. 2*C*). Thus CPY treatment together with HPLC fractionation enabled the detection and identification of SL8 extended at its C terminus with Ile as well as with Ala, Val, or Thr (data not shown) in complex cell extracts.

C-terminally extended SL8 analogs are presented by K^b in cells expressing minimal precursors

The observation that the C-terminally extended analogs of SL8 bind to K^b led us to ask whether cells expressing the appropriate precursors could process and present these longer peptide/K^b complexes. We extracted peptides from COS cells transfected with the M-SL8-I construct and analyzed the HPLC-fractionated peptides for T cell-stimulating activity. Each fraction was treated with CPY or left untreated prior to the T cell stimulation assay. As expected from previous analysis of M-SL8-I/K^b transfectants (40), only a single peak corresponding to the SL8 octapeptide was found in untreated fractions. In contrast, fractions treated with CPY contained an additional peak of activity that coeluted with the SL8-I nonapeptide, demonstrating that cells can generate both SL8 and SL8-I from the M-SL8-I precursor (Fig. 3*A*). Neither SL8 nor SL8-I was found in extracts from cells lacking K^b, indicating that the activity peaks in the M-SL8-I/K^b transfected cells represent K^b-bound peptides (Fig. 3*B*), consistent with previous observations that antigenic peptides must be bound to a stabilizing MHC molecule in order to be present in detectable quantities (9, 44). Analysis of peptides in anti-K^b immunoprecipitates, as well as in acid eluates of intact cells (45), confirmed that these peptides were bound to K^b and were present on the cell surface (data not shown).

Furthermore, K^b-transfected cells expressing other precursors, such as M-SL8-V, M-SL8-A or M-SL8-T also generated SL8-V, SL8-A, and SL8-T peptides, respectively, in addition to SL8 (Fig. 3, *C* to *E*), showing that, in living cells, TAP can transport and K^b can bind peptides with a number of different amino acids at the C terminus. This observation is consistent with the recent analysis of TAP function in vivo (46). Thus, both SL8 and its C-terminally extended SL8-X analogs are contained within the pool of naturally processed peptides in cells expressing the M-SL8-X constructs.

The generation of the SL8-I/K^b complexes is abrogated by additional C- but not N-terminal flanking residues

The discovery of an extended analog of an antigenic peptide bound to K^b was surprising given the results from peptide pool sequencing, which showed that the vast majority of peptides presented by K^b are precisely cleaved octamers (10, 47). We speculated that the generation of SL8-I from longer precursors might be limited by protease specificity in the processing pathway. To answer this question, we generated full-length OVA constructs in which Thr, the natural C-terminal flanking residue of SL8, was replaced by Ile. This change was introduced because SL8-I and SL8 were well resolved by reverse phase HPLC, allowing us to easily differentiate between these two potential products. TFA extracts of K^b-expressing COS cells transfected with the modified OVA (T265I) construct were fractionated by HPLC, treated with CPY, and assayed for T cell-stimulating activity. As shown previously, the SL8 octapeptide was readily detectable in HPLC fractions derived from cells expressing the full length precursor (8, 9). However, in contrast to the results with the minimal precursors, the SL8-I nonapeptide was not generated in detectable levels (Fig. 4*A*). Estimation of peptide amounts based on standard curves run in parallel indicated that SL8-I, if generated at all, was present at less than 0.1% of the SL8 level in cells expressing the OVA(T265I) precursor. This is opposed to the results from the M-SL8-I precursor, which yielded roughly equivalent amounts of SL8 and SL8-I peptides (Fig. 3*A*). Thus, in contrast to the M-SL8-I precursor, processing of OVA(T265I) yielded exclusively the SL8 octapeptide
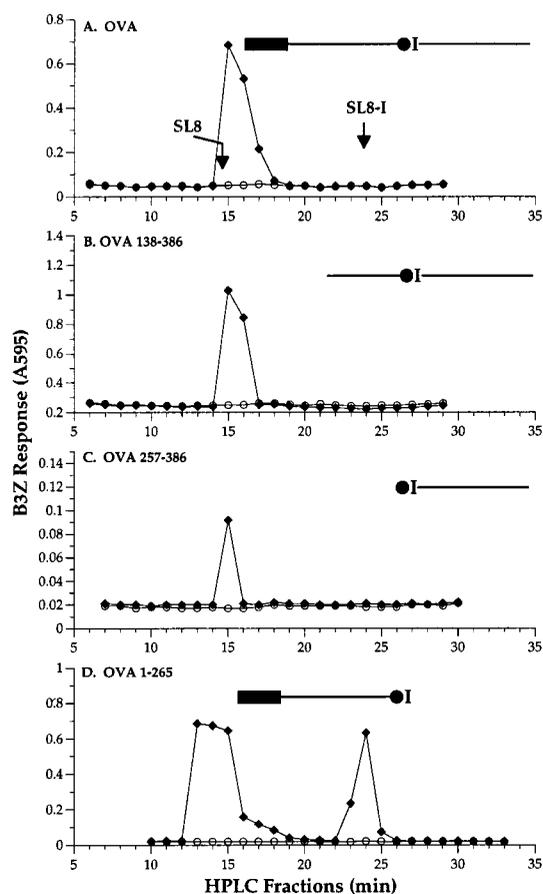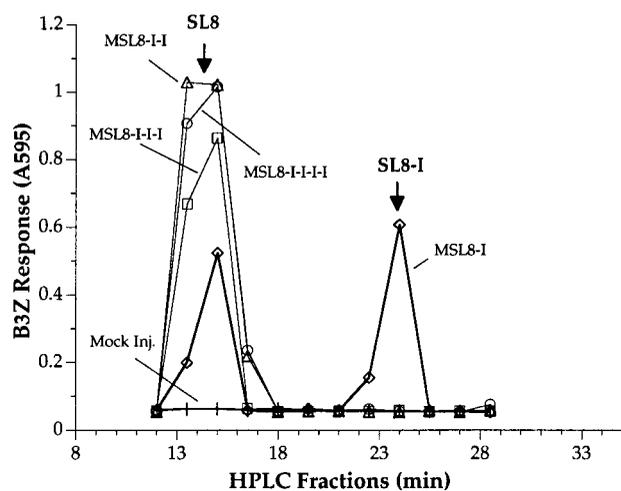
FIGURE 4. Naturally processed SL8-I is generated only from precursors with N- but not C-terminal flanking residues. TFA extracts, from COS cells transfected with cDNAs encoding K^b and different precursors, were fractionated by HPLC and tested for their B3Z-stimulating activity after CPY treatment as described in Materials and Methods and in the legend to Fig. 2. *A* Full length OVA(T265I), *B*, OVA$_{139-386}$(T265I), *C*, OVA$_{257-386}$ (T265I), and *D*, OVA$_{1-265}$(T265I). Each construct is also shown schematically indicating the location of the SL8-I sequence (shown as I) relative to the N- and C-terminal flanking residues (lines). The ER translocation signal at the N terminus is indicated by a shaded box. B3Z-stimulating activity in fractions from buffer samples injected before each run are shown by open circles.


FIGURE 5. The generation of SL8-I peptide can be abrogated by even one additional C-terminal flanking residue. TFA extracts, from COS cells transfected 2 days earlier with cDNAs encoding K^b and different precursors, were fractionated by HPLC and tested for their B3Z-stimulating activity after CPY treatment as described in Materials and Methods and in the legend to Fig. 2. M-SL8-I (*A*), M-SL8-I-I (*C*), M-SL8-I-I-I (*G*), or M-SL8-I-I-I-I (*E*). The elution time of synthetic SL8 and SL8-I peptides are shown by vertical arrows. The lack of B3Z-stimulating activity in fractions from buffer-alone samples (Mock Inj) run before the experimental runs indicates absence of cross-contamination between samples.

product, demonstrating a clear difference in the processing outcomes from the two precursors. Similarly, SL8, but not SL8-V, was obtained with Val as the C-terminal-flanking residue in full length OVA (Data not shown). We conclude that the intracellular pool of peptides generated from full length OVA protein was highly biased and represents only a subset of all the potential peptides capable of being presented by MHC class I molecules.

Generation of the SL8-I/K^b complex depends upon the absence of C- but not N-terminal flanking residues

Why was SL8-I generated in cells expressing M-SL8-I but not in cells expressing full length OVA(T265I)? One possibility was that the generation of SL8-X was dependent upon the intracellular location of the precursor, since full-length OVA is a secreted protein that is cotranslationally translocated into the ER (39, 48), while the M-SL8-X decapeptide does not contain an ER translocation signal and is therefore expected to reside in the cytoplasm. To test this possibility, a deletion construct lacking the ER translocation signal, OVA$_{139-386}$(T265I), was cotransfected into COS cells with K^b MHC molecule and the peptides were acid extracted from these

cells, separated by HPLC, and analyzed for the presence of both SL8 and SL8-I. The cytosolic precursor was similar to the secreted OVA(T265I) in yielding only the SL8, but no detectable SL8-I peptide (Fig. 4*B*). Therefore, differences in intracellular location of the precursor did not account for the inability to generate the processed SL8-I peptide.

Next, we determined which flanking residues affected the generation of the SL8-I peptide by testing constructs in which all the N- or the C-terminal flanking residues were eliminated. Removal of the N-terminal flanking residues from whole OVA(T265I), although decreasing the overall recovery of the SL8 peptide, did not yield detectable amounts of the SL8-I nonapeptide (Fig. 4 *C*). In contrast, the precursor OVA$_{1-265}$(T265I), which lacks all C-terminal flanking residues, was processed to both SL8 and SL8-I (Fig. 4*D*). Likewise, other cytosolic constructs such as OVA$_{138-265}$ (T265I) or a 1025 residue fusion protein β-galactosidase-SL8-I also yielded both SL8 and SL8-I (data not shown). We conclude that generation of SL8-I product was prevented by additional C-terminal flanking residues, but was not affected by even a thousand additional N-terminal flanking residues.

It was possible that either the length of the C-terminal extension or the nature of the flanking amino acids induced the specific cleavage at the C terminus of SL8. To determine the length of C-terminal extension required for defining the specificity of cleavage at the C terminus, we analyzed peptide extracts from cells expressing K^b plus a series of constructs containing one, two, three, or four flanking Ile residues. In striking contrast to M-SL8-I, which was processed to both SL8 and SL8-I, other precursors containing one or more additional flanking residues (M-SL8-I-I, M-SL8-I-I-I, and M-SL8-I-I-I-I) were all processed specifically to SL8, but not to SL8-I. The amount of SL8 generated was also greater from M-SL8-I-I, -I-I-I, and -I-I-I-I, suggesting that the length of the C-terminal extension can affect the efficiency of cleavage at the C terminus (Fig. 5). Similar results were obtained when SL8 was extended by T, TE, TEW, or TEWT as in native OVA (data not shown). Since alternate products such as SL8-I-I,
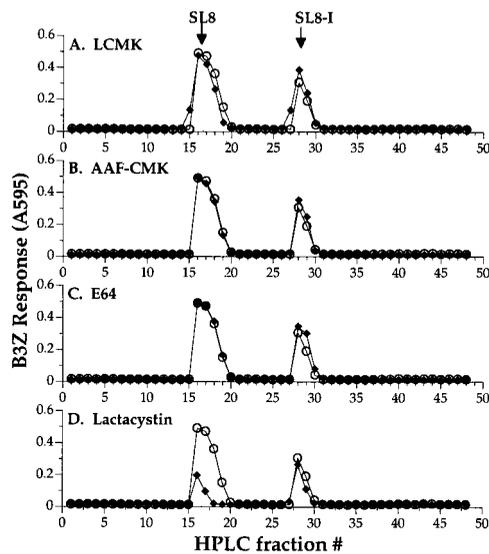
FIGURE 6. Generation of SL8, but not SL8-I, from $OVA_{139-265}(T265I)$ is blocked by the proteasome inhibitor, lactacystin. COS cells transfected with $OVA_{139-265}I$ and K^b were left untreated (open symbols) or were treated with the indicated inhibitors at 4 h posttransfection for 12 h (closed symbols). Cells were treated with 10 μM LCMK, (*A*), 10 μM AAF-CMK (*B*), 50 μM E64 (*C*), or 10 μM lactacystin (*D*), Peptide extracts from these cells were separated by HPLC and fractions were treated with CPY. Graphs represent the B3Z-stimulating activity of each HPLC fraction.

SL8-I-I-I, AND SL8-I-I-I-I might not be detectable by CPY treatment alone, the existence of these peptides in the extracts is presently uncertain. Nevertheless, these results indicate that generation of SL8-I is blocked by the presence of even a single C-terminal flanking residue, suggesting that the C terminus of SL8 is generated by an endopeptidase, which preferably cleaves at its C-terminal leucine.

Proteasome inhibitors block generation of SL8, but not SL8-I

The preceding results indicated that a specific cleavage generates the C terminus of the SL8 peptide. To investigate which protease might make this specific cleavage, we tested the effects of different protease inhibitors on the generation of both SL8 and SL8-I. COS cells were transfected with K^b as well as the cytoplasmic $OVA_{138-265}(T265I)$ precursor. Four hours posttransfection, protease inhibitors were added and the cells were cultured for an additional 8 h before processed peptides were acid extracted and analyzed by HPLC fractionation. Lactacystin, an inhibitor with specificity for the proteasome (49), almost entirely blocked the generation of SL8, but failed to block generation of SL8-I (Fig. 6). Similar effects were seen with the peptide aldehyde inhibitors of the proteasome, LLnL and MG132 (data not shown). In contrast, neither the serine protease inhibitor AAF-CMK, the cysteine protease inhibitor E64, or the aminopeptidase inhibitor LCMK had any measurable effect on the generation of SL8 or SL8-I. These results indicate that the C terminus of SL8 is generated by a specific proteasomal cleavage. Generation of SL8-I, however, is not inhibited by the proteasome inhibitors or any of the other inhibitors tested here, suggesting that it is made by a different protease or proteases that are either resistant or inaccessible to these inhibitors.

Discussion

We have analyzed the complexity of the peptide pool supplied to MHC class I molecules by monitoring the differential generation of a minimal antigenic peptide and its C-terminally extended analog.

In order to detect C-terminally extended, poorly antigenic peptides, we digested samples with CPY, converting peptides with single amino acid extensions to the optimally active SL8 peptide and thus enabling their detection with high sensitivity. Using this technique, we found that peptides with single C-terminal amino acid extensions could be generated in cells and presented by K^b MHC class I molecules. Interestingly, however, these peptides were not generated from precursors containing additional C-terminal flanking residues. Furthermore, the generation of SL8, but not SL8-I, was blocked by specific inhibitors of the proteasome. These results indicate that the pool of peptides supplied to MHC class I molecules is biased, and that this bias is largely due to the specificity of the proteases involved in the generation of the peptides.

Studies of MHC class I-bound peptides have revealed that they are cleaved to specific lengths dictated by the particular MHC class I molecules that present them (4–7). In this study, however, we found that the C-terminally extended SL8-I, A, V, and T nonamers can be presented by K^b, and appear to be able to bind relatively well to K^b compared with the minimal SL8 peptide. The existence of extended peptides bound to K^b in this study suggests that the predominance of SL8 presented by K^b is due largely to the specificity of the proteases that cleave OVA, rather than to the exclusive binding ability of SL8 to K^b.

Our previous studies showed that peptide precursors containing single residue C-terminal, but not N-terminal, extensions were inefficiently processed to SL8 when expressed in cells (40). This can now be explained at least partially by the fact that the C-terminally extended peptides can bind and be presented by K^b, thereby preventing them from conversion to SL8. It is unclear, however, whether all C-terminally extended forms of SL8 can bind to K^b, partly because the mode of the binding of the extended peptides is not known. It is possible that they lie in an extended conformation with the C terminus sticking out of the groove. Alternatively, the peptides could sit in the groove in a "bulge" conformation with both ends buried in the conserved pockets. Both of these binding modes have been observed in crystal structures of peptide/MHC class I complexes (50–52), and would likely alter the conformation of the MHC/peptide complex so that it would be inefficiently recognized by the SL8/K^b-specific T cells. Whatever the binding mode of C-terminally extended peptides, their binding apparently precludes further processing, and therefore prevents generation of the optimal SL8 peptide.

Interestingly, while SL8-I is capable of binding K^b, it was not generated from the full length precursor, or from any precursor containing even a single additional C-terminal flanking residue. The failure to generate SL8-I from precursors with additional C-terminal flanking residues indicates that SL8-I or any N-terminally extended form of SL8-I is not an intermediate in the generation of SL8, since such intermediates would also lead to the generation of K^b/SL8-I complexes. The lack of generation of SL8-I from M-SL8-I-I is also consistent with reports suggesting that there is little if any carboxypeptidase activity in the Ag processing pathway (33, 53). Since the vast majority of MHC class I-bound peptides identified to date are derived from precursors with both N- and C-terminal extensions, the cleavage specificities of the proteases involved may limit the generation of extended versions of antigenic peptides from most precursors. While this decreases the diversity of potentially antigenic peptides, biased cleavages would also be expected to increase the overall abundance of the peptides that are presented. Indeed, as shown by Pamer and colleagues (54), the efficiency of Ag processing can be quite high, on the order of one antigenic peptide displayed on the surface for every five protein molecules degraded.

Which proteases generate the antigenic peptides? With a panel of protease inhibitors, we determined that processing of a C-terminally extended precursor, $OVA_{139-265}$(T265I), to SL8 was almost entirely blocked by the proteasome inhibitor, lactacystin. The small amount of SL8 that was found in cells treated with lactacystin may have been generated because the proteasome was not completely inhibited during the entire course of the transfection. Alternatively, it is also possible that another protease is capable of generating SL8 in the inhibitor-treated cells, albeit inefficiently. In contrast to SL8, the generation of SL8-I from the truncated OVA precursor, which required only N-terminal cleavages, was not affected by proteasome inhibitors. While the proteolytic activity of the proteasome is not necessary to generate SL8-I, the proteasome or its associated proteins may still play a role in the generation of SL8-I by unfolding the precursor or by bringing it into proximity of other proteolytic activities. Thus there appear to be at least two different proteases in the cytoplasm that generate peptides available to MHC class I molecules. In the case of OVA, one of these proteases, the proteasome, generates the C terminus of the peptide. The other proteolytic activity is capable of generating the N terminus of the SL8 peptide. This protease appears to be resistant to all the inhibitors tested. However, it is conceivable that these inhibitors may not have had access to these proteases. Alternatively, it is also possible that there is more than one proteolytic activity capable of generating the C-terminally extended peptide, and that testing multiple inhibitors in combination will be necessary to block its generation. These results are consistent with the findings of Craiu et al, who inferred from functional T cell stimulation assays that the proteasome is necessary for generation of SL8 from a panel of C-terminally extended precursors, but not from N-terminally extended precursors (55). Our biochemical analysis of the naturally processed peptides in living cells now confirms and extends this analysis. Furthermore, the fact that the N-terminal extension in the precursors tested here are much longer than the extensions tested in that study indicates that significant nonproteasomal cleavages do occur in the cytoplasm during Ag processing.

There are a number of studies indicating MHC class I ligands can be generated in the cytosol independently of the proteasome (56–61). In this study, the specificity of the nonproteasomal protease that generated SL8-I differs from that of the proteasome in that it does not cleave after the C terminus of SL8. However, it clearly cleaves at other positions in OVA and may be an important source of antigenic peptides from other precursors. We speculate that the protease proteases that generated SL8-I in these experiments are likely to generate other peptides that can bind MHC class I molecules, and may account for the observation that proteasomes are not exclusively required for supplying processed peptides to MHC class I molecules (11, 56, 57).

The analysis of the proteolytic steps in the MHC class I Ag-processing pathway has been hindered by the low sensitivity of the methods available to detect potential extended analogues of naturally processed peptides. As shown here, converting the extended analogs to the optimally active antigenic peptides can alleviate this problem. Future refinements of this technique could allow analysis of peptides with yet longer N- and C-terminal extensions, and thus allow a deeper understanding of the nature of the peptide pool available to the MHC molecules in the ER.

Acknowledgments

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