The Role of MHC Class I Molecules in the Generation of Endogenous Peptide/MHC Complexes[1]

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Cellular proteins undergo proteolysis to yield peptide/MHC class I complexes for display on the APC surface. During this process it is not clear whether MHC molecules bind to and stabilize independently generated peptides, or whether they are involved in the peptide cleavage events. In this study, we analyzed the role of MHC molecules in Ag processing by characterizing the naturally processed peptide analogues of OVA (OVA257–264, SL8) in APC. DNA constructs encoding SL8 precursors were transfected into cells that varied in their MHC expression. By HPLC fractionation of cell extracts and with sensitive T cell assays for both the processed SL8 and its minimal Met-SL8 (MSL8) precursor, we determined that expression of K^b MHC molecule was essential for detecting processed peptides in living cells. Curiously, although the translated MSL8 nonapeptide precursor itself could bind K^b as well as the SL8 octapeptide, and MSL8 was available to MHC, only the SL8 peptide was found in K^b cell extracts. The presence of naturally processed SL8, but not MSL8 peptide in K^b-expressing cells suggests that the precise identity of endogenously processed peptides is also strongly influenced by the MHC molecules. *The Journal of Immunology,* 1995, 154: 585–598.

Ag processing and presentation is the series of events that result in the display of peptide fragments derived from cellular proteins on the cell surface by MHC class I molecules (reviewed in Refs. 1–3). Peptide/MHC class I complexes expressed on the surface of target cells are recognized by the TCRs of $CD8^+$ cytotoxic T cells that lyse target cells bearing foreign peptide/MHC complexes resulting from viral infection or tumorigenic transformation.

MHC class I molecules bind to and display thousands of naturally processed peptides derived from intracellular proteins (4–7). Amino acid sequences of several processed peptides have revealed that for a given MHC molecule, the set of bound peptides share a consensus motif defined by length (8–11aa) and by conserved "anchor" residues (5–7). Recently crystal structures of several peptide-bound MHC complexes have been solved (8, 9). These structures have explained, at atomic resolution, how polymorphic residues of MHC molecules bind tightly to a multitude of peptides with these consensus motifs (10, 11).

The mechanism by which these peptide/MHC complexes arise within cells, however, remains obscure. Current models of Ag presentation envisage generation of peptides in the cytosol, before their transport into the lumen of the endoplasmic reticulum (ER)[3]. Stable peptide/MHC complexes are assembled in the ER, and transported to the cell surface for display (reviewed in Refs. 12–14). This view is consistent with recent discoveries of genes encoded within the MHC that share sequence similarity with proteasome subunits (LMP2 and LMP7) (15–17), and with those serving transport functions (TAP1 and TAP2) (18–21). Thus, it has been postulated that LMP2 and LMP7 are components of the proteasome that generates processed peptides in the cytoplasm (12, 13). The processed peptides are then transported via the TAP1/TAP2 heterodimer to the ER for binding to the MHC. Evidence for this attractive model is, however, largely circumstantial. Currently available data indicate that MHC-encoded LMP2 and LMP7 subunits are not essential for generating processed peptides (22, 23). On the other hand, the TAP1/TAP2 complex does determine stable expression of endogenous peptide/MHC complexes on the cell surface (24–28) and does allow peptide

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[3] Abbreviations used in this paper: ER, endoplasmic reticulum; CPRG, chlorophenol red β-galactoside; CHO, Chinese hamster ovary; TFA, trifluoroacetic acid.

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transport into the ER (29, 30). However, the natural peptide substrates that are transported by TAP1/TAP2 from the cytoplasm into the ER are not known. Therefore, the temporal order, the subcellular location, and the mediators of events that yield processed peptide/MHC complexes are not established.

The role of MHC molecules in Ag processing is also controversial. Analysis of APC extracts showed that processed peptides were predominantly present in cells that also expressed appropriate MHC molecules (31, 32). This result suggested that a generation of processed peptides may not occur independently as proposed in the model discussed above, but could be regulated by MHC molecules. Alternatively, if processed peptides were short lived because of their rapid proteolysis in APC, the requirement for MHC molecules could reflect their critical role in preventing peptide degradation (33, 34). Neither of these hypotheses have been tested because intermediate steps between full-length antigenic proteins, and their final cleaved peptide products are not known. Furthermore, direct analysis of naturally processed peptides and their putative precursors has been difficult because they are present in very small amounts, and their activity in immunologic assays is profoundly diminished by increase in peptide length (35–37). Thus, the role of MHC molecules in Ag processing is not clear.

In this study, we analyzed the role of MHC molecules in the generation of processed OVA peptides in APC. With OVA/K^b-specific T cells, we established that OVA257–264 (abbreviated as SL8) is the naturally processed product of OVA, and that its presence in APC extracts requires coexpression of K^b MHC. We designed the minimal SL8-encoding construct, Met-SL8 (MSL8) to directly analyze both the precursor and its processed SL8 product. By using this model, we established a direct role for MHC molecules in stabilizing processed peptides. Surprisingly, we also discovered that the same precursors yielded either octapeptide or nonapeptide products depending on the coexpressed K^b or D^b MHC molecules. These results establish the role for MHC in stabilizing intracellular peptides and provide insights into the mechanism that generates endogenous peptide/MHC complexes.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO-K1) cells expressing either OVA, K^b MHC, or OVA and K^b MHC were obtained by electroporating OVA cDNA and/or K^b genomic DNA constructs as described (37, 38). Stable cell lines were selected in 1 mg/mlG418 and were subcloned by limiting dilution. Derivation of K^b- or D^b-expressing COS cells, OVA257–264/K^b- or NP366–374/D^b-specific, lacZ-inducible B3Z and DBFZ.25 T hybrids have been described elsewhere (37–39). T2 and its derivative T2-K^b cell lines were a kind gift of Dr. P. Cresswell (Yale University, New Haven, CT). All mammalian cells were maintained in RPMI 1640 medium (Cellgro, Mediatech, Washington, DC) supplemented with 2 mM glutamine, 1 mM pyruvate, 50 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (HyClone, Logan, UT), at 37°C in 5% CO_2/air. Murine $K^b/\beta 2$ M or $D^b/\beta 2$ M transfected drosophila cells were a kind gift of Drs. M. R. Jackson and P. A. Peterson (Scripps Research Institute, La Jolla, CA) and were maintained in Schneider cell medium (Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum at room temperature in air as described (40).

Immunoprecipitations

^{35}S metabolic labeling of cells and immunoprecipitation of K^b MHC and OVA were performed as described (41). Briefly, CHO cells (2.5×10^6/ml) were labeled for 3 h with 0.1 mCi/ml ^{35}S methionine and cysteine (Trans-label, ICN Biochemicals, Cleveland, OH). mAbs Y3 (anti-K^b) (42), or polyclonal rabbit anti-OVA (Cappel Laboratories, Durham, NC) were used with protein A-Sepharose beads (Sigma Chemical Co., St. Louis, MO) for immunoprecipitations. Precipitates were boiled in sample buffer and run on 12% SDS-PAGE gels (Bio-Rad Laboratories, Richmond, CA) under reducing conditions. Gels were soaked in Amplify (Amersham, Arlington Heights, IL) plus 4% glycerol before overnight exposure to Hyperfilm (Amersham) with intensifying screen at −70°.

DNA constructs and peptides

Genomic and cDNA clones encoding mouse K^b (a kind gift of R. Flavell) (43), and OVA cDNA (pcDNAI/neo-OVA) have been described (37). Constructs for expressing MSIINFEKL (MSL8), MIINFEKL (ML8), and MKSIINFEKL (MKSL8) peptides were prepared by using complementary synthetic oligonucleotides encoding these residues in the pcDNAI vector (Invitrogen, San Diego, CA) (37). In addition, the sequences contained nucleotides complementary to restriction site overhangs (*Bst*X1 or *Eco*RV for ML8) at the 5′ end and a termination codon and *Xba*I site overhang at the 3′ end. MSIINFEKL, 5′TGTGATGAGTATAATCAAC TTTGAAAAACTCTAGA; MIINFEKL, 5′ATGATAATCAACTTTGA AAAACTCTAGA, MKSIINFEKL, 5′TGTGATGAAGAGTATAATCA ACTTTGAAAAACTCTAGA. Each construct was confirmed by DNA sequencing. Solid phase synthesis of SIINFEKL peptide (37), and its analogues MSIINFEKL, MIINFEKL, KSIINFEKL was conducted by using Applied Biosystems (Foster City, CA) Model 421 automated synthesizer. Peptides were purified by HPLC, and their synthesis confirmed by mass spectrometry. Purity of each peptide was estimated to be >96% by analytical HPLC and by mass spectrometry. The FITC derivative of SL8 was prepared as described (41). Briefly, 100 nm of SL8 was incubated with 1.5 molar excess of FITC (Molecular Probes, Eugene, OR) in 100 mM sodium bicarbonate for 2 h at 37°. The FITC-SL8 derivative was separated from uncoupled FITC by size exclusion chromatography on P-2 beads (Bio-Rad) and was used to analyze peptide binding to T2-K^b cells by FACS as described below.

Transient transfections

COS cells, or their K^b or D^b derivatives, were transfected with CsCl-purified plasmid DNAs by the DEAE-dextran and chloroquin procedure as described (37). Analytical transfections were performed with 2×10^4 cells per well of a 96-well tissue culture plates. For preparative extractions, 1 to 1.5×10^7 cells were transfected in tissue culture flasks. Two days later, transfected cells were either directly tested for expression of peptide/MHC complexes by overnight coculture with 3×10^4 T-hybridoma cells or were used for peptide extraction described below.

T cell activation assay

Peptide/MHC ligand-specific LacZ activity induced in OVA/K^b (B3Z)- or NP/D^b (DBFZ.25)-specific T cell hybrids was determined by using the lacZ substrate chlorophenol red β-galactoside (CPRG) as described (37, 39). The lacZ-mediated conversion of CPRG to chlorophenol red, in each well of the 96-well plate, was measured at 595 nm and 635 nm as reference wavelength, with the Bio-Rad Model 3550 plate reader. For peptide assays, 3×10^4 K^b expressing L-cell transfectant (K89.7.5, (37)) were cocultured overnight with 3×10^4 B3Z T cells with synthetic peptides or aliquots from HPLC fractions. The specific B3Z response is shown as mean absorbance (A595) of duplicate wells.

FACS analysis

Drosophila Schneider cells, transfected with murine β_2-microglobulin and either murine K^b ($K^b/\beta 2$ M-SC) or D^b ($D^b/\beta 2$ M-SC) MHC genes

under transcriptional control of the metallothionin promoter were used to assess peptide binding capacity of these MHC molecules as described (40). Briefly, expression of transfected genes was induced by 1 mM copper sulfate during 24-h culture, at room temperature, in serum-free insect cell culture medium (Sf900; Life Technologies). Cells were then incubated at 37° for 5 h in medium alone or with 25 μM peptide, washed, and stained with either anti-K^b (Y3, (42)) or anti-D^b (B22.249.R1, (44)) mAbs. FITC-conjugated goat anti-mouse Ig (Cappel) was used as secondary Ab.

Inhibition of FITC-SL8 binding to empty K^b MHC on T2-K^b cells by unlabeled SL8 analogues was used to measure their relative K^b MHC binding ability. T2-K^b cells were incubated first with varying concentrations of either SL8 or MSL8 peptides for 30 min on ice, and then with approximately 100 pmol of FITC-SL8 derivative for another 30 min on ice. Cells were washed three times with PBS and analyzed by FACS. FACS analysis was conducted using the Becton Dickinson Instrument (FACS IV; Mountain View, CA) or with the Coulter Epics (Coulter, Hialeah, FL). Each profile represents a histogram of 10^4 events.

Peptide extractions and HPLC analysis

Total acid soluble peptide pool in stable or transiently transfected cells was extracted by trifluoroacetic acid (TFA) as described (31). Briefly, 10^7 to 10^8 cells were washed with PBS, lysed in 2 ml 0.1% TFA in water, and homogenized by ultrasonication. The homogenate was spun at 12,000 × *g* for 30 min. The supernatant was passed through a 10-kDa Ultra Free-MC filter (Millipore Corporation, Bedford, MA). The filtrate was dried in a vacuum centrifuge, resuspended in 100 μl 0.1% TFA, and fractionated by HPLC. T2 or T2-K^b cells (45), expressing empty MHC molecules were used to characterize the peptides bound by K^b MHC. Then, 3×10^6 T2-K^b cells were incubated on ice with 1 μM SL8 or MSL8 synthetic peptide in serum-free RPMI. After 20 min, cells were washed and lysed in 1 ml of lysis buffer (20 mM Tris-Cl, 150 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 0.01% azide, 50 μg/ml bestatin, 1.1 mg/ml puromycin, 0.1 mg/ml pepstatin A, 34 μg/ml TLCK, 10 μg/ml aprotinin, 92 μg/ml iodoacetamide, and 1 μM PMSF). K^b molecules were immunoprecipitated with 10 μl Y3 ascites and protein A-Sepharose. The beads were washed, and bound peptides were eluted with 0.1% TFA, passed over a 10-kDa cutoff filter, dried in a vacuum centrifuge, and analyzed either directly in a T cell stimulation assay or after HPLC fractionation.

Extracted peptides were fractionated by using the Hewlett Packard 1050 HPLC system controlled by HP ChemStation software. Reverse phase C18 columns (Vydac, 4.6 × 250 mm, 5 μm, 300 Å) were run in 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The gradient used for all separations was as follows: 0 to 5 min, 100% A; 5 to 65 min, linear increase of B to 60%; 65 to 70 min, rapid linear increase of B to 100%; 70 to 75 min, 100% B; 75 to 85 min, decrease of B to 0%. Flow rate was maintained at 1 ml/min, and fractions of indicated volume (0.25 to 1.0 ml) were collected by using the programmable Frac100 fraction collector (Pharmacia, Piscataway, NJ). Mock runs, without injecting peptide samples, were performed and assayed under identical conditions to ensure absence of cross-contamination between samples. Fractions were dried in a vacuum centrifuge (Savant Instruments, Inc., Farmingdale, NY) and resuspended in 100 μl complete RPMI medium, and aliquots were assayed (1:15 dilution) for stimulating B3Z T cells by using K^b-L cells as APC as described above.

Results

Processing of OVA yields the SL8 octapeptide in K^{b+} cells

To analyze processing of OVA to its peptide fragments, we generated CHO cell transfectants expressing OVA (CHO-OVA), K^b MHC (CHO-K^b), or both OVA and K^b (CHO-(OVA + K^b)). Each transfectant was tested for its ability to stimulate the OVA257–264 (SL8)/K^b-specific B3Z T cell hybrid (37, 38). Only CHO-(OVA + K^b) cells constitutively expressed the OVA/K^b ligand recognized by B3Z T cells (Fig. 1*A*). K^b-CHO cells stimulated B3Z T cells only in presence of the exogenous SL8 peptide. However, expression of OVA alone, without K^b, was insufficient to stimulate B3Z cells with or without the SL8 peptide.

The processed OVA peptide(s) that stimulate B3Z T-cells were identified in the TFA cell extracts (31). The acid-soluble peptide pool was fractionated by reverse-phase HPLC and assayed for B3Z-stimulating activity by using K^b-L cells as APC (37). The B3Z-stimulating activity in CHO-(OVA + K^b) extracts eluted as a single, sharp peak (Fig. 1*B*, *left*). This peak was identified as SL8 peptide by its identical elution profile with synthetic SL8 peptide. No B3Z-stimulating activity was, however, detected in extracts of CHO-OVA cells (Fig. 1*B*, *right*), despite the fact that both CHO-(OVA + K^b) and CHO-OVA cells synthesized comparable amounts of OVA, as determined by immunoprecipitation of metabolically labeled cells (Fig. 1*C*), as well as by measurement of OVA secreted in culture supernatants (data not shown). Because the same parental CHO cells were transfected with either OVA, and/or K^b MHC genes, directly establishes that generation of SL8 in CHO cells depends on the expression of OVA as well as K^b MHC. This result confirms and extends the original observations of Rammensee et al. (31, 46) made with unknown minor histocompatibility Ags.

Two different hypotheses can explain why SL8 was absent in extracts of cells expressing OVA alone but present in cells expressing both OVA and K^b MHC. First, processing of OVA to SL8 could have occurred in CHO-OVA cells. Without K^b MHC to stabilize this independently generated SL8, it may not have been detected because of its rapid degradation. Alternatively, if processing of OVA to SL8 was somehow dependent on K^b MHC, SL8 could not possibly exist in cells expressing OVA but lacking K^b MHC. These two hypotheses have not been resolved because of difficulties in analysis of precursors that yield processed peptides. If proteolytic fragments of OVA, unlike the final SL8 peptide product (Fig. 1*B*), do not represent a homogeneous species, they would be unlikely to elute in a single fraction, but would be diluted out among many fractions. This could effectively lower their concentration below the sensitivity threshold of the T cell assay. Yet, even if only a single homogeneous intermediate existed and eluted in a single fraction, its necessarily larger size and consequently low activity would again render it undetectable in the assay (37). Thus, to analyze the role of MHC in Ag processing, it was essential to circumvent these difficulties.

Minimal precursors for expression of SL8/K^b complex in APC

We designed SL8 encoding precursors with two goals: 1) to minimize or to eliminate putative processing intermediates between the 386 residue OVA protein and its

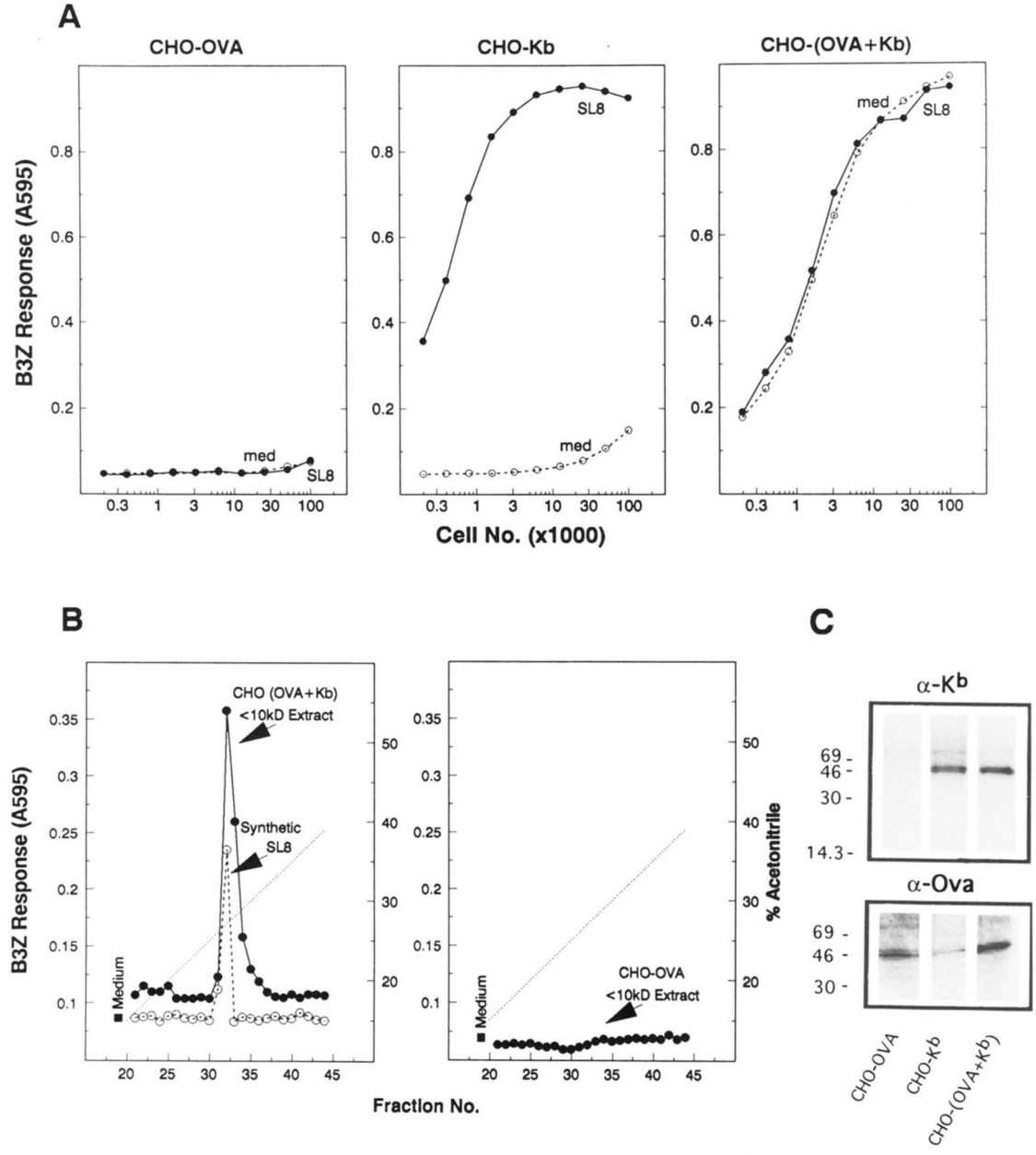

FIGURE 1. *A*) SL8/K^b-specific, B3Z T cells are stimulated by OVA + K^b MHC- transfected CHO cells in medium alone, but require exogenous SL8 peptide for stimulation by K^b MHC-transfected CHO cells. OVA-transfected CHO cells fail to stimulate B3Z T-cells with or without the SL8 peptide. *B*) identification of processed B3Z stimulating activity by HPLC fractionation of TFA extracts prepared from either CHO-(OVA + K^b) transfectants (*left*) or CHO-OVA transfectants (*right*). Synthetic SL8 (○) elutes with the same retention time as the activity eluted from CHO (OVA + K^b) extracts (●). Each point represents an individual fraction collected at 1-min intervals. Diagonal dotted line indicates composition of the acetonitrile gradient shown on the right scale. *C*) immunoprecipitation analysis of K^b MHC (*top*) and of OVA (*bottom*) polypeptides expressed in each of the CHO transfectants shown below the autoradiograms. Molecular weight markers are indicated on the left. *A*) indicated number of stable CHO cell transfectants expressing either OVA (CHO-OVA), K^b MHC (CHO-K^b), or both OVA and K^b MHC (CHO-OVA + K^b) were incubated in medium alone (○) or with 5 nM OVA257–264 (SL8) peptide (●) for 2 h at 37°C. Cells were washed and cocultured with 1×10^5 B3Z T-cells. After overnight incubation, lacZ activity induced in B3Z cells was determined in culture lysates by using the lacZ substrate CPRG, as described in experimental procedures (37). *B*) 10^8 CHO-(OVA + K^b) or CHO-OVA transfectants were extracted with TFA as described (31). Low m.w. (<10 kDa) material was fractionated by HPLC by using a C18 reverse phase column run in 0.1% TFA and an acetonitrile gradient. Each fraction was dried, resuspended in medium, and assayed for its B3Z T cell-stimulating activity. See *Materials and Methods* for details. *C*) lysates of [^{35}S]Met metabolically labeled CHO cell transfectants were immunoprecipitated either with anti-K^b monoclonal Y3, or with polyclonal anti-OVA Abs. Samples were run on 12% SDS-PAGE gels and autoradiographed.

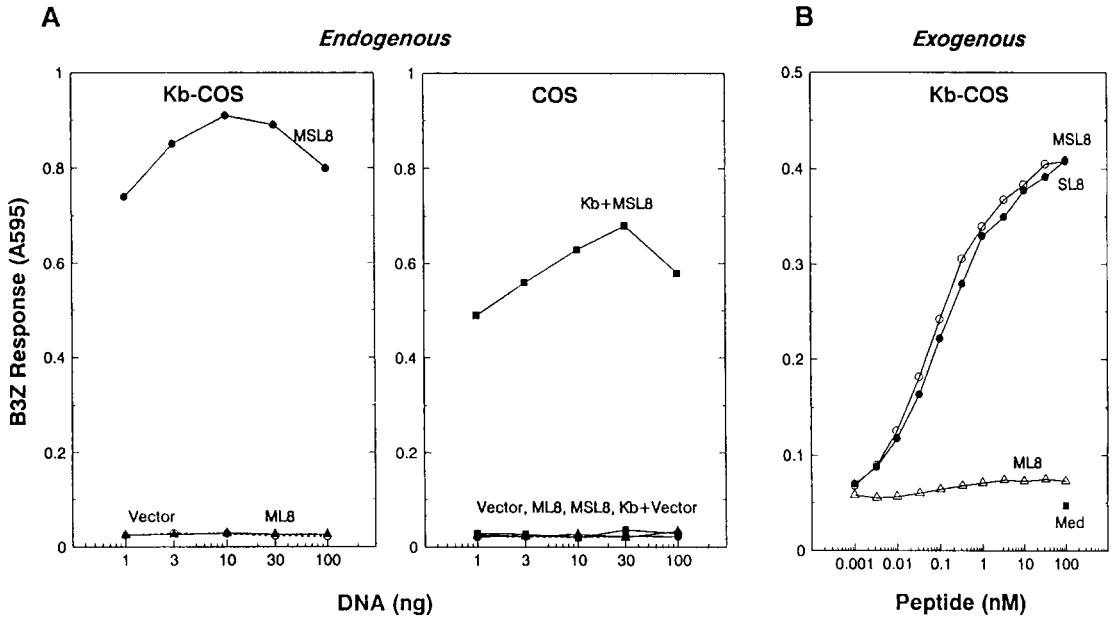

FIGURE 2. Minimal precursors for generating endogenous or exogenous peptide/K^b MHC complexes that stimulate B3Z T cell response in COS APC. *A*) B3Z response to K^b-COS (*left panel*) or parental COS cells (*right panel*) transfected with indicated DNA constructs encoding MSIINFEKL (MSL8), MIINFEKL (ML8), or vector control. Parental COS cells lacking K^b expression were also cotransfected with K^b cDNA plus vector or MSL8 DNAs. *B*) B3Z-stimulating activity of synthetic MSL8 nonapeptide is identical with that of synthetic SL8 octapeptide, whereas synthetic ML8 peptide has negligible activity. A total of 2×10^4 COS or K^b-COS cells were transfected with varying amounts of plasmid DNAs, by the DEAE-dextran and chloroquin procedure as described (37). Two days later, transfectants were tested for APC function by coculture with 3×10^4 B3Z T cells by the lacZ assay. For exogenous peptide titrations, 3×10^4 B3Z T cells were cocultured overnight with 3×10^4 K^b-COS cells in medium (Med) alone or with indicated concentrations of synthetic peptides.

SL8 product; and 2) to develop an assay for directly measuring SL8 precursors in cell extracts. The smallest open reading frame encoding SL8 is the nonapeptide Met-SL8 (MSIINFEKL, abbreviated as MSL8). However, if the SL8/K^b- specific B3Z T cells could tolerate a Ser to Met substitution at the first residue, the octapeptide Met-IL7 (MIINFEKL, ML8) could also conceivably serve as a precursor for ML8/K^b complex, without any intermediates.

DNA constructs encoding MSL8 or ML8 residues, as well as synthetic peptides corresponding to these two gene products were prepared. These were tested for their ability to generate a B3Z-stimulating peptide/K^b complex by either transfecting K^b-COS cells or by exogenous addition of synthetic peptides. In K^b-COS cells, transfection of MSL8 DNA construct or addition of MSL8 synthetic peptide stimulated B3Z T cells very efficiently (Fig. 2, *A* and *C*). However, expression of the same constructs in parental COS cells lacking K^b MHC, did not yield any B3Z T cell response (Fig. 2*B*). To confirm that the MSL8 peptide was nevertheless synthesized in COS cells, they were cotransfected with the K^b cDNA plus the MSL8 construct. K^b + MSL8-cotransfected COS cells showed strong B3Z stimulation activity (Fig. 2*B*). In contrast with MSL8, either endogenously synthesized or exogenously added ML8 peptide yielded negligible B3Z-stimulating activity (Fig. 2, *A* and *C*). This indicated either that the single Ser to Met substitution in SL8 abrogated K^b MHC binding function of SL8 octapeptide, or more likely, that Ser is a critical contact residue for the B3Z TCR. The MSL8 peptide was thus identified as the minimal endogenous precursor that yielded the peptide/K^b MHC complex recognized by B3Z T cells. Most importantly, the exogenous peptide dose-response curve of MSL8 peptide was superimposible with that of SL8 peptide (Fig. 2*C*), providing us with a sensitive assay for both the MSL8 precursor and processed SL8 in cell extracts.

Identification of processed peptides in cell extracts

The processed peptides were extracted from either K^b-COS or COS cells transfected with the minimal MSL8 construct. The peptides were fractionated by HPLC, and tested for their B3Z T cell-stimulating activity as described above for CHO transfectants. Again, a single peak of activity was found in extracts of MSL8 transfected K^b-COS cells (Fig. 3*A*). As in CHO-(OVA + K^b) cells, this activity represented the SL8 peptide, because synthetic SL8 migrated with an identical elution profile (Fig. 3*A*).

Unlike K^b-COS extracts, no B3Z-stimulating activity was detected in MSL8-transfected COS cells that do not express K^b MHC (Fig. 3*B*). The absence of this activity was not caused by differences in transfection efficiency

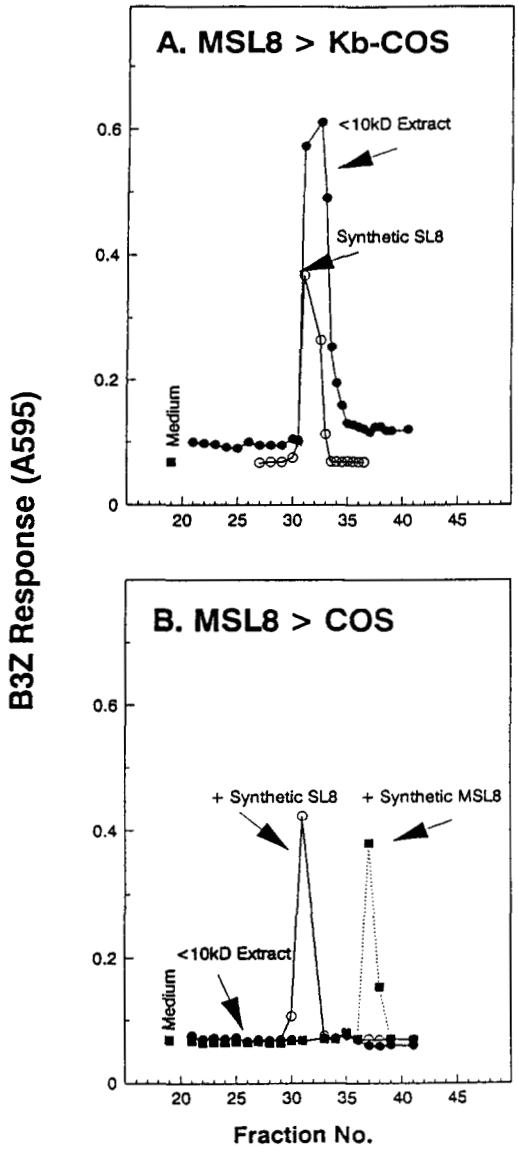

FIGURE 3. Identification of processed peptides generated in MSL8-transfected (*A*) K^b-COS cells or (*B*) parental COS cells by HPLC analysis of TFA extracts. The single peak of activity in K^b-COS extracts (●) comigrates with synthetic SL8 peptide (○). No B3Z-stimulating activity was detected in extracts of MSL8-transfected COS cells. Extracts of COS cells to which 200 fmol of either synthetic SL8 (○) or synthetic MSL8 (■) peptides was added before extraction do contain B3Z-stimulating activity. See legend to Figure 1 and *Materials and Methods* section for details of transfection, extractions, HPLC fractionation, and assay of eluted peptides. Data shown were obtained from the same RP C18 column run under identical conditions to ensure reproducibility of peptide retention times. Fractions were collected at 1-min intervals.

among K^b-COS and COS cells, because expression of transfected β-galactosidase reporter construct was comparable in both cells (data not shown). Furthermore, as shown above, COS cells cotransfected with constructs encoding K^b and MSL8 stimulated B3Z T cells very efficiently, directly establishing that MSL8 was indeed translated in these cells (Fig. 2*B*). The possibility that these peptides were somehow inactivated or sequestered during the extraction procedure was also ruled out by determining that synthetic SL8 or MSL8, when added to cells before extraction, were readily recovered and identified by their typical HPLC elution profiles (Fig. 3*B*).

Thus, in cells expressing MSL8, unlike those expressing full-length OVA, the absence of SL8 cannot be attributed to either the inability of APC to synthesize the precursor, to unknown intermediates, or to artifacts of the extraction procedure. We therefore conclude that expression of K^b MHC is essential for obtaining processed peptides in cell extracts. To our knowledge, these results are the first direct test of peptide stability in vivo, and strongly support the notion that MHC molecules serve a direct role in stabilizing processed peptides that are otherwise rapidly degraded.

The search for MSL8 nonapeptide

We were intrigued by the absence of the MSL8 precursor peptide in K^b-COS extracts. Clearly, both the SL8 octa- and MSL8 nonapeptides were comparable in their K^b-mediated B3Z T cell-stimulating activity, as judged by their superimposible dose-response curves (Fig. 2*C*). Thus, if the capacity of K^b MHC to bind available intracellular peptides was the only condition necessary and sufficient to generate peptide/K^b complexes, then MSL8 should have been present in extracts of MSL8 transfected K^b-COS cells. Alternatively, the exclusive recovery of SL8 could be explained by a complete post-translational removal of methionine from MSL8 to yield SL8 alone (as the sole intracellular precursor), a stringent preference of K^b MHC for binding SL8 over MSL8, or a K^b-dependent cleavage of methionine residue.

We first assessed the K^b binding capacity for SL8 and MSL8 peptides by an independent method with *Drosophila* cells that express empty murine MHC and β2-microglobulin complexes on their surface (40). MHC molecules in these cells are thermolabile in absence of appropriate peptides, but their conformation can be stabilized in presence of appropriate peptides (40). FACS analysis with anti-K^b monoclonal Y3 (42) showed that this K^b epitope was stabilized by both SL8 octapeptides and MSL8 nonapeptides (Fig. 4, *left panels*). More importantly, the K^b-stabilizing activity of either peptide was maintained over a one million-fold concentration range (Fig. 5). Thus, both SL8 and MSL8 were apparently capable of binding K^b on the cell surface.

Two further controls were performed to rule out the formal possibility that K^b stabilization by MSL8 was artifactual. It could be argued that trace proteases present in the serum-free medium could have caused conversion of MSL8 to SL8 during the short incubation period, and that SL8 was the actual peptide bound to K^b MHC. First, we

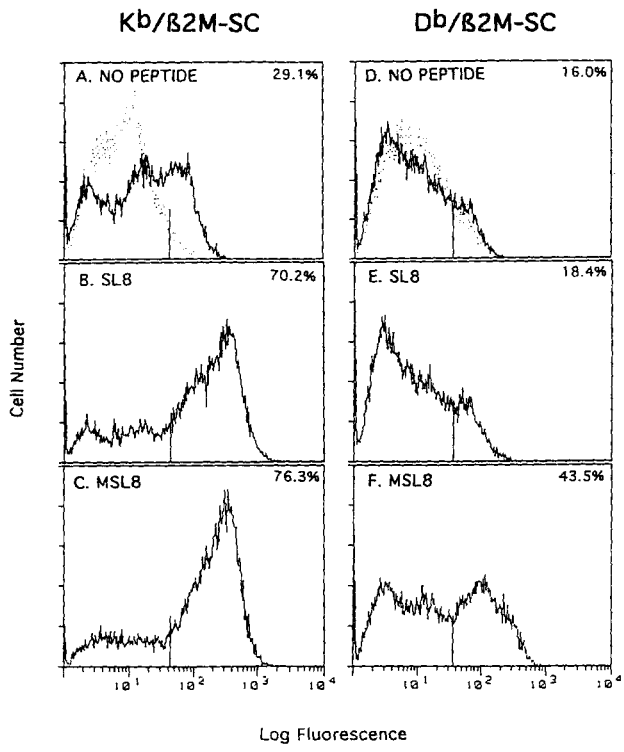

FIGURE 4. K^b or D^b MHC binding capacity of synthetic SL8 or MSL8 peptides. Stable *Drosophila* Schneider cell transfectants ($K^b/\beta 2$ M-SC or $D^b/\beta 2$ M-SC) (40) were incubated (room temperature), in serum-free medium with copper sulfate to express empty murine K^b (*panels A* through *C*) or D^b (*panels D* through *F*) MHC on cell surface. Cells were further incubated with either synthetic SL8 or MSL8 peptides (25 μM) at 37°, and stained with anti-K^b monoclonal Y3, or with anti-D^b monoclonal B22.249.R1, followed by FITC goat anti-mouse Abs. Each FACS profile represents analysis of 10^4 events and shows cell number vs fluorescence intensity on a logarithmic scale. Vertical lines indicate the background staining of uninduced cells (*panels A* and *D*, dotted lines) and were used to determine the indicated percentage of cells with higher fluorescence intensity.

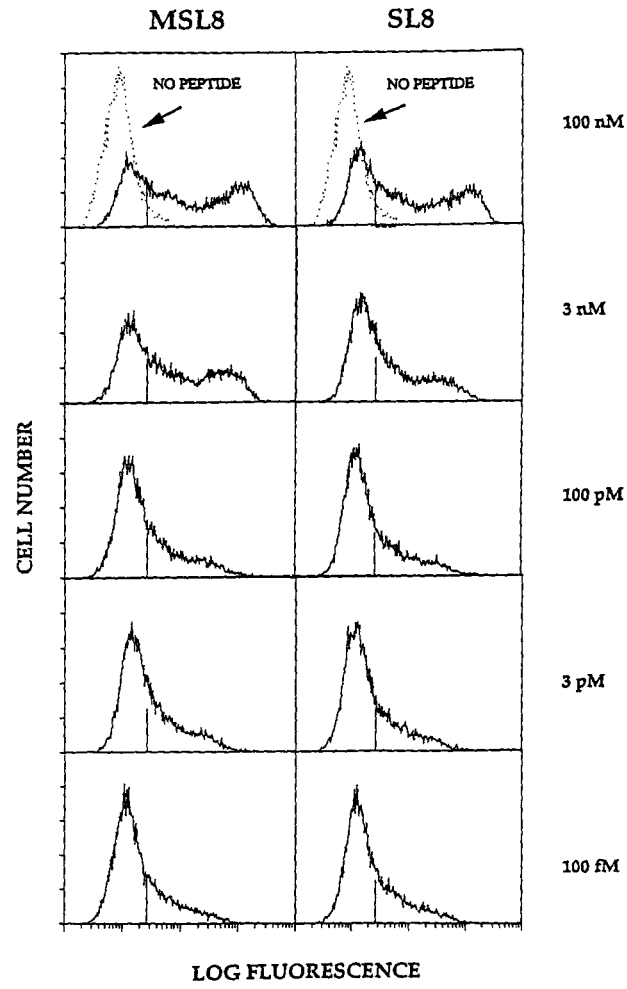

FIGURE 5. Both the SL8 octapeptides and MSL8 nonapeptides are comparable in their ability to stabilize empty K^b MHC. $K^b/\beta 2$ M-SC cells were incubated with indicated concentrations of either SL8, MSL8, or no peptide and analyzed by FACS by using the Y3 anti-K^b mAb as described in the legend to Figure 4. Vertical bar indicates the fluorescence staining of cells without addition of peptide.

tested the ability of SL8 and MSL8 to stabilize D^b expression. A unique ridge in the peptide binding groove of D^b MHC causes the peptide backbone to arch, and, as a consequence, D^b MHC prefer nonapeptides rather than octapeptides (47). The fortuitous presence of the D^b MHC-binding motif in MSL8 (see below) allowed us to test the D^b stabilization property of MSL8 and SL8 peptides. In contrast with K^b, which was stabilized by both SL8 and MSL8, D^b was stabilized only by MSL8 and not by SL8 (Fig. 4, compare *left* with *right panels*). These data show that MSL8 and SL8 peptides could be distinguished by D^b but not by K^b MHC and argue against the possibility that MSL8 was converted by proteolytic cleavage to SL8 before binding to K^b MHC.

To directly establish that MSL8 peptide was actually bound to K^b we used the T2-K^b cell line. Because these cells lack the TAP1/TAP2 complex, they express empty MHC molecules that are available for binding exogenous peptides (45). T2-K^b cells were incubated on ice for 20 min with either SL8 or MSL8 in serum-free medium, and the K^b-bound peptides were extracted from immunoprecipitated K^b MHC molecules. Again, comparable amounts of T cell-stimulating peptides were recovered from K^b molecules from cells pulsed with either SL8 or MSL8 (Fig. 6*A*). No T cell-stimulating activity was recovered from peptide-pulsed T2 cells (data not shown). Most importantly, the major activity peak in HPLC-fractionated extracts of MSL8-pulsed cells corresponded to the synthetic MSL8 peptide (Fig. 6*B*). Quantitative estimates with synthetic peptide standards showed that less than 10% of the T cell-stimulating activity could be attributed to the SL8 peptide, thus providing direct evidence that MSL8

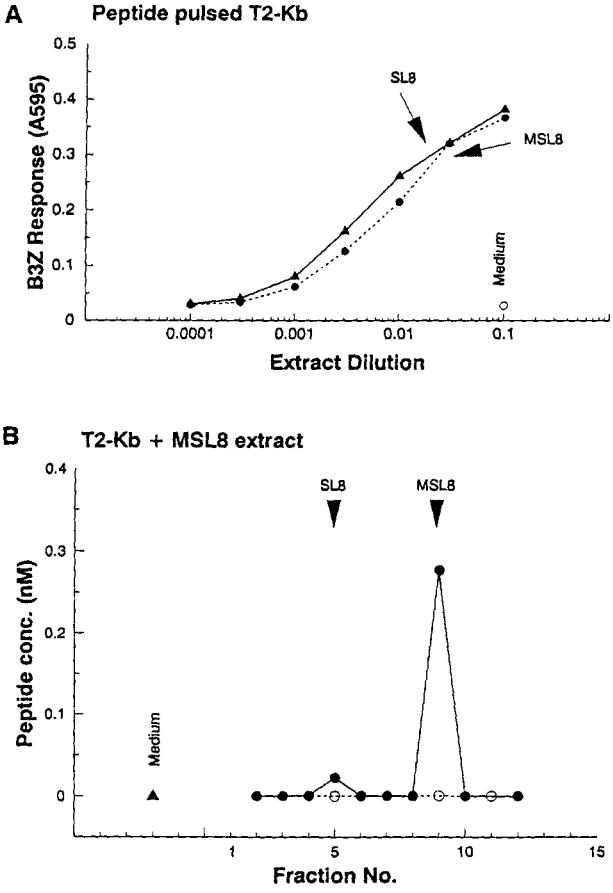

FIGURE 6. Analysis of K^b-bound peptides recovered from peptide pulsed T2-K^b cells. *A*) K^b molecules bind comparable amounts of SL8 or MSL8 peptide on T2-K^b cells. A total of 2×10^6 cells were incubated with 1 μM SL8 or MSL8 peptides on ice for 20 min in serum-free medium. K^b-bound peptides were extracted from Y3 immunoprecipitates as described in *Materials and Methods*. *B*) HPLC analysis of K^b-bound peptides extracted from MSL8 pulsed T2-K^b cells. Closed and open circles represent the MSL8-pulsed T2-K^b extracts and fractions from a mock injection, respectively. Each fraction (1 ml) was assayed for B3Z-stimulating activity, and the concentration of peptides recovered was determined by comparison with synthetic standards. Arrows on the top mark the elution times of synthetic SL8 and MSL8 peptides.

itself, rather than its degradation product SL8 was actually bound to K^b MHC molecules.

To quantitatively assess the peptide binding to K^b MHC, a FACS-based inhibition assay was devised by using FITC-labeled SL8 peptide and T2-K^b cells expressing empty MHC molecules. The FITC-SL8 derivative bound T2-K^b, but not T2 cells and the influenza nucleoprotein peptide amino acids 366–374 (ASNENMETM) that does not conform to the K^b motif did not inhibit the binding even at 100 μM concentration (data not shown). Thus, the relative fluoresence intensity of FITC-SL8-bound T2-K^b cells provided a measure of peptide binding by K^b MHC on the cell surface. Varying concentrations of synthetic SL8 or MSL8 peptides were used to inhibit the binding of FITC-SL8. The downward shifts in the peak fluorescence intensity showed that both SL8 or the MSL8 peptides inhibited the binding of FITC-SL8 probe to K^b MHC (Fig. 7*A*). Plotting the peak fluorescence vs peptide concentration showed that both curves were virtually superimposible (Fig. 7*B*). However, without an accurate estimate of specific activity of FITC-SL8, the exact K^b association constants could not be computed. The concentrations of SL8 or MSL8 peptides required to obtain 50% inhibition of staining were 64 nM and 46 nM, respectively, indicating their similar affinity for K^b MHC. These values are in good agreement with the 4.1 nM k_d for SL8/soluble K^b complex (48). These data are also consistent with the independent evidence that K^b MHC can bind both octapeptides (VSV NP52–59) and nonapeptides (Sendai virus NP324–332) from crystals of these peptide/K^b complexes (9, 11), as well as with measurements of affinity constants by using soluble K^b MHC and a panel of VSV or OVA peptides (48). We conclude that, at least on the cell surface, both the SL8 octapeptides and MSL8 nonapeptides were comparable in their K^b-MHC-binding capacity. Thus, the absence of MSL8 in the cell extracts cannot be simply explained by its inability to bind K^b MHC and prompted us to search for the presence of MSL8 peptide in transfected APCs.

MSL8 nonapeptide is present in D^b-COS extracts

To address the issue of whether MSL8 precursor was actually present in the cells and was available for binding to MHC, we took advantage of the D^b MHC-binding motif, which overlaps the K^b MHC-binding motif in the MSL8 sequence (Fig. 8*A*). Unlike K^b MHC-bound peptides, which conform to the octapeptide consensus motif xxxx[F/Y]xx[M,I,L], D^b MHC- bound peptides are nonamers with the consensus motif xxxx[N]xxx[M,I,L] ((4, 5), P. Scott and N. Shastri, unpublished observations). We reasoned that, because MSL8 matches the D^b motif perfectly, and does bind D^b (Fig. 4), it could be present within the APC as the MSL8/D^b complex. However, unlike K^b-COS cells, MSL8 transfected D^b-COS cells cannot stimulate the SL8/K^b-specific B3Z T cells (Fig. 8*B*). As the reciprocal control, transfection of D^b-COS, but not K^b-COS cells with the Flu NP construct stimulated the NP/D^b-specific DBFZ.25 T cells (Fig. 8*C*). Thus, D^b-COS cells were competent in expressing transfected genes and in presenting endogenously synthesized Ags.

To determine whether processed "SL8" activity was nevertheless present in transfected D^b-COS cells, HPLC-fractionated extracts were tested for their B3Z T cell-stimulating activity by using K^b APC. Despite the inability of MSL8 transfected D^b-COS to stimulate SL8/K^b-specific B3Z cells (Fig. 8*B*), their extracts contained peptides that stimulated B3Z T cells when tested with K^b APC (Fig. 9*A*).

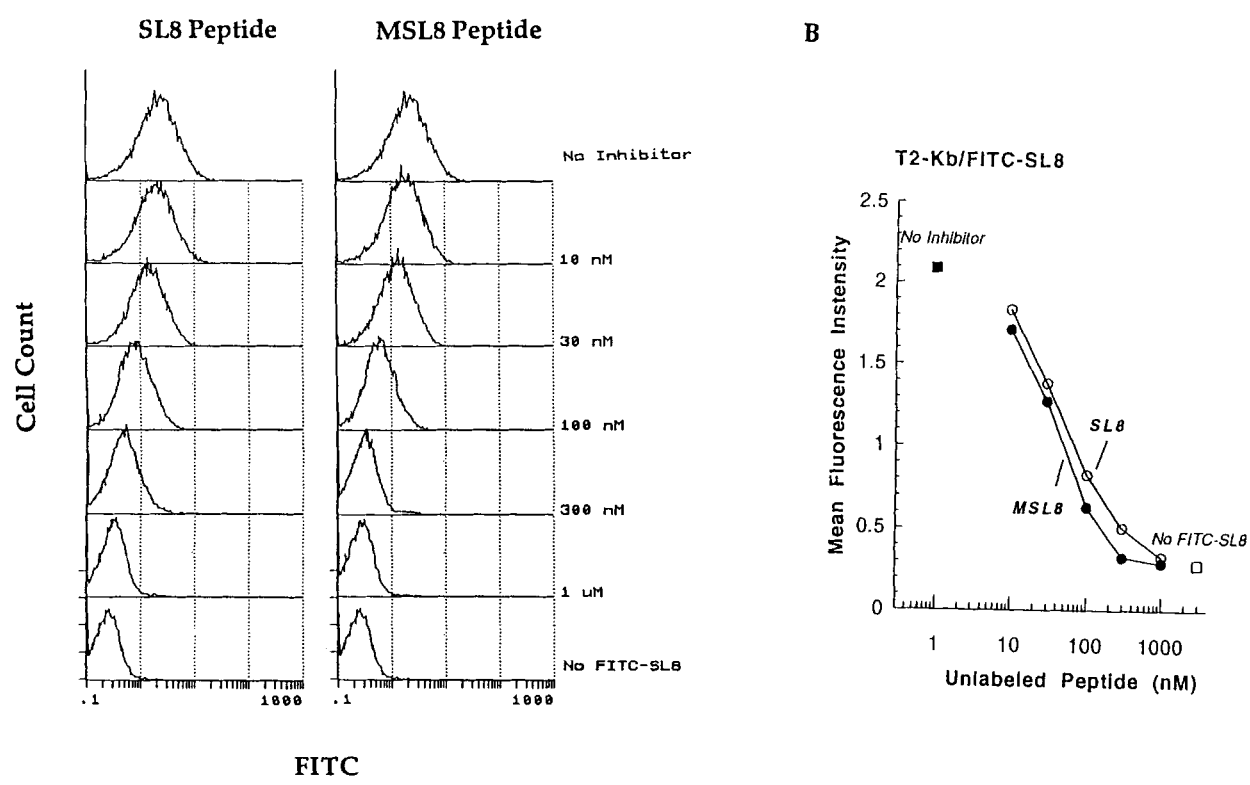

FIGURE 7. Inhibition of K^b MHC binding by SL8 or MSL8 peptides. T2-K^b cells were incubated (30 min on ice) with indicated concentrations of either SL8 or MSL8 synthetic peptides, followed by further incubation (30 min on ice) with FITC-SL8 peptide, and were analyzed by FACS. *A*) FACS profiles indicating that both SL8 and MSL8 peptides inhibit the binding of FITC-SL8 to T2-K^b cells in a dose-dependent manner. *B*) the mean fluorescence intensity of labeled cells plotted against peptide concentration. Fifty percent inhibition of FITC-SL8 binding was obtained with 64 nM SL8 (○) and and 46 nM MSL8 (●), respectively. Fluorescence of control cells not incubated with FITC-SL8 (□) or incubated with FITC-SL8 alone (■) are also indicated.

Significantly, in contrast with extracts of MSL8-transfected K^b-COS that contained only the SL8 peptide (Fig. 9*B*), the activity recovered from D^b-COS eluted in a different peak that was identical with synthetic MSL8 peptide (Fig. 9*A*). The MSL8 peptide was therefore present in the cells and was available to the MHC molecules. Thus, the absence of MSL8 peptide in K^b cell extracts cannot be explained as a post-translational cleavage of the methionine residue and suggests that the precise identity of processed peptides was strongly influenced by (K^b vs D^b) the MHC.

Finally, to further establish that recovery of processed peptides was MHC-dependent, we tested the still larger M-K-SL8 (MKSIINFEKL) decapeptide as a precursor. Again, in K^b-COS cells, but not in D^b-COS cells, endogenous expression of M-K-SL8 construct stimulated B3Z T cells as efficiently as MSL8 (Fig. 8*B*). As with MSL8-transfected K^b-COS cells, the single activity peak in HPLC-fractionated extracts of M-K-SL8 transfected cells was identical with synthetic SL8 octapeptide (compare Fig. 9, *B* and *C*). However, the activity in extracts of D^b-COS cells transfected with the same M-K-SL8 construct eluted in fractions distinct from either SL8 or MSL8 (Fig. 9*D*). This activity peak represented the KSL8 nonapeptide as shown by its identical elution profile with synthetic KSL8 nonapeptide. Thus, both MSL8 and KSL8 nonapeptides were available intracellularly, and were comparable with SL8 in their binding to K^b MHC (Figs. 2, 5, and 6; and data not shown). Nevertheless, the exclusive recovery of SL8, but not MSL8 or KSL8, peptides from K^b-COS cell extracts indicates that MHC also play a role in determining the identity of endogenous peptide/MHC complexes.

Discussion

In this study, we analyze the role of MHC class I molecules in Ag processing. First, we provide evidence that MHC molecules stabilize processed peptides in living cells. Second, we show that endogenous generation of processed peptide/MHC complexes is also strongly influenced by MHC. These findings resolve earlier questions raised as to the fate of processed peptides in cells and provide insights into the cellular mechanism for Ag processing.

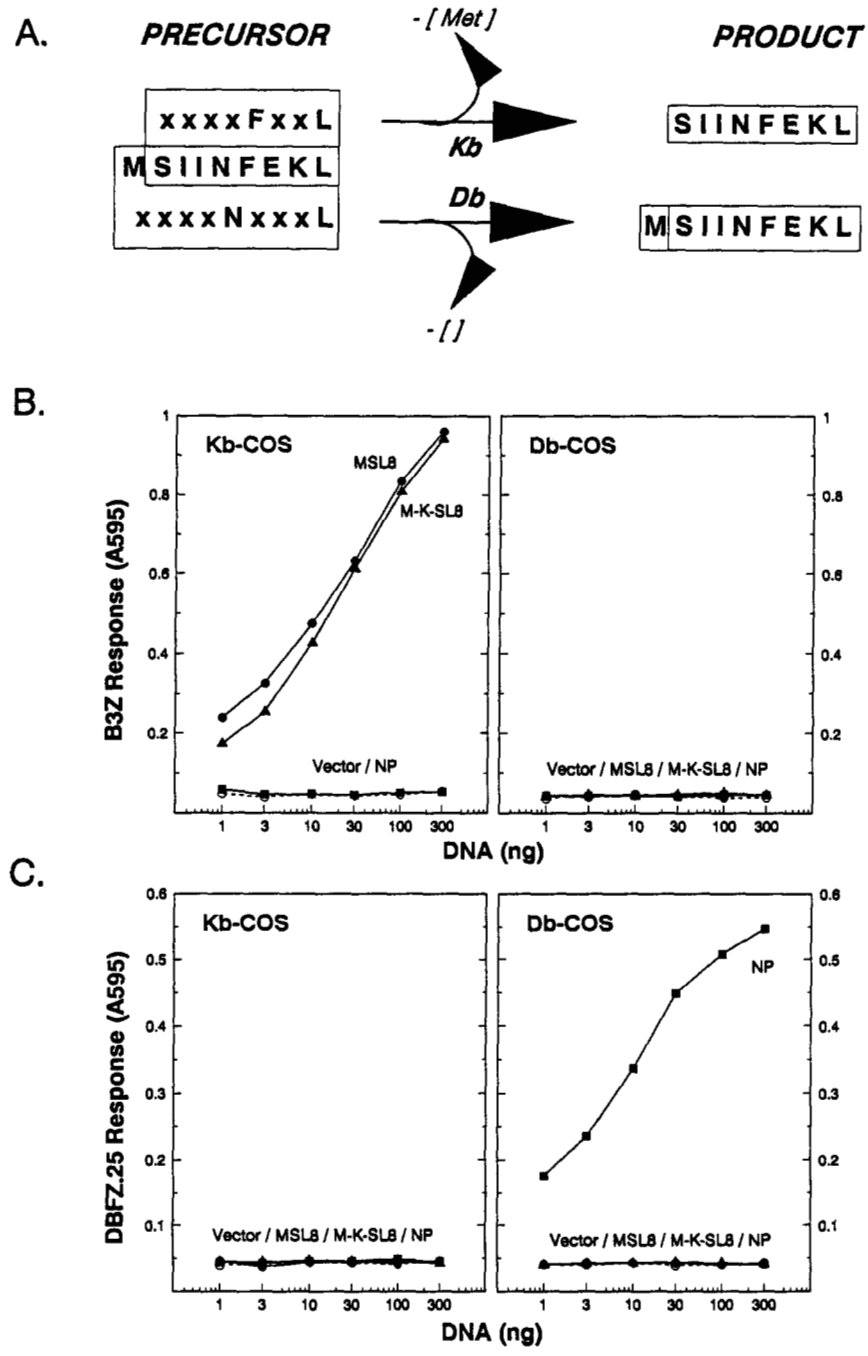

FIGURE 8. *A*) K^b and D^b MHC binding octa- and nonapeptide motifs contained within the MSL8 precursor peptide (5). The processed peptide products expected in K^b or D^b cells are shown on the *right*. *B* and *C*) stimulation of T cell response requires expression of appropriate endogenous Ag and MHC molecules. K^b-COS (*left panels*) or D^b-COS (*right panels*) was transfected with either vector alone (○), MSL8 (●), M-K-SL8 (▲), or NP (■) plasmid DNAs. Two days later, transfectants were tested for their APC function with (*B*) B3Z (SL8/K^b) or (*C*) DBFZ.25 (NP/D^b) T cell hybrids. See legend to Figure 2 for other details.

Role of MHC in stabilizing processed peptides

OVA257–264 (SL8) octapeptide is the naturally processed peptide derived from OVA or its analogues in K^b APC. In this study, we show that this process- ing event occurs in K^b-expressing CHO cells and in simian COS cells (Figs. 1, 3). Taken together with earlier results in OVA/K^b-expressing mouse EL-4 and human Hela cells (49, 50), this finding estab- lishes that the Ag-processing mechanism for gene- rating the SL8 octapeptide is conserved among these species. Nevertheless, in absence of K^b MHC, expression of OVA alone is insufficient for recovery of processed

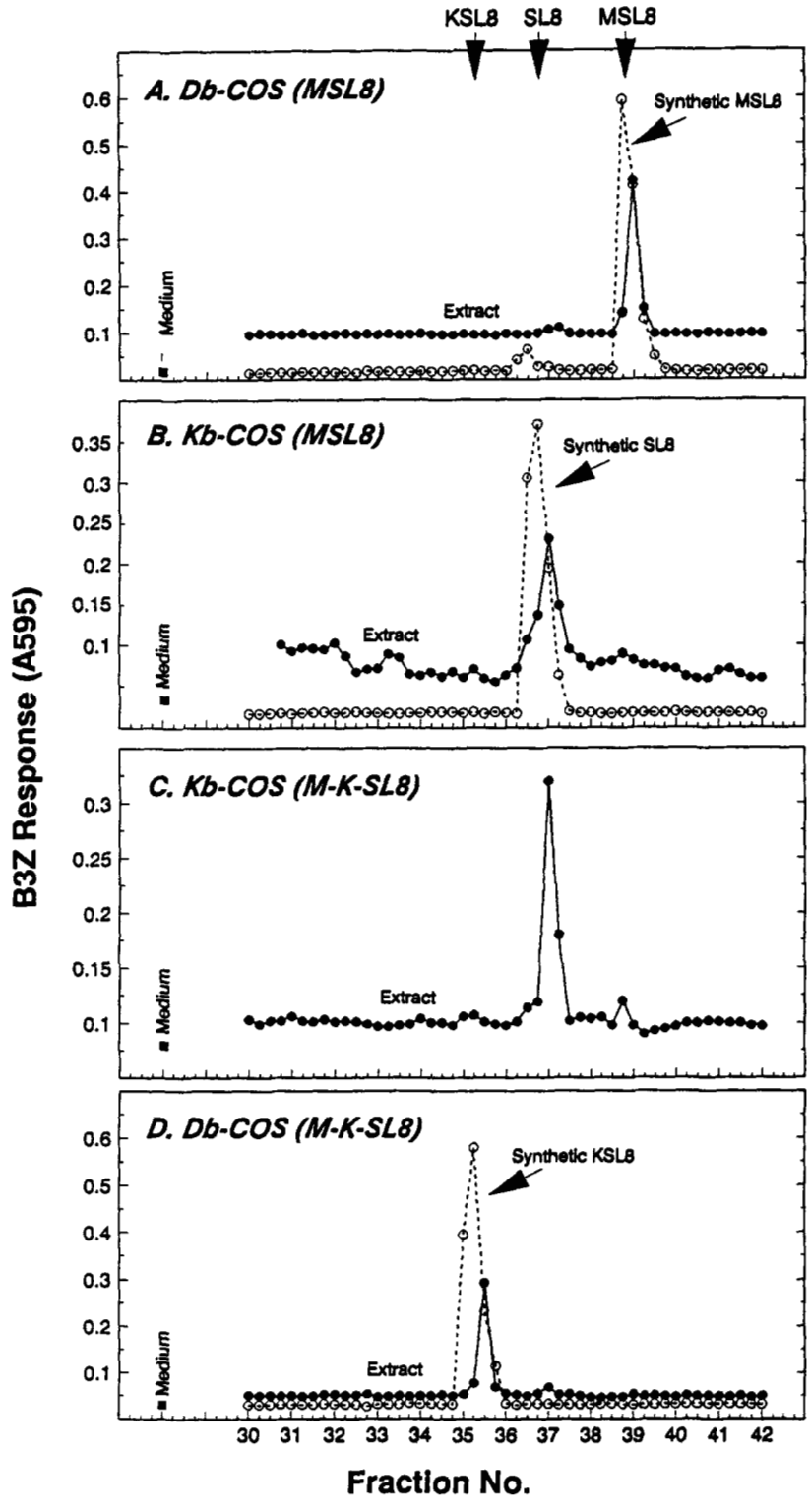

FIGURE 9. Identification of processed peptides in extracts of either D^b-COS or K^b-COS cells expressing either MSL8 or M-K-SL8 precursors. Each extract (●, solid lines) and indicated synthetic peptides (○, dashed lines) were fractionated by the same RP C18 column run under identical conditions. Each fraction, collected at 0.25-min intervals, was tested for presence of B3Z T cell-stimulating activity with K^b-L cells as in the legend to Figure 1. HPLC runs of synthetic MSL8, SL8, or KSL8 peptides shown were conducted under identical conditions to determine their elution profiles. Arrowheads in *panel A* indicate the retention times for each synthetic peptide.

SL8 in cell extracts. How is SL8 generated from OVA, and what is the role of K^b MHC in this process?

Our analysis of the role of MHC in Ag processing differs in significant detail from earlier studies (31, 32). First, by designing the minimal MSL8 construct as a precursor for processed SL8, we circumvented considerations of putative intermediates, which could exist when SL8 is generated from full-length OVA. Second, and most importantly, the fact that synthetic MSL8 peptide was similar to naturally processed SL8 in its high K^b binding and B3Z T

cell-stimulating activity, allowed us to assay the MSL8 peptide in complex cell extracts by its biologic activity. These two key features of the MSL8 precursor, taken together with the ability to readily resolve SL8 and MSL8 peptides by reverse-phase HPLC formed the basis of our analysis.

Expression of K^b or of D^b MHC was essential for recovery of processed peptide in COS cell extracts. In parental COS cells lacking either MHC molecule, the amount of MSL8 precursor or of processed SL8 peptide remained below detection. Note that we cannot formally dismiss the possibility that these processed peptides were actually present but excluded from the acid-soluble peptide pool. The simplest interpretation of this clear result, however, is that, without appropriate MHC capable of binding processed peptides, they are rapidly degraded. This conclusion is supported by two facts. First, each peptide was recovered in extracts of COS cells spiked with synthetic SL8 or MSL8 (Fig. 3*B*), indicating that absence of these peptides was not an artifact of the extraction or analysis procedures, but rather a result of their fate within living cells. Second, and most importantly, endogenously translated SL8 or MSL8 peptides were actually recovered in extracts of K^b or D^b-COS transfectants (Fig. 9), indicating that the fate of these peptides within cells depends on appropriate MHC molecules. Taken together, these findings strongly support the view that MHC molecules play an essential role in stabilizing processed peptides. They also suggest that the previously puzzling, MHC-dependent existence of processed peptides in cell extracts (31, 32) could be a manifestation of this intracellular peptide-stabilizing function of MHC. Considering the generally high levels of gene expression obtained in COS cells, our inability to detect either MSL8 or SL8 peptides (>300-fold) also indicates that peptide degradation must be a rather rapid process. The intracellular site of degradation and its mechanism are presently unknown.

Stability vs cleavage of processed peptides

The most intriguing finding of this study is that the MHC molecules determined not only the existence, but also the identity of processed peptides detected in cell extracts. With MSL8 and MKSL8 constructs, we showed that both precursors yielded only SL8 octapeptide in K^b-COS extracts, but yielded either unmodified MSL8 or the processed KSL8 nonapeptides in D^b-COS extracts (Fig. 9). Note that the recovery of MSL8 in D^b-COS cells also suggests that no obligatory cleavage steps are necessary between the translated precursor and the processed peptide, with the caveat that it is not known whether the MSL8/D^b complex is actually present on the cell surface. Most importantly this result provides evidence that MSL8 nonapeptide was indeed available to MHC and raises the issue of why MSL8 was absent in K^b-COS cells.

How can we account for the absence of MSL8 in K^b-COS cells? It is clear that the selection of SL8, but not of MSL8, in K^b-COS extracts cannot be simply explained by a superior capacity of K^b MHC to bind the SL8 over the MSL8 peptide, as judged by functional and direct peptide binding assays with K^b MHC on the cell surface (Figs. 4, 5, and 6). However, it can be argued that the MHC-binding capacity determined with synthetic peptides on the cell surface cannot be extrapolated to peptides synthesized and bound by MHC in the ER. It is conceivable that both MSL8 and independently processed SL8 could have been available to K^b MHC, but in the intracellular ER environment, SL8 could have been strongly preferred over MSL8 for binding by K^b MHC. Given that D^b MHC prefer nonapeptides (5, 47) (Fig. 4), the availability of SL8 would go unnoticed in D^b-COS cells. Alternatively, it can also be argued that, after transport of the translated MSL8 peptide into the ER, it was bound by K^b MHC and rapidly cleaved to yield the SL8/K^b complex. Accordingly, the longer M-K-SL8 precursor would, by cleavage of both methionine and lysine residues, yield SL8 in K^b APC and, by cleavage of methionine alone, yield KSL8 in D^b APC (Fig. 9).

Without knowing the precise identity of the polypeptides that enter into the Ag-processing pathway we cannot distinguish among the above hypotheses. However, the notion that trimming of larger antigenic precursors can occur in the ER is consistent with some puzzling observations in the literature. Transport of peptides longer than their processed counterparts into the ER has indeed been observed (29, 30, 51, 52). The idea that residues flanking the optimal binding peptides can be cleaved in the ER is also supported by the intriguing finding that an ER form of angiotensin-converting enzyme dramatically improved the presentation of a longer antigenic precursor (53) and, independently, by the recovery of peptides longer than the usual 8 to 10 mers bound to MHC on the cell surface (54–56). The relevance of these findings to normal processing events is presently unclear, because the cytoplasmic precursors and their processed products have not as yet been characterized, and that the angiotensin-converting enzyme is unlikely to be the normal protease involved in Ag processing. Future experiments are aimed at characterizing the sequence of events that generate these well-defined cleavages and elucidating the role of MHC molecules in this process.

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