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# Discrete Proteolytic Intermediates in the MHC Class I Antigen Processing Pathway and MHC I–Dependent Peptide Trimming in the ER

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## Summary

**The antigen processing pathway generates the peptides displayed by MHC I molecules on the cell surface. Whether these peptides are generated in the cytosol or from longer intermediates transported into the ER is unclear, because peptides other than those bound to MHC I have been difficult to find. Using a novel assay, we show that N-terminally extended antigenic analogs were associated with high–molecular weight material in the cytosol and were transported by TAP. In the ER, a nonapeptide was predominant that was converted to the final octapeptide only in presence of the appropriate MHC I molecule. The existence of extended peptides and their MHC I–dependent trimming suggest a mechanism for efficiently satisfying the distinct sequence preferences of polymorphic MHC I molecules.**

## Introduction

Antigen processing and presentation is a series of events that culminates in the display of peptides bound to major histocompatibility complex class I (MHC I) on the cell surface. The peptide/MHC I complexes serve as the ligands for CD8 T cells that recognize target cells displaying peptides originating from viral or bacterial pathogens, tumor-specific genes, or polymorphic histocompatibility loci. Most antigen precursors are initially fragmented into peptides in the cytosol by the multicatalytic proteasome as well as other proteases (Rock and Goldberg, 1999). Cytosolic peptides that meet the size and sequence requirements of the peptide transporter TAP are then delivered into the lumen of the endoplasmic reticulum (ER), where they are loaded onto appropriate MHC I molecules. (York and Rock, 1996; Pamer and Cresswell, 1998). Most empty MHC I molecules, associated with calreticulin and the ERp57 molecule, are tethered to TAP by tapasin (Sadasivan et al., 1996; Ortmann et al., 1997; Hughes and Cresswell, 1998). Upon peptide binding, the MHC I molecules are released from this multimeric complex and transported to the cell surface through the secretory pathway. The peptide loading event, centered at the TAP1/TAP2 heterodimer, thus represents the convergence of two separate pathways: one generating the antigenic peptides and another generating the peptide-receptive MHC I molecules. In contrast to our considerable understanding of how peptide receptive MHC I molecules are generated, far less is known about the pre- and postTAP steps that antigenic precursors undergo to yield the final peptide products presented by MHC I molecules.

Each MHC I molecule displays a large set of peptides that is defined by size and conserved sequence motifs (Rammensee et al., 1995). For a given antigenic precursor, a particular MHC I molecule generally presents a precisely cleaved, homogenous peptide on the cell surface (Van Bleek and Nathenson, 1990; Falk et al., 1991). For example, in cells expressing the ovalbumin protein, the $K^b$ MHC I presents the SIINFEKL (SL8) octapeptide (Rotzschke et al., 1991; Malarkannan et al., 1995). The SL8 peptide shares the sequence motif xxxx(F,Y)x-x(I,M,L) with many other peptides presented by the same $K^b$ MHC. The $D^b$ MHC I molecule, on the other hand, generally presents nonamer peptides that conform to the motif xxxx(N)xxx(I,L,M) (Falk et al., 1991). In principle, the cells could generate these sets of exactly cleaved 8- to 11-mer peptides either in the cytosol itself, where the antigen processing pathway begins (Monaco and Nandi, 1995), or they could generate a set of extended peptides that, following TAP transport, could be trimmed in the ER to the appropriate length (Falk et al., 1990). Support for the former model comes from in vitro assays using model substrates which showed that optimally cleaved peptide fragments can, albeit inefficiently, be generated by the cytosolic proteases (Niedermann et al., 1995; Dick et al., 1996; Beninga et al., 1998), and that these peptides are within the size range suitable for TAP-mediated transport into microsomes (Neefjes et al., 1993; Androlewicz and Cresswell, 1994; Heemels and Ploegh, 1994). However, whether these perfectly cleaved peptides are actually produced in the cytosol and transported by TAP in living cells has not been established. To the contrary, attempts to isolate naturally processed peptides from cells expressing the antigenic precursor in the absence of the restricting MHC I molecule have consistently failed (Falk et al., 1990; Malarkannan et al., 1995), with the single exception, to our knowledge, of the $K^b$-restricted NP peptide that was isolated from VSV-infected H-$2^d$ cells (Nieland et al., 1996). Assuming that the antigenic peptides were not simply inaccessible to the extraction procedures used, these results imply that the MHC I molecules exert a profound influence on the cellular composition of antigenic peptides (Falk et al., 1990). Whether the role of MHC I is to protect the extraordinarily labile peptides from further destruction or to guide the trimming of extra flanking residues to generate the precisely cleaved peptides seen in HPLC fractionated cell extracts or in peptide/MHC crystals has not been resolved. Furthermore, without the ability to detect the proteolytic intermediates in the antigen processing pathway, it is not known which peptide fragments are actually generated from a given antigenic precursor, whether these proteolytic intermediates are bound or free in the cytosol, and what their fate is in the ER in the presence or absence of the restricting MHC I molecules.

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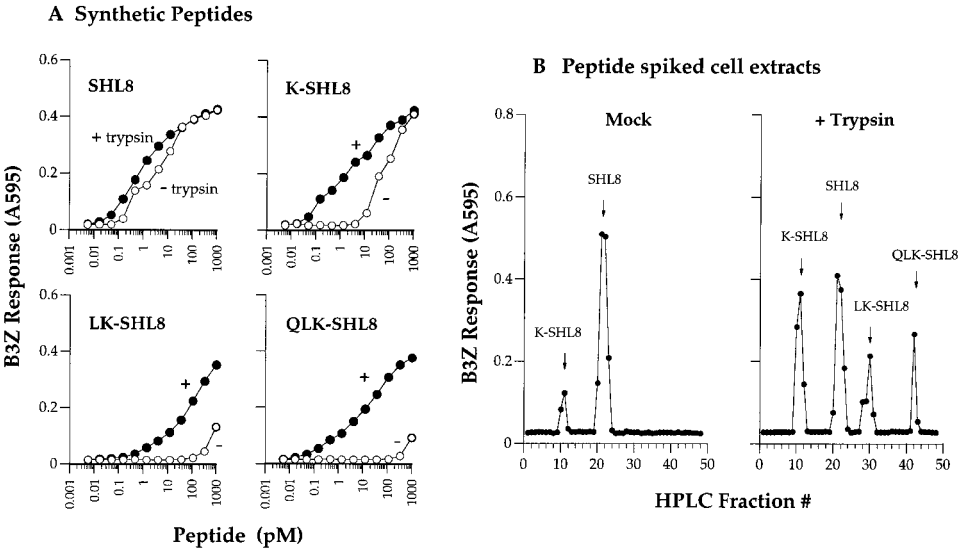


Figure 1. Trypsin Treatment Dramatically Enhances the Antigenic Activity of N-Terminally Extended ∼K-SHL8 Analogs and Allows Their Detection in a Complex Peptide Extract

(A) The SIINFEHL (SHL8) synthetic peptide analogs, with one to three additional N-terminal residues indicated in single letter amino acid code, were digested with active (plus) or DEPC-inactivated trypsin (minus) for 3 hr at 37° and added to $3 \times 10^4$ $K^b$-L cells as APC and $1 \times 10^5$ SHL8/$K^b$-specific B3Z T cell hybridoma cells. After overnight culture, the activation of the B3Z cells was measured as the induction of β-galactosidase activity using the chlorophenol red β-pyranoside substrate. The absorbance of the colored product was measured at 595 nm.

(B) Two femtomoles of each of the indicated peptides was added to an acid extract of COS cells, passed through a 10 kDa cut-off filter, and fractionated by reverse-phase HPLC as described in Experimental Procedures. Individual fractions were treated with inactivated ("Mock") or active trypsin ("+Trypsin") and assayed as in (A). The reproducible elution time for each peptide, marked by vertical arrows, was determined with synthetic peptides that were run and assayed under identical conditions.

To address these questions, we have devised enzymatic methods to analyze the intracellular, naturally processed peptide pool in the antigen processing pathway (Serwold and Shastri, 1999). The conventional T cell activation assays used for detecting antigenic peptides are remarkably insensitive when peptide length is increased by even a single residue. We reasoned that the sensitivity of this assay could be increased if the optimally active peptide was enzymatically released from its extended analogs. This was accomplished by introducing a trypsin cleavage site at the N terminus of the naturally processed peptide in its precursor and treating the HPLC-fractionated cell extracts with trypsin before the T cell assay. Analysis of cell extracts by this HPLC/trypsin method revealed several distinct proteolytic intermediates that were generated in the cytosol and were bound to high–molecular weight material. Some of these fragments were selectively transported via TAP and were further trimmed in the ER. Strikingly, in the ER the conversion of the predominant nonamer to the final octapeptide product occurred only in the presence of the appropriate MHC I molecule. These findings show that proteolysis of antigenic precursors occurs both in the cytosol and in the ER and suggest a mechanism for efficiently satisfying the widely varying peptide-binding preferences of the polymorphic MHC I molecules.

## Results

### An Assay for Detecting Proteolytic Intermediates

In the presence of appropriate APC, antigenic peptides bind to MHC I molecules on the cell surface and can be detected by their ability to stimulate CD8 T cell responses. In contrast to the naturally processed peptides eluted from MHC I, which are active at subpicomolar concentrations in the conventional T cell assay, their analogs with additional N- or C-terminal residues are essentially inactive (Shastri and Gonzalez, 1993; Serwold and Shastri, 1999). This remarkable difference in activity of the optimal versus longer peptides has made it very difficult to detect the putative proteolytic intermediates in cell extracts, because they are necessarily longer than the precisely cleaved final peptide product. To overcome this limitation, we developed an enzymatic method for detecting antigenic analogs of the ovalbumin-derived SIINFEKL (SL8) octapeptide that is the naturally processed product presented by the $K^b$ MHC I molecule. First, the p7 lysine (K) residue was replaced with the histidine (H) residue in the SL8 sequence to yield the SIINFEHL (SHL8) peptide. This K7H substitution within the antigenic SL8 peptide does not affect the recognition of the SHL8/$K^b$ complex by the B3Z T cell and also makes the SHL8 peptide refractory to trypsin cleavage. Second, the natural glutamic acid residue at the N-terminal flanking position was replaced with the lysine (K) residue. These substitutions were designed to allow the release of the optimally active SHL8 peptide from its poorly active analogs containing additional N-terminal flanking residues after treatment with trypsin, which specifically cleaves at the carboxyl end of lysine residues. Indeed, this strategy allowed a 100- to 10,000-fold increase in the sensitivity of the B3Z T cell activation assay for detecting the peptides with one to three additional N-terminal flanking residues (Figure 1A). Further, when a cell extract spiked with only 2 fmol of each of

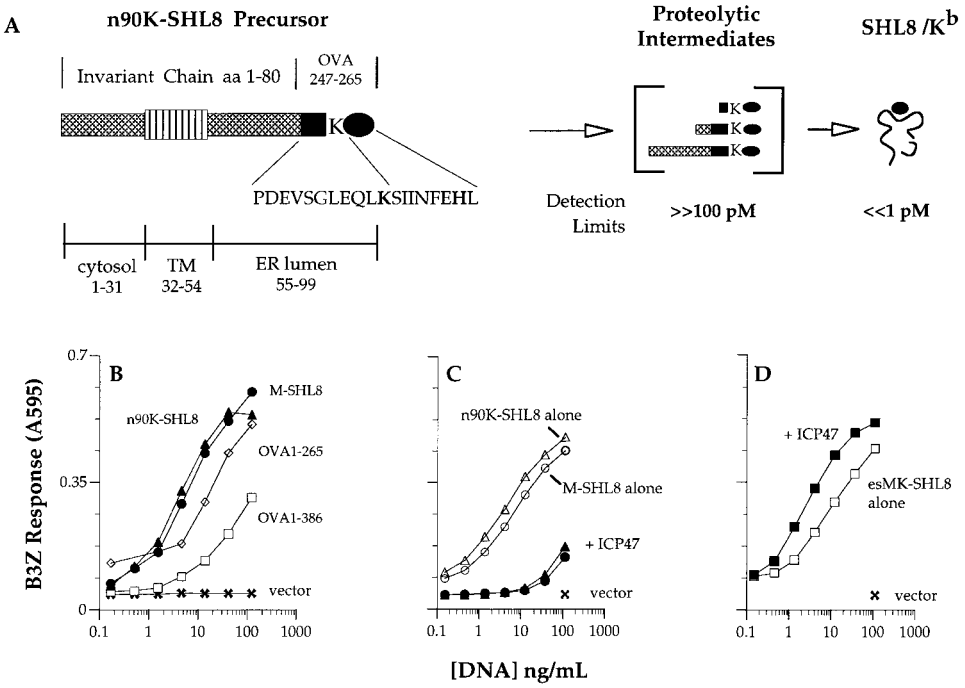


Figure 2. Schematic Representation of the n90K-SHL8 Precursor and Its Efficient Processing to the SHL8/$K^b$ Complex in a TAP-Dependent Manner

(A) Schematic representation of the n90K-SHL8 precursor, a fusion between the invariant chain (aa 1–80) and ovalbumin (OVA) residues 247–265. The cytosolic tail, transmembrane, and ER lumenal regions are indicated. The OVA residues were substituted (E257K, K264H) to allow the release of the optimally active SIINFEHL (SHL8) peptide from poorly active putative proteolytic intermediates (~K-SHL8) by trypsin treatment.

(B–D) Presentation of SHL8/$K^b$ complex to B3Z T cells in transfected COS cells without or with the TAP inhibitor ICP47. COS cells ($5 \times 10^4$) were transfected with 50 ng/ml $K^b$ cDNA and varying concentrations of vector DNA or the indicated antigen DNA constructs (full-length OVA1–386, OVA1–265, Met-SHL8, n90K-SHL8, or the ER-targeted esMK-SHL8) in 96-well plates. Two days later, B3Z ($1 \times 10^5$) T cells were added to each culture, and after an overnight incubation, their lacZ activity was measured as in the legend for Figure 1. In (C) and (D), the TAP inhibitor ICP47 expression plasmid (1 μg/ml, filled symbols) or vector DNA (open symbols) was included during transfection.

the four peptides was fractionated by reverse-phase HPLC and treated with inactive trypsin, only the SHL8 and the K-SHL8 peptides were detected in the T cell assay (Figure 1B). However, after each HPLC fraction was treated with active trypsin, all four peptides were detected in appropriate fractions. Thus, trypsin treatment of HPLC fractions overcomes the limitations in detecting the poorly active, N-terminally extended SHL8 analogs and allows their identification based upon their reproducible HPLC retention times.

### The n90K-SHL8 Is a TAP-Dependent Precursor for Generating the SHL8/$K^b$ Complex

With the goal of analyzing putative proteolytic intermediates generated during antigen processing using the HPLC/trypsin method, we tested different antigenic precursors in an endogenous presentation assay. COS cells were cotransfected with $K^b$ cDNA together with the plasmid vector alone, the minigene M-SHL8, full-length OVA, the OVA1–265 fragment, or a fusion protein containing the SHL8 peptide at the carboxyl end of the invariant chain fragment (n90-K-SHL8) (Figure 2A). The highest B3Z T cell–stimulating activity was observed with constructs encoding the minimal M-SHL8 peptide and the n90-K-SHL8 fusion protein (Figure 2B). To determine whether the n90-K-SHL8 precursor, as a type 2 transmembrane protein (Sanderson et al., 1995), was processed in the ER or in the cytosol, the cells were also transfected with the TAP inhibitor ICP47 (Fruh et al., 1995; Hill et al., 1995). Coexpression of ICP47 inhibited the B3Z T cell stimulation activity generated from both the cytosolic M-SHL8 minigene and the n90-K-SHL8 constructs by over 100-fold (Figure 2C). In contrast, the B3Z stimulating activity of cells expressing the ER-targeted construct esM-K-SHL8 was not inhibited and actually increased in the presence of ICP47 (Figure 2D). Presentation of the SHL8/$K^b$ complex from the ER-targeted n90-K-SHL8 precursor was therefore TAP dependent, indicating that it was processed in the cytosol, either because a fraction of the translated protein failed to translocate into the ER or because it was retrieved from the ER for degradation in the cytosol (Skipper et al., 1996; Snyder et al., 1997). The n90-K-SHL8 was chosen as a model precursor because it was processed efficiently in a conventional TAP-dependent manner and because its SHL8-containing putative proteolytic intermediates could be detected with the HPLC/trypsin method described above.

### Proteolytic Intermediates in the Cytosol and Membrane Compartments

To determine whether proteolytic intermediates actually exist in the MHC I antigen processing pathway, we generated stable FO-1 transfectants expressing the n90-K-SHL8 construct. The FO-1 cell line is derived

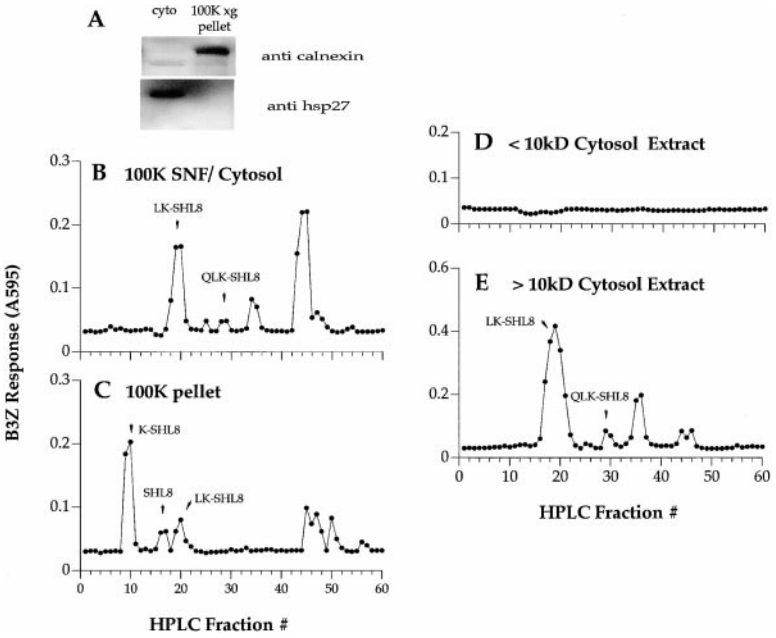


Figure 3. Discrete Proteolytic Intermediates Are Generated from the n90K-SHL8 Precursor in the Cytosol and in the ER

The cytosolic peptides are recovered as high–molecular weight species. The n90K-SHL8-transfected FO-1 cells were homogenized and centrifuged at 100,000 × g to separate the supernatant (SNF/cytosol) from the membrane compartments (100K pellet).

(A) Western blot of the supernatant and pellet fractions with antibodies specific for the ER-resident calnexin or the cytosolic hsp27 proteins. Each lane represents $10^5$ cell equivalents.

(B and C) Antigenic peptides were acid extracted from the cytosol or the pellet by boiling in 10% formic acid, passed through a 10 kDa molecular weight cut-off filter, and fractionated by reverse phase HPLC. Each fraction was digested with trypsin and assayed for B3Z T cell stimulating activity as described in the legend to Figure 1. The arrows indicate the elution time of synthetic peptides run under identical conditions.

(D and E) The cytosol fraction was passed over the 10 kDa cut-off filter without acid extraction to separate free from bound peptides. Peptides were then extracted from the filtrate (<10 kDa cytosol) and the retentate (>10 kDa cytosol), fractionated by HPLC, and analyzed for antigenic activity.

from a human melanoma and does not express endogenous β2-microglobulin (D'Urso et al., 1991). As a consequence, the endogenous MHC I molecules cannot assemble in FO-1 cells, which permits analysis of antigen processing events in the complete absence of functional MHC I molecules. To analyze putative peptide intermediates, the FO-1 cells were homogenized and separated into the 100,000 × g (100K) pellet containing the membrane compartments and the 100K supernatant representing soluble cytosol. Western blot analysis confirmed that, as expected, the 100K pellet was enriched for calnexin, an ER-resident protein, while the cytosolic hsp27 protein was detected only in the supernatant fraction (Figure 3A).

The antigenic peptides in the 100K pellet and the cytosol were extracted by boiling in 10% formic acid, passed through a 10 kDa molecular weight cut-off filter, and analyzed for the presence of B3Z-stimulating SHL8 analogs by the HPLC/trypsin method described above. Two dominant and three smaller activity peaks were detected in the cytosol extract (Figure 3B). One of the major (fractions 18–20) and a minor (fractions 28–29) peak of activity coeluted with the LK-SHL8 10-mer and the QLK-SHL8 11-mer peptides, respectively. The intermediate (fractions 34–35) and late-eluting activity peaks (fractions 43–50) have not yet been assigned. Notably, the optimally active SHL8 as well as the K-SHL8 nonapeptide were not detected in the cytosol. In contrast, the K-SHL8 peptide was the predominant peptide in the extract of the 100K pellet that includes the ER compartment (Figure 3C). Small amounts of antigenic activity were also detected in the late HPLC fractions as well as in peaks coeluting with SHL8 and LK-SHL8 peptides. The HPLC/trypsin method thus revealed the existence of proteolytic intermediates in both the cytosol and in the ER that were generated in the absence of any functional MHC I molecules. Most importantly, the peptide composition of the cytosol was distinct from that of the membrane fraction, with little if any SHL8 peptide in either compartment. Similar results were also obtained in human HeLa and mouse P815 (H-$2^d$) cells transfected with the same precursor (data not shown), indicating that the composition of the cytosolic and ER peptides was similar in human and mouse cells and was not influenced by a variety of different MHC I molecules expressed in these cells. Additionally, N-terminally extended antigenic peptides but not the naturally processed analog were also recovered from FO-1 and COS cells expressing the n90-K-precursor appended with another $K^b$-restricted peptide.

### Cytosolic Peptides Are Associated with High–Molecular Weight Material

The ability to detect the LK-SHL8 and QLK-SHL8 peptides in the cytosol provided an opportunity to characterize the naturally processed preTAP peptides. To determine if these cytosolic peptides existed in a free or bound state, the cytosol was passed through a 10 kDa molecular weight cut-off filter before acid extraction and analysis of the peptides by the HPLC/trypsin method. Free peptides should have flowed through the 10 kDa filter, while the bound peptides would have remained in the >10 kDa retentate. Significantly, no peptides were detected in the <10 kDa filtrate (Figure 3D), and virtually all the peaks observed in the total cytosolic extract were present in the extract of the >10 kDa material (Figures 3E versus 3B). We conclude that none of the cytosolic peptides detected were free but were bound to high–molecular weight species.

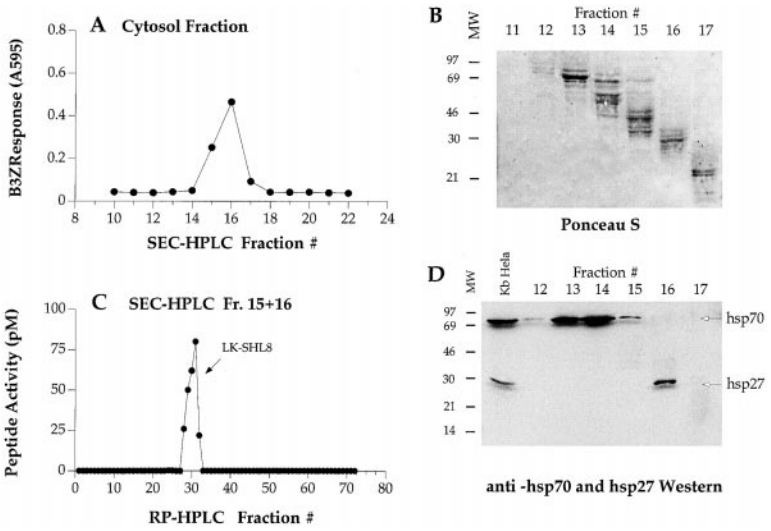


Figure 4. The LK-SHL8 Peptide Is the Predominant Antigenic Activity in the Cytosol and Is Associated with 30–60 kDa Species

The cytosol from $K^b$-HeLa cells expressing the n90K-SHL8 precursor was prepared as in Figure 3, resuspended in 0.5% NP-40, and fractionated by size-exclusion HPLC (SEC-HPLC).

(A and B) Each fraction was tested for (A) B3Z-stimulating activity after acid-extraction and (B) the molecular weight of the constituent proteins by running an aliquot in a 12% SDS-PAGE gel. The proteins were transferred onto a nitrocellulose and stained with Ponceau S.

(C) Fractions 15 and 16 from the SEC-HPLC run were pooled, acid extracted, and fractionated by reverse-phase HPLC (RP-HPLC). Serial dilutions of each fraction were treated with trypsin, and the concentration of antigenic peptides was determined by comparison with synthetic LK-SHL8 peptide standard curve.

(D) The Western blot in (B) was probed with anti-hsp70 and anti-hsp27 antibodies. Molecular weight standards are indicated on the left, and the hsp70 and hsp27 bands (arrows) are indicated on the right.

Previous studies have indicated that antigenic peptides are associated with the cytosolic heat shock proteins (Udono and Srivastava, 1993; Srivastava et al., 1998). However, we were unable to detect antigenic peptides in extracts of hsp70 or hsp90 immunoprecipitates (data not shown). Because immunoprecipitated material could be biased by the specificity of the antibodies used, we directly characterized the cytosolic high–molecular weight species by size-exclusion chromatography (SEC-HPLC, Figure 4A). Each size-exclusion fraction was acid extracted and analyzed for the presence of antigenic peptides. The antigenic activity was detected in fractions 15 and 16 (Figure 4A). These fractions contained material in the 30–60 kDa range as shown by Ponceau S staining of fractionated proteins after denaturing polyacrylamide gel electrophoresis (Figure 4B). Interestingly, Western blot analysis of the same fractions showed that the bulk of hsp70 eluted in fractions 13–14, which did not contain detectable antigenic activity (Figure 4D). However, hsp27, another cytosolic heat shock protein, eluted primarily in fraction 16, which did contain the antigenic activity. But the antigenic activity was not detected in anti-hsp27 immunoprecipitates (data not shown), making it less likely that hsp27 serves as a chaperone for cytosolic antigenic peptides. Further analysis of the antigenic activity in fractions 15 and 16 of the size-exclusion column by reverse-phase HPLC showed that this activity coeluted with the predominant LK-SHL8 decapeptide found in the unfractionated cytosol. The other peptide activities detected in the unfractionated cytosol (Figure 3B) were not detected in fractions 15–16 of the size-exclusion chromatography. It is uncertain whether the other cytosolic peptides were bound to the same chaperone(s) but were lost during experimental manipulations or were bound to yet other molecules. We conclude that the predominant cytosolic LK-SHL8 proteolytic intermediate was associated with a high–molecular weight species.

### TAP Activity Influences Both the Cytosolic and the ER Peptide Pool

To establish that the peptide fragments were part of the antigen presentation pathway and to assess potential relationships between the cytosolic and ER peptides, we analyzed cells with or without functional TAP. COS cells were transfected with the n90-K-SHL8 antigenic precursor in the absence or presence of the TAP inhibitor ICP47. Analysis of the whole cell extracts by the HPLC/trypsin method revealed the same cytosolic and ER peptides as found in the FO-1 and HeLa cells above: K-SHL8, LK-SHL8, QLK-SHL8, as well as the late peaks (Figure 5A). When TAP transport was blocked by coexpression of ICP47, there was a dramatic reduction in the K-SHL8 activity and a concomitant increase in the LK-SHL8 and QLK-SHL8 peaks as well as a shoulder on the late peak, while the bulk of the late-eluting activity in fractions 38–40 remained unchanged (Figure 5B). There was no detectable change in the SHL8 peptide peak, suggesting that this material may represent the 1% activity detected in the absence of TAP function (Figure 2C), or it could have been generated during the extraction procedure. The relative amounts of antigenic peptides were estimated by comparison of serial dilutions of each fraction with appropriate synthetic peptide standard curves assayed under identical conditions. TAP inhibition caused a 6- to 10-fold increase in LK-SHL8 and QLK-SHL8 peptide concentrations and a similar decrease in the K-SHL8 peptide concentration recovered in the fractions (Figure 5C). We therefore infer that the LK-SHL8 and the QLK-SHL8 peptides are cytosolic substrates for TAP and that the K-SHL8 peptide is their TAP-dependent product in the ER.

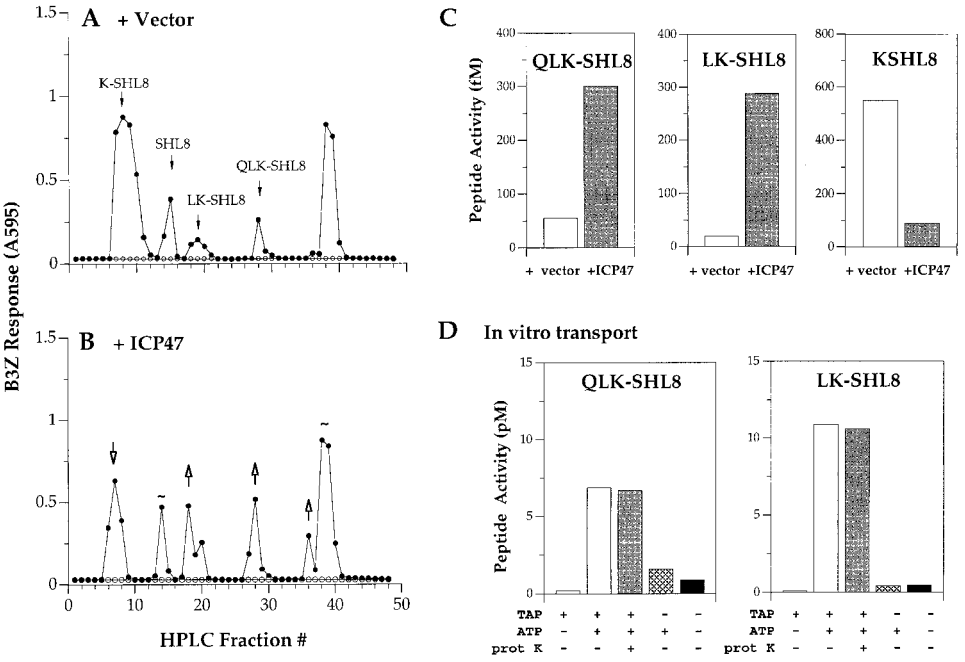


Figure 5. Inhibition of TAP Activity Causes an Increase in Concentration of Cytosolic Peptides and a Decrease in Concentration of ER Peptides

The cytosolic QLK-SHL8 and LK-SHL8 peptides are transported into microsomes in a TAP- and ATP-dependent manner.

(A and B) COS cells were transfected with the n90K-SHL8 antigen precursor together with either (A) vector DNA or (B) the TAP inhibitor ICP47 encoding DNA by electroporation and were harvested 48 hr later. Whole-cell peptide extracts were prepared by boiling in 0.5 mL 10% formic acid. Peptides smaller than 10 kDa were fractionated by HPLC and after trypsin treatment were assayed for B3Z T cell–stimulating activity. In cells expressing ICP47, an increase or decrease in antigenic activity is indicated by the direction of arrowheads. No detectable change is indicated by "∼."

(C) The total relative concentration of QLK-SHL8, LK-SHL8, or K-SHL8 peptides recovered in the absence or presence of ICP47 was computed from serial dilutions of active fractions with the SHL8 peptide as a standard.

(D) In vitro transport of QLK-SHL8 and LK-SHL8 synthetic peptides (100 fmol) was measured using microsomes prepared from wild-type $TAP^+$ or $TAP^{-/-}$ animals with or without ATP. After 10 min, the microsomes were washed and digested with proteinase K or mock digested. Translocated peptides in the microsomes were acid extracted and analyzed for B3Z-stimulating activity.

### TAP Transports the Cytosolic Proteolytic Intermediates

To directly establish that the cytosolic LK-SHL8 and the QLK-SHL8 peptides were TAP substrates, we tested these peptides in an in vitro transport assay (Shepherd et al., 1993). Microsomes were prepared from wild-type $TAP^+$ and TAP1 knockout mice and used for transport of synthetic LK-SHL8 and QLK-SHL8 peptides as described in Experimental Procedures. Transport of these peptides into the microsomes required both ATP and a functional TAP molecule (Figure 5D). Most importantly, the peptide recovery, representing ∼10% of the original 100 fmol input, was completely resistant to treatment of the microsomes with proteinase-K, demonstrating that the peptides were actually translocated into the lumen of the microsomes. This in vitro result therefore supports the analysis of the whole cell extracts and subcellular fractions above and establishes that both the LK-SHL8 and QLK-SHL8 proteolytic intermediates were bonafide substrates for TAP-mediated transport into the lumen of the ER.

### $K^b$ MHC Profoundly Influences Recovery of the K-SHL8 and the SHL8 Peptides

The K-SHL8 nonapeptide was by far the predominant antigenic species detected in the ER (Figure 3C). To assess the relationship between the K-SHL8 peptide and its potential SHL8 cleavage product presented by $K^b$ MHC I, we analyzed extracts from cells coexpressing the antigenic precursor and the vector alone, the irrelevant $K^d$ MHC I, or the $K^b$ MHC I. In striking contrast to the virtually identical antigenic peptide profiles in cells expressing the vector alone or the irrelevant $K^d$ MHC I, where the SHL8 peptide was barely detected (Figures 6A and 6C), the SHL8 peptide peak became the most abundant species in cells expressing $K^b$, even spilling over into the later fractions (Figure 6B). By assaying 1:100 dilution of each fraction, we determined that 98% of the SHL8 activity eluted in fractions 14–15, but the spillover of the remaining 2% obscured all other antigenic peptides, including the LK-SHL8 and QLK-SHL8 peptides, that elute in the later fractions (Figure 6C, inset). Most interestingly, the K-SHL8 peptide peak, which was the predominant species in cells without $K^b$ MHC I, was dramatically reduced in the presence of $K^b$ MHC I (Figure 6B versus Figures 6A and 6C). Occasionally a new peak (marked by an asterisk, Figure 6B) was also observed to elute in fraction 10–11, between the K-SHL8 and the SHL8 peaks. The peptide structure corresponding to this activity is not known because none of the SHL8 analogs with up to six additional amino acids elute at this position. Because shorter SHL8

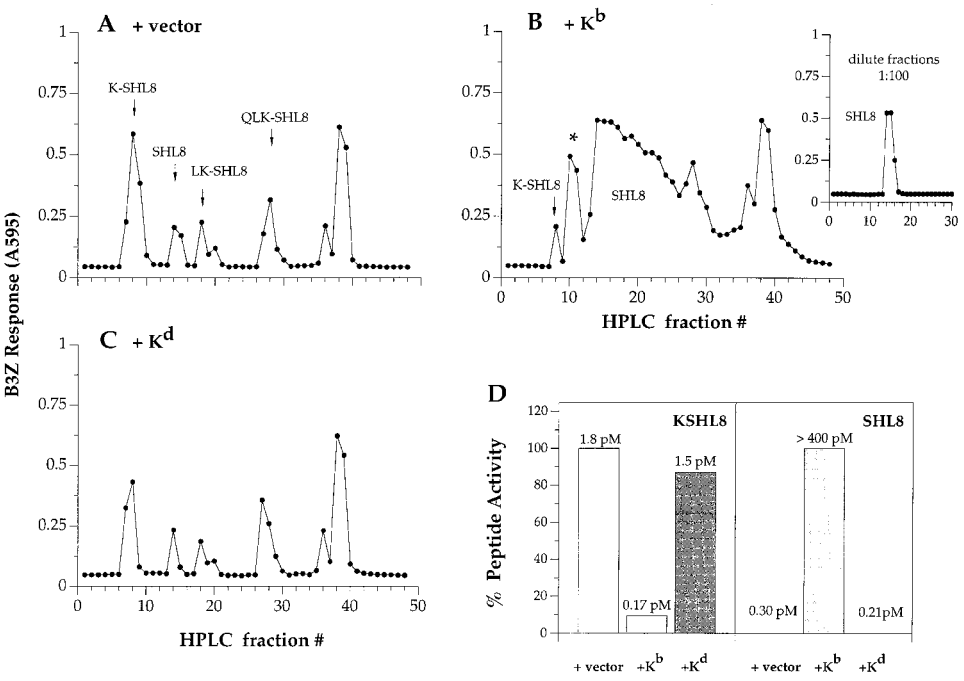


Figure 6. Coexpression of $K^b$ Specifically Affects the Yield of K−SHL8 and SHL8 Peptides

(A–C) Peptides were extracted from COS cells transfected with the n90K-SHL8 construct together with (A) vector alone, (B) $K^b$ MHC I, or (C) $K^d$ MHC I cDNA and analyzed as in Figure 3. The inset in (B) depicts the antigenic activity in the 1:100 dilution of the same HPLC fractions. (D) The total concentration of K-SHL8 and SHL8 peptides recovered from cells cotransfected with the n90-K-SHL8 precursor and the vector, $K^b$ or $K^d$ cDNAs. Serial dilutions of appropriate fractions were compared with trypsin-treated K-SHL8 and SHL8 synthetic peptides as standards, respectively.

analogs are inactive in the T cell assay, the asterisk activity could be due to some other chemical modification. Notably, no significant differences were detected in the cytosolic QLK-SHL8 or the late peaks. We conclude that the profound reciprocal changes in the K-SHL8 and SHL8 peptides were specific to $K^b$ and occurred in the ER.

To quantitate the change in the recovery of the K-SHL8 and SHL8 peptides, serial dilutions of each HPLC fraction were assayed in parallel with appropriate standard curves. The results confirmed the reciprocal relationship in the recovery of the K-SHL8 and SHL8 peptides in the absence or presence of $K^b$ MHC I (Figure 6D). Relative to cells expressing the antigenic precursor with the vector alone or the irrelevant $K^d$ MHC I, in $K^b$ cells there was a 10-fold reduction in the recovery of K-SHL8 peptide and at least a 1000-fold enhancement in the recovery of SHL8 peptide. We conclude that the expression of $K^b$ MHC I correlated not only with the expected increase in the SHL8 octapeptide, but also with a decrease in the K-SHL8 nonapeptide.

To determine if this profound influence of $K^b$ MHC I on the reciprocal recovery of the K-SHL8 and SHL8 peptides required the intact MHC I molecule or if it could be mediated by the $K^b$ heavy chain alone, we introduced into the n90-K-SHL8-expressing, β2-microglobulin-negative FO-1 cells the $K^b$ only or both $K^b$ and β2-microglobulin. The cell extracts were then analyzed by the HPLC/trypsin method for the K-SHL8 and SHL8 peptides that elute within the first 24 fractions. As seen below, an extract of FO-1 cells lacking $K^b$ contained the K-SHL8 peptide, but the SHL8 activity was not detected (Figure 7A). There was little change in this profile when $K^b$ α chain was also expressed in the cells (Figure 7B), despite the fact that the $K^b$ heavy chain was readily detected by Western blot (Figure 7D). By contrast, when both the $K^b$ heavy chain and the β2-microglobulin were expressed in the FO-1 cells, the K-SHL8 peptide was no longer detected and only the SHL8 peptide was recovered in the extract. Taken together, these results support the notion that the K-SHL8 peptide was converted to the SHL8 peptide and that this conversion occurred only in presence of the intact $K^b$ MHC I molecule.

## Discussion

Using a novel method to analyze antigenic peptides, we found that the antigen processing pathway generated a heterogeneous set of proteolytic intermediates in the cytosol that was associated with high–molecular weight material. The N-terminally extended peptides were transported via TAP into the ER and were cleaved to the optimal peptide in the presence of the restricting MHC I molecule. Antigen processing therefore occurs both in the cytosolic and the ER compartments and may have evolved to efficiently satisfy the varying peptide binding preferences of polymorphic MHC I molecules.

### Assays for Proteolytic Intermediates in the Antigen Processing Pathway

Biochemical analysis of the pathway that makes the processed peptides available to the peptide-receptive

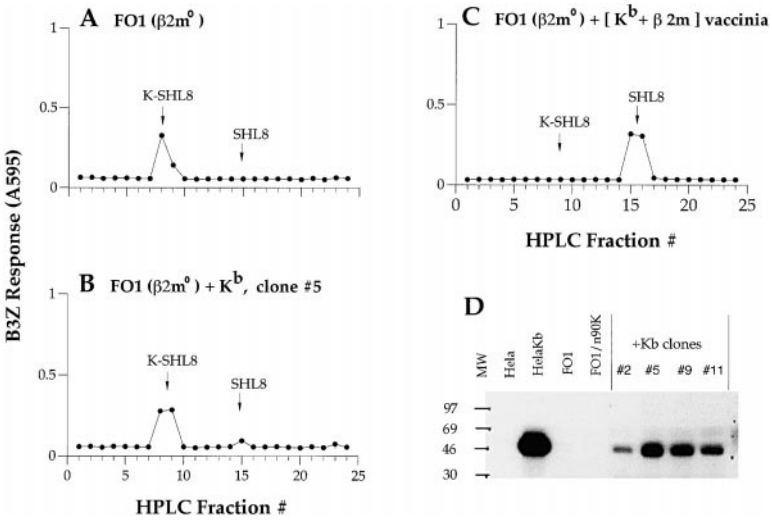


Figure 7. The Intact $K^b$/β2-Microglobulin Heterodimer Is Required for Generation of the SHL8 from Its K-SHL8 Precursor

(A and B) Peptide extracts from FO-1 cells stably expressing n90K-SHL8 antigen (A) without or (B) with the $K^b$ heavy chain were prepared and analyzed as described in Figure 3.

(C) Analysis of peptide extracts from the n90-K-SHL8 transfected FO-1 cells that were coinfected with recombinant vaccinia expressing $K^b$ and β2-microglobulin.

(D) Western blot analysis for $K^b$ α chain expression in four different FO-1/n90-K-SHL8 clones transfected with $K^b$ heavy chain. The indicated cells were lysed in NP-40 and $10^5$ cell equivalents were separated by 12% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes and probed with a $K^b$ specific polyclonal rabbit antiserum. Molecular weight markers are shown on left.

MHC I in the ER has so far been limited by the sensitivity of conventional methods available for detecting antigenic peptides. The standard assays based upon T cell activation are adequate for analysis of the final MHC I–bound processed peptide products but are remarkably insensitive for detecting antigenic analogs with additional flanking residues (Figure 1). This bias is expected because the T cells are elicited by the final processed peptide/MHC I complexes in the animals and was the apparent cause for the failure of previous attempts to identify these proteolytic intermediates (Falk et al., 1990; Malarkannan et al., 1995). The HPLC/trypsin method, as well as the design of the antigenic precursor where the optimal SHL8 peptide could be released in a single step, increased the sensitivity of the T cell assays by several orders of magnitude and allowed the detection of the elusive proteolytic intermediates in living cells. We anticipate that further improvements in this methodology will also permit the detection of proteolytic intermediates when the antigenic peptides are located in sequence contexts containing both N- and C-terminal flanking residues (T. Serwold and N. S., unpublished data).

**Antigen Processing in the Cytosol**

At least six discrete proteolytic intermediates were detected in the cytosol (Figure 3B). Notably, this heterogeneous set of cytosolic peptides did not include the SHL8 peptide, which is one of the most widely studied peptides presented by the $K^b$ MHC molecule. This result clearly shows that proteolysis of the antigenic precursor in the cytosol does not generate detectable amounts of the exactly cleaved peptides that are eventually presented by the MHC I molecule, and it could explain why these peptides have been difficult to find even in prolonged in vitro digests with the proteasome (Dick et al., 1994; Niedermann et al., 1995). Our findings are also consistent with a previous study that indicated that the final peptide products presented by the MHC I are often poor substrates in in vitro transport assays without additional flanking residues (Neisig et al., 1995). We show that the LK-SHL8 and the QLK-SHL8 peptides were the actual substrates for TAP transport because their concentrations increased in cell extracts when TAP was blocked by ICP47 in vivo (Figure 5). Moreover, these peptides were directly transported into the microsomal lumen in vitro in a TAP- and ATP-dependent manner (Figure 5). Notably, except for a change in the shoulder, blocking TAP did not affect the antigenic peptides that eluted later in the HPLC profiles, indicating that TAP does not transport all proteolytic intermediates. Determination of the molecular structures of the peptides contained within these late peaks should reveal whether the failure to be transported was due to their size, which may have exceeded the capacity of TAP, or their inability to associate with the putative cytosolic chaperone(s). Alternatively, it is possible that the longer peptides were the immediate precursors to the LK-SHL8 and QLK-SHL8 peptides. The potential precursor/product relationship between the late peaks and the 10- to 11-mer TAP substrates was not addressed in this analysis but is an attractive and testable hypothesis which suggests that cytosolic antigen processing may be a multistep process involving different proteolytic mechanisms (Craiu et al., 1997; Glas et al., 1998; Serwold and Shastri, 1999).

The antigenic peptides detected in the cytosol were not free but were recovered in a high–molecular weight fraction, which did not include the previously described heat shock proteins (Figures 3 and 4). Ignoring technical reasons such as the differences in cell types, the antigenic peptides analyzed, or the sensitivity of the assays employed to detect them, two interesting possibilities may account for this disparity. First, proteolytic intermediates generated from different antigenic precursors may associate with distinct cytosolic chaperones. One might expect a difference between transmembrane proteins such as the n90-K-SHL8 precursor, which could be retrieved from the ER for proteolysis in the cytosol, and other proteins that are synthesized and degraded in the cytosol itself (Skipper et al., 1996; Snyder et al., 1997). Second, distinct chaperone(s) may carry out different functions, such as ferrying the proteolytic fragments between different proteases and from the protease(s) to the TAP complex. Because the LK-SHL8 peptide was actually transported by TAP, we speculate

that the high–molecular weight material it was associated with could be the cytosolic chaperone that transports peptides to TAP, although the possibility that this is a protease cannot be ruled out. Efforts to identify this material are in progress.

### Role of MHC I during Antigen Processing in the ER

The influence of the $K^b$ MHC I on the recovery of the final SHL8 octapeptide product versus the longer K-SHL8 nonapeptide found exclusively in the ER was striking. Coexpression of the $K^b$ but not $K^d$ or other MHC I molecules in cells expressing the antigenic precursor led to a dramatic increase in SHL8 octapeptide and a concomitant decrease in the K-SHL8 nonapeptide in cell extracts (Figure 6). The effect of $K^b$ was specific for the K-SHL8 peptide found exclusively in the ER and did not affect the cytosolic QLK-SHL8 peptide nor the peptides contained within the late peaks. Furthermore, the loss of K-SHL8 peptide occurred only in the presence of the $K^b$ $\alpha$ chain and $\beta$2-microglobulin, indicating that this effect required the complete $K^b$ heterodimer. In other experiments, a similar increase in SHL8 and a decrease in K-SHL8 recovery was observed with mutant $K^b$ MHC I molecules lacking the cytoplasmic tail or the transmembrane region (data not shown). Taken together, these findings strongly suggest that the K-SHL8 peptide is the immediate precursor for the SHL8 octapeptide and that its conversion depends upon the antigen-binding function of the $K^b$ MHC I molecule.

How does $K^b$ MHC I influence the conversion of the K-SHL8 to the SHL8 peptide? The simplest explanation for this result is that the MHC I molecules serve as templates for the final proteolytic event, as was suggested by Rammensee's group almost a decade ago when they first showed that antigenic peptides in cell extracts were detected only in presence of appropriate MHC I molecules (Falk et al., 1990). Since then, several studies have inferred from functional assays that N-terminal rather than C-terminal flanking residues can be cleaved from antigenic precursors in the ER (Snyder et al., 1994; Powis et al., 1996; Yellen-Shaw et al., 1997). Our results provide definitive identification of proteolytic intermediates in the cytosol and the ER during antigen processing. Changes in their concentration and structure provide direct evidence that not only does N-terminal trimming actually occur in the ER but that this aminopeptidase activity is profoundly influenced by the MHC I molecules. We have also observed that similar MHC I-dependent cleavages occur in isolated microsomes (N. B. and N. S., unpublished data), which may allow access to the molecules that mediate these rapid proteolytic events. It should also be interesting to test the potential role of the ERp57 molecule in ER trimming events, because it was recently discovered to be a member of the TAP/tapasin/MHC I complex (Hughes and Cresswell, 1998) and has been reported to possess protease activity (Otsu et al., 1995). Furthermore, note that the large, $>$100-fold difference in the steady state concentrations of the K-SHL8 versus the $K^b$-bound SHL8 peptides demonstrates that in the absence of peptide binding to MHC I, the proteolytic intermediates do not accumulate in the cells. Therefore, another mechanism must exist that rapidly degrades the antigenic precursors that fail to find a niche in the antigen-binding groove of the available MHC I molecules.

### Compartmentalized Proteolysis Can Improve Antigen Processing Efficiency

The distinct proteolytic products described here suggest a solution to the efficiency conundrum posed by the postulate that peptides presented by MHC I are generated in the cytosol prior to their transport and binding to MHC I in the ER. Generating the exactly cleaved peptides in the cytosol, without knowledge of which polymorphic MHC I molecules await their arrival in the ER, can be an expensive undertaking. Considering an extreme example, a set of 379 random 8-mers must be generated from the 386 residue ovalbumin protein to ensure that the exact SL8 octapeptide is among those available for binding to $K^b$ MHC I in the ER. Yet, because the same SL8 peptide with an additional N-terminal residue is also presented by the $D^b$ MHC I molecule (Malarkannan et al., 1995), the cells must also generate an additional 378 random 9-mers to ensure that the X-SL8 nonamer will be available if the $D^b$ MHC I was in the ER. Given that cells simultaneously process thousands of different proteins, generating sets of exact 8- to 11-mer peptides in the cytosol would result in an enormous and eventually wasteful pool of peptides. However, this waste can be largely obviated if longer peptides were generated in the cytosol and trimmed to fit the available MHC molecules in the ER. In the model system described here, the LK-SHL8 peptide could yield either the SHL8 or the K-SHL8 peptides depending upon whether the $K^b$ or $D^b$ MHC I were available in the ER. Thus, MHC I–dependent cleavage of extra flanking residues can significantly increase the efficiency with which optimal peptide/MHC complexes can be generated from a given precursor.

#### Experimental Procedures

##### Cell Lines

The SHL8/$K^b$-specific, lacZ-inducible, B3Z T hybridoma, $K^b$ L, and COS cell lines have been described (Karttunen et al., 1992). The $\beta$2m negative FO1 melanoma cell line was generously provided by Dr. S. Ferrone (D'Urso et al., 1991). FO1 and HeLa cells expressing the n90K-SHL8 $\pm$ $K^b$ were generated by electroporation. Positive clones were identified by their ability to stimulate B3Z T cells directly or when transfected with $K^b$ cDNA or infected with vaccinia (a kind gift of J. Yewdell) expressing $K^b$ and $\beta$2m at a multiplicity of infection of 1.

##### Plasmid DNAs, Synthetic Peptides, and Antibodies

Plasmids encoding OVA, OVA253–386, n90K-SHL8, M-SIINFEHL, or es-SHL8 have been described or were prepared using synthetic oligonucleotides (Shastri and Gonzalez, 1993). The $K^d$ and ICP47 cDNAs (kind gifts of Drs. T. Hansen and D. Johnson, respectively) were subcloned into the pcDNAI vector. Synthetic peptides were prepared by Dr. David King (UC-Berkeley, CA), purified ($>$99.9%) by HPLC, and confirmed by mass spectrometry. The rabbit anti-$K^b$ was kindly provided by Dr. E. Song.

##### T Cell Activation Assays

SHL8/$K^b$-specific B3Z response was measured as the $\beta$-galactosidase (lacZ) activity induced upon T cell activation. B3Z cells ($1 \times 10^5$) were cocultured overnight with 3–5 $\times$ $10^4$ transfected APC or $K^b$ L cells and synthetic peptides or HPLC fractions. To detect N-terminally extended SHL8 analogs, synthetic peptides or HPLC

fractions were digested in 50 μl PBS with 20 μg/ml trypsin for 3 hr at 37°C. DEPC-inactivated trypsin was used for mock-trypsin digestion. LacZ activity was measured by the cleavage of chlorophenol red β-pyranoside to chlorophenol red at 595 nm wavelength (Shastri and Gonzalez, 1993).

### Transient Transfections

For endogenous presentation, $5 \times 10^4$ COS cells were transiently transfected with varying antigen DNA concentrations and 50 ng/ml $K^b$ cDNA (Shastri and Gonzalez, 1993; Sanderson et al., 1995). TAP dependence of presentation was determined by including 1 μg/ml ICP47 DNA during the transfection. For peptide extracts, $2 \times 10^7$ COS cells were electroporated with 10 μg antigen DNA (Karttunen et al., 1992), and 15 μg MHC I cDNA ($K^b$ or $K^d$) or ICP47 were included for testing the role of MHC I or TAP. In all transfections, total DNA amounts were equalized with vector DNA.

### Peptide Extraction and RP-HPLC Analysis

Peptide extracts were prepared by boiling cell pellets in 500 μl 10% formic or acetic acid and 2 μM irrelevant peptide for 5 min. After removing particulate debris by centrifugation, the supernatant was passed through a 10 kDa cut-off filter and injected into the HPLC. Cell extracts, synthetic peptides, and fractions from SE-HPLC were separated using the Hewlett Packard 1050 HPLC on a 2.1 × 250 mm, 5 μm, 300 Å pore C18 column (Vydac). Peptides were separated using 0.1% TFA in H20 (buffer A) and 0.1% TFA in acetonitrile (buffer B): 0–5 min, 23% B; 5–49 min, 23%–45% B; 49–52 min, 45%–77% B at a flow rate of 0.25 ml/min. Five drop fractions were collected in flat-bottom 96-well plates and dried in a vacuum centrifuge. The trypsin-digested fractions were tested for antigenic activity as described above. HPLC runs from mock injections were routinely analyzed to rule out cross-contamination among samples.

### Subcellular Fractionation

Cell pellets of $\sim 2.0 \times 10^8$ FO1/N90K-SHL8 were dounced with 15 strokes in ice-cold homogenization buffer (0.25 M sucrose, 10 mM Tris-Cl [pH 7.4] plus 1 mM PMSF, 5 mM iodoacetamide, 0.2 TIU/ml aprotinin, and 100 μM LLnL). Intact cells and nuclei were pelleted by centrifugation at 1000 × g for 10 min at 4°C and dounced again. The supernatants were pooled and the cytosol separated from membrane fraction by centrifugation at 100,000 × g for 1 hr at 4°C. Anti-calnexin (AF8 antibody kindly provided by Dr. M. Brenner) or anti-hsp27 (MS-101P, NeoMarkers) antibodies were used to confirm the purity of cytosol preparation in the 100,000 × g supernatant. The cytosol was extracted as described above and fractionated by reverse-phase HPLC to analyze the antigenic peptides or resuspended in 0.5% NP40/PBS + protease inhibitor cocktail for size-exclusion HPLC. The cytosol was injected into a 7.8 mm × 30 cm size-exclusion column with 5 μm particle size (TSK-GEL 08541, TosoHass). Fractions of 0.5 ml were collected in PBS at a flow rate of 1 ml/min. Ten microliters of each fraction ($10^5$ cell equivalent) was analyzed by SDS-PAGE and proteins visualized by Ponceau S staining or by Western Blot with anti-hsp70 (SPA810, Stressgen) and anti-hsp27.

### In Vitro Peptide Translocation Assay

Microsomes from C57BL/6 ($TAP^{+/+}$) or $TAP^{-/-}$ mice were prepared as described (Shepherd et al., 1993). Microsome preparations were snap frozen in 25 μl aliquots in 50 mM HEPES, 250 mM sucrose, and 1 mM DTT (pH 7.3), stored at −70°C, and used only once after thawing. Translocation assays with 5 μl freshly thawed microsomes were performed in 100 μl in transport buffer (50 mM HEPES [pH 7.5], 150 mM KOAc, 5 mM MgOAc, 250 mM sucrose, and 1 mM DTT) with or without ATP-regenerating system (50 μM ATP, 2.5 mM phosphocreatine, and 0.05 U/ml creatine-phosphokinase). Synthetic peptides (100 fmol) were added to the transport mix and incubated at RT for 10 min. Samples were treated with 100 μg/ml of Proteinase K for 30 min on ice and stopped by the addition of 1 mM PMSF. The reactions were spun over a sucrose cushion (0.5 M sucrose in 1 M KOAc, 50 mM HEPES, and 10 μM irrelevant peptide) for 15 min at 140,000 × g to pellet the microsomes. Transported peptides were then extracted and analyzed for antigenic activity as above.

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