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# MHC Class I Molecules Can Direct Proteolytic Cleavage of Antigenic Precursors in the Endoplasmic Reticulum

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## Summary

**The large set of peptides presented by MHC (major histocompatibility complex) class I molecules are generated by proteolysis of diverse precursors in the cytoplasm and possibly in the endoplasmic reticulum (ER). To define the potential peptide trimming events in the ER, we analyzed proteolytic products generated in isolated microsomes. The residues flanking the N terminus of the final antigenic peptide were rapidly removed within the microsomes but only in the presence of appropriate MHC molecules. Remarkably, the precursor peptide was bound to the MHC molecules in a distinct conformation and required an aminopeptidase activity to generate the optimal peptide. The MHC molecules are therefore not only the final repositories of antigenic peptides, but they can also direct their excision from longer precursors.**

## Introduction

Cytotoxic T cells detect abnormal intracellular events by the presence of unique peptides displayed by MHC (major histocompatibility complex) class I molecules (Falk et al., 1991; Madden et al., 1991). These peptides are generated by fragmentation of the diverse pool of newly synthesized intracellular proteins (Rock and Goldberg, 1999; Reits et al., 2000; Schubert et al., 2000). The peptide/MHC complex thus represents the convergence of two independent pathways: one for generating peptide-receptive MHC molecules, and another for generating candidate peptides for potential binding to the MHC. Peptide-receptive MHC molecules are generated in the endoplasmic reticulum (ER) by mechanisms involving general housekeeping components, such as the chaperones calnexin and/or calreticulin, that enable proper folding of the MHC subunits as well as specialized components, such as tapasin, which tethers the empty MHC molecules to the TAP transporter (Cresswell et al., 1999). On the other hand, antigenic precursors are degraded in the cytosol, primarily by the multicatalytic proteosome and possibly other proteases (Rock and Goldberg, 1999; Stoltze et al., 2000). The peptide fragments are then transported by TAP into the ER where they can be tested for their ability to bind the available MHC molecules.

The topological separation between the sites for peptide generation in the cytosol and MHC loading in the ER raises the dilemma of how cytosolic proteolysis alone can efficiently generate appropriate peptides, with strictly defined length and conserved sequence motifs, without knowledge of which set of polymorphic MHC molecules await their arrival in the ER. A possible solution to this conundrum was recently suggested by the discovery that cytosolic proteolysis of a model antigenic protein yielded a mixture of proteolytic intermediates rather than the single, precisely cleaved peptide that was ultimately presented by the MHC molecule on the cell surface (Paz et al., 1999). A subset of these extended peptides was transported by TAP into the ER in this and in another recent study (Lauvau et al., 1999). Intriguingly, the yields of the extended precursor and that of the optimally trimmed peptide were inversely proportional and were dramatically influenced by the presence of the appropriate MHC molecule (Paz et al., 1999). These findings suggested the existence of a precursor/product relationship between the extended and optimally cleaved peptides that was somehow influenced by the MHC molecules. They also provided an explanation for earlier reports which had shown that naturally processed peptides were detected only in the presence of appropriate MHC molecules (Falk et al., 1990; Malarkannan et al., 1995). Furthermore, the evidence for the inverse relationship between the MHC dependent recovery of the precursor and its peptide products supported the original hypothesis of the Rammensee group, which suggested that MHC molecules may also direct the generation of the optimally cleaved antigenic peptides that they subsequently display on the cell surface (Falk et al., 1990). The most attractive feature of this model was that it relieved the cytosol of the sole responsibility of generating the large sets of precisely cleaved peptides required by the ER-resident polymorphic MHC molecules (Rammensee et al., 1995). Rather, the model postulated that antigenic precursors were fragmented not only in the cytosol, but also in the ER where the final peptide trimming events could be efficiently guided by the MHC molecules (Paz et al., 1999).

Our attempts to test this hypothesis and to define how the MHC molecules determined the outcome of the antigen processing reactions, however, failed in living cells. The failures were due to (1) the high complexity of the mixture of proteolytic intermediates generated in living cells, which made it difficult to study precursor/product relationships, and (2) an inability to selectively inhibit the ER trimming reactions in living cells to capture putative reaction intermediates. To overcome these difficulties, we developed a novel in vitro model for analysis of peptide trimming reactions in murine microsomes. The microsomes have previously been established as a model for the study of selective peptide transport by TAP (Neefjes et al., 1993; Shepherd et al., 1993; Momburg et al., 1994; van Endert et al., 1994) and peptide binding molecules (Marusina et al., 1997; Spee and Neefjes, 1997). Using a natural TAP substrate and a highly sensitive assay to detect proteolytic intermediates, we show here that antigenic peptides are trimmed in the microsomes by an aminopeptidase and that the MHC molecules can direct this process.

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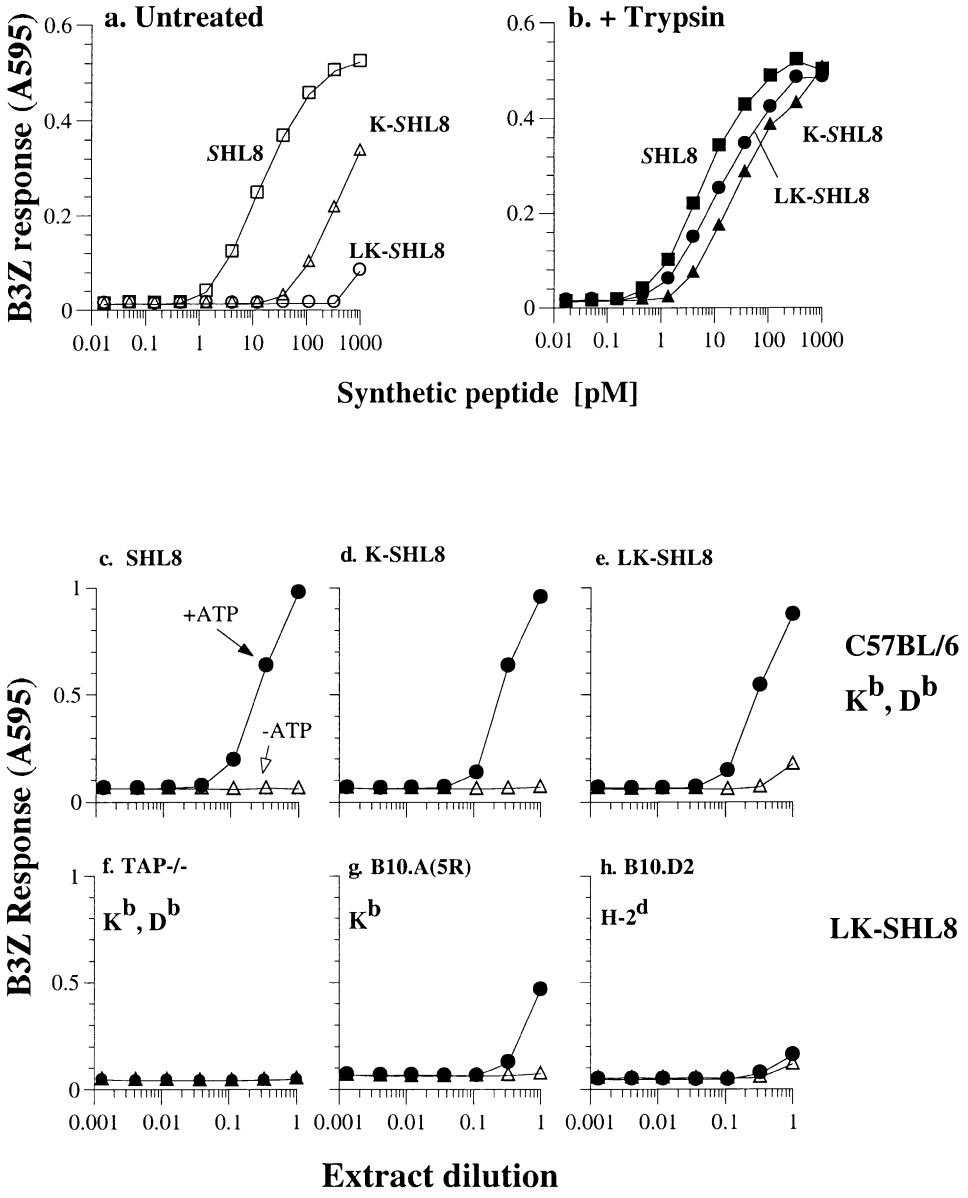


Figure 1. Activity of the N-Terminally Extended SHL8 (SIINFEHL) Analogs K-SHL8 and LK-SHL8 is Greatly Enhanced by Trypsin Treatment

Varying concentration of synthetic SHL8, K-SHL8, and LK-SHL8 peptides were tested (a) as such or (b) after treatment with trypsin to stimulate SHL8/$K^b$-specific B3Z T cell hybridoma. $K^b$-L cells were used as antigen-presenting cells (APC). After overnight incubation, the lacZ response of B3Z T cells was assayed as the β-galactosidase activity by the conversion of the substrate chlorophenol red β-D-pyrannoside measured at 595 nm.

Antigenic peptide activity can be recovered from microsomes after TAP- and ATP-dependent transport and in presence of appropriate $K^b/D^b$ MHC class I molecules. SHL8/$K^b$-specific B3Z T cell response to dilutions of total microsomal extracts after incubation with C57BL/6 microsomes and 50 fmol of (c) SHL8, (d) K-SHL8, and (e–h) LK-SHL8 peptides in the absence (c) or presence of ATP (J). B3Z T cell responses to total extracts prepared after incubation of the LK-SHL8 peptide with microsomes prepared from (f) $TAP^{-/-}$, (g) B10.A(5R), and (h) B10.D2 mice. The presence of the relevant $K^b$ and $D^b$ MHC class I molecules in the microsomes is indicated. All samples were treated with trypsin before the assay to release the optimally active SHL8 peptide from the extended precursor (Paz et al., 1999). $K^b$-L cells were used as APC, and the antigen-specific B3Z T cell response was measured as above.

## Results & Discussion

### In Vitro Model for Peptide Trimming in the ER

To determine if trimming of antigenic peptides occurred in the ER, we developed an in vitro model for antigen processing in murine microsomes. An N-terminally extended version of the naturally processed OVA257-264 (SIINFEHL, SHL8) peptide, LK-SIINFEHL (LK-SHL8), was used as a precursor because we had earlier shown that this peptide was a natural substrate for TAP translocation in intact cells (Paz et al., 1999). Most importantly, because the LK-SHL8 peptide contains a single lysine (K) residue flanking the SHL8 peptide, treatment of LK-SHL8 or the K-SHL8 peptides with trypsin, which

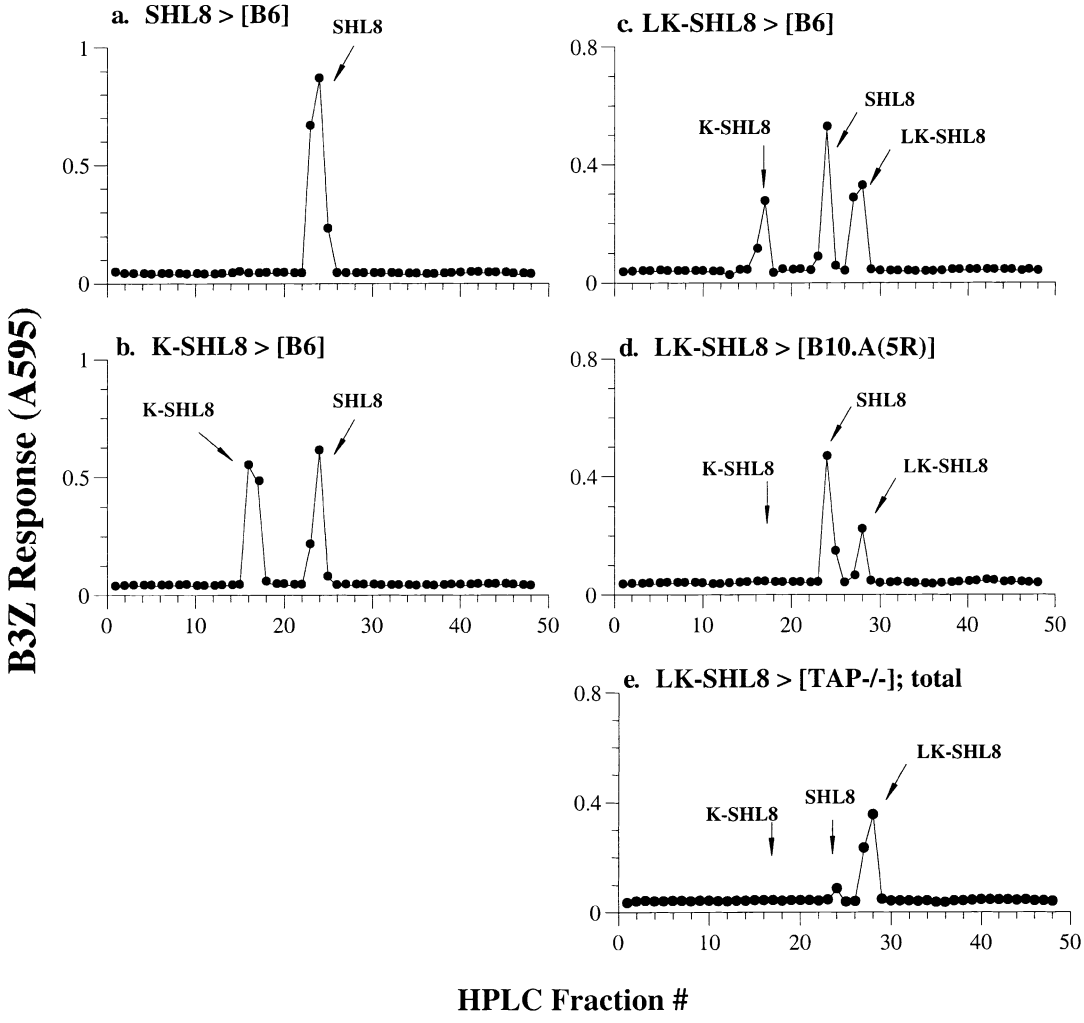


Figure 2. Precursor Peptides Trimmed to Optimal MHC Binding Peptides within the Microsomes

Acid extracts prepared from (a–c) C57BL/6 and (d) B10.A(5R) microsomes after incubation with (a) SHL8, (b) K-SHL8, or (c–e) LK-SHL8 peptides were fractionated by HPLC and assayed for B3Z T cell stimulating activity as in legend to Figure 1. (e) The entire reaction mixture was extracted after incubation of the $TAP^{-/-}$ microsomes with the LK-SHL8 peptide. All HPLC fractions were treated with trypsin before the assay to release the optimally active SHL8 peptide from the extended precursor. Arrows mark the peak HPLC retention time of the indicated synthetic peptides run and assayed under identical conditions.

cleaves at the carboxy terminus of lysines, releases the optimally active SHL8 peptide (Figures 1a and 1b). This 100- to 1000-fold increase in activity makes it possible to detect these poorly antigenic N-terminally extended analogs of the SHL8 peptide in complex extracts using the standard SL8/$K^b$-specific B3Z T cell activation assay. Furthermore, when the extracts were first fractionated by HPLC and the fractions were treated with trypsin *prior* to the T cell assay the conversion of the LK-SHL8 and K-SHL8 peptides into SHL8 revealed their presence as well as their identity as activity peaks that reproducibly elute in characteristic fractions (Paz et al., 1999).

We first tested whether LK-SHL8 and its proteolytic products K-SHL8 and SHL8 could serve as substrates for TAP transport in vitro in isolated murine microsomes. The "SHL8" antigenic activity was recovered after incubating the peptides with microsomes from C57BL/6 (B6) mice in the presence of ATP (Figures 1c–1e) but not with microsomes from TAP-deficient mice (Figure 1f) (Neefjes et al., 1993; Shepherd et al., 1993). Most significantly, the antigenic activity was detected in microsomal extracts only when the appropriate MHC molecules were also present in the microsomes prepared from the C57BL/6 ($K^b$, $D^b$) or the B10.A(5R) ($K^b$) mice, but not from the B10.D2 ($H\text{-}2^d$) mice (Figures 1g and 1h). Presently, it is unclear why the precursor LK-SHL8 peptide was not recovered from the B10.D2 microsomes that lack the relevant $K^b$ or $D^b$ MHC molecules. It is possible that the peptide was rapidly degraded (Paz et al., 1999) or was fluxed out of the microsomes (Schumacher et al., 1994; Koopmann et al., 2000). Another possibility is that the LK-SHL8 may have been bound to ER chaperones, such as gp96 (Srivastava et al., 1998), and could have remained inaccessible to the extraction procedure. The latter possibility is, however, rendered less likely because the association of the LK-SHL8 peptide with the

chaperones would have to be resistant to a harsh 5 min incubation with 10% formic acid at 95°C.

To determine if the antigenic activity recovered from the microsomes represented the input peptides themselves or their proteolytic products, the microsomes were isolated from the reaction mixture by a spin through a sucrose cushion and were extracted with acetic acid. The microsomal extracts were then fractionated by reverse phase HPLC, and the fractions were analyzed for presence of T cell-activating peptides after trypsin treatment. The activity profiles showed that while the input SHL8 peptide was recovered as such, the N-terminal residues of both the K-SHL8 and LK-SHL8 peptides were trimmed to yield the optimal SHL8 octapeptide (Figures 2a–2c). The LK-SHL8 decapeptide also yielded the K-SHL8 nonapeptide in $K^b$ and $D^b$ containing B6 microsomes, but not in B10.A(5R) microsomes which contain only $K^b$ MHC (Figure 2d). This result is consistent with the previous demonstrations that the SHL8 and K-SHL8 peptides bind optimally to $K^b$ and $D^b$ MHC, respectively (Buchholz et al., 1995; Malarkannan et al., 1995). Generation of SHL8 and K-SHL8 occurred within the microsomes because the trimmed peptides were not detected when the LK-SHL8 precursor was incubated with the $TAP^{-/-}$ microsomes in the presence of ATP and the entire mixture was extracted and analyzed by HPLC (Figure 2e). Furthermore, consistent with their lumenal location, the peptide recovery was not inhibited by proteinase K treatment of the microsomes (Paz et al., 1999; data not shown). We conclude that, as observed earlier in intact cells (Paz et al., 1999), within isolated microsomes an N-terminally extended precursor peptide was transported via TAP, and its optimally trimmed products were recovered only in the presence of the relevant MHC molecules.

The requirement for the MHC molecules in allowing the recovery of optimally cleaved peptides in this in vitro model is similar to what has been consistently observed in intact cells (Falk et al., 1990; Malarkannan et al., 1995; Paz et al., 1999). However, whether this outcome was due to the requirement of MHC molecules to stabilize the optimally cleaved peptides or was due to an active role for the MHC in the peptide trimming reactions has remained difficult to resolve in intact cells. A key prediction of the latter hypothesis is that a longer precursor should be associated with the MHC molecules as an intermediate for the generation of the final peptide/MHC complex. Because such an intermediate has never been observed in intact cells, we searched for its existence in this readily manipulated in vitro model system.

### Peptide Trimming in the Microsomes Requires Aminopeptidase Activity and Occurs Rapidly

To detect this elusive intermediate, we initially optimized conditions in which peptide trimming could be blocked. First, we tested a panel of protease inhibitors for their ability to block the generation of SHL8 and K-SHL8 products from the LK-SHL8 precursor in B6 microsomes (Figures 3a–3c). Inclusion of the aminopeptidase inhibitor leucine chloromethylketone (LCMK) during the incubation completely inhibited peptide trimming, whereas another aminopeptidase inhibitor (bestatin) specifically inhibited the generation of the K-SHL8 but not the SHL8

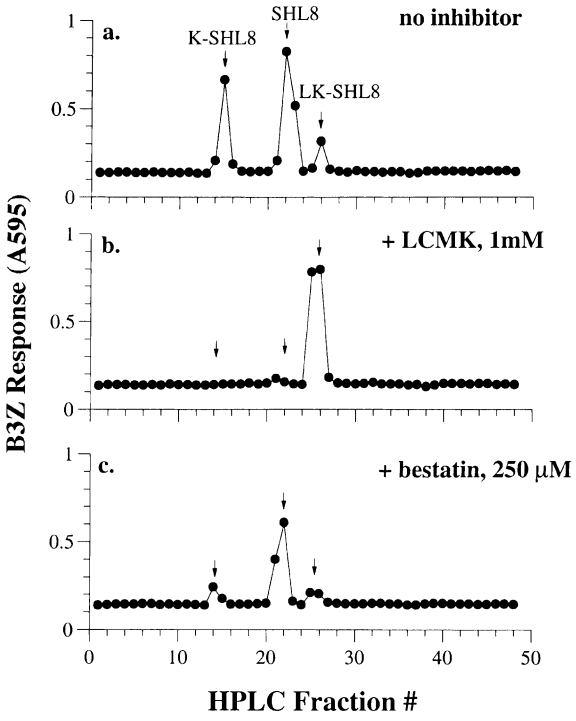


Figure 3. Microsomal Trimming of LK-SHL8 Peptide Is Blocked by Aminopeptidase Inhibitors

B3Z T cell responses to HPLC fractionated extracts of C57BL/6 microsomes after incubation with LK-SHL8 peptide in (a) the absence or (b and c) presence of aminopeptidase inhibitors Leucinechloromethylketone (LCMK) or bestatin. HPLC fractions were analyzed as in legend to Figure 2. The arrows indicate the peak retention times of the indicated peptides shown in part (a).

peptide. Inhibitors of cysteine-proteases (FK029 and E64), of serine proteases (dichloroisocoumarin), and of proteasomes (LLnL and lactacystin) did not significantly affect the cleavage of LK-SHL8 in the microsomes (data not shown). These results implicated an aminopeptidase(s) in the peptide trimming reaction.

Second, we modified the in vitro cleavage reaction in which the peptide was added to permeabilized rather than intact microsomes. This critical modification made it possible to decrease the incubation temperature and time, which otherwise dramatically reduced peptide translocation and consequently the yield of trimmed peptides in intact microsomes. The cleavage of LK-SHL8 precursor peptide to its final SHL8 product in $K^b$-expressing B10.A(5R) microsomes occurred rapidly and was essentially complete in 10 min at 10°C or just 2 min at 37°C (Figures 4c and 4e). Decreasing the incubation temperature to 0°C partially blocked peptide trimming (Figures 4a and 4d). Addition of the aminopeptidase inhibitors LCMK or bestatin under these conditions also inhibited peptide cleavages, as was observed with the intact microsomes above (data not shown).

Third, we also tested whether the presence of an excess of a $K^b$ binding peptide in the reaction could inhibit peptide trimming. We compared the amount of SHL8 peptide recovered from B10.A(5R) microsomes incubated with the LK-SHL8 peptide in the presence of another $K^b$ binding SFV8 or the irrelevant control NP pep-

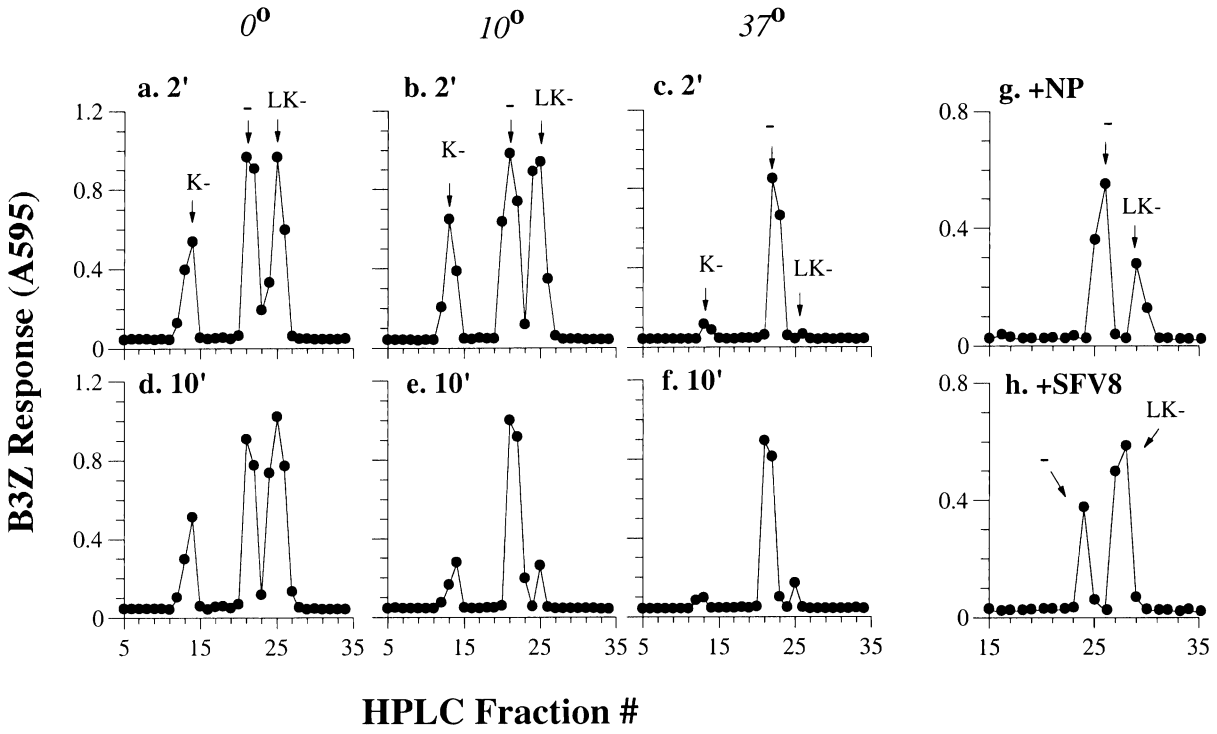


Figure 4. Trimming of LK-SHL8 (LK-) to K-SHL8 (K-) or SHL8 (-) within the Microsomes Occurs Rapidly and Is Temperature Dependent

B3Z T cell response to HPLC fractions of permeabilized B10.A(5R) microsomes extracted after incubation with the LK-SHL8 peptide (a–c) for 2 min or (d–f) for 10 min at either 0°, 10°, or 37°C. Processing of LK-SHL8 within microsomes was inhibited by $K^b$ binding peptides. B3Z T cell response to HPLC fractionated extracts of permeabilized B10.A(5R) microsomes incubated with LK-SHL8 and excess of the (g) $D^b$ binding NP peptide, or the (h) $K^b$ binding SFV8 peptide. HPLC fractions were analyzed as in Figure 2.

tide (Figures 4g and 4h). The yield of the SHL8 peptide was 0.85 fmol in presence of the SFV8 peptide compared to 2.3 fmol recovered in the presence of the NP peptide, which represents approximately 65% inhibition of the cleavage reaction. This result supports the notion that the $K^b$ peptide binding site was required for the generation of SHL8. Together, these data indicate that peptide trimming was a rapid reaction which could nevertheless be inhibited by lowering the incubation temperature, decreasing the incubation time, and including certain aminopeptidase inhibitors.

### LK-SHL8/$K^b$ Complex Can Be Immunoprecipitated from Microsomes

To determine if the precursor peptides were bound to the MHC molecules when their proteolysis was inhibited, the $K^b$ MHC molecule was immunoprecipitated with a panel of antibodies and the eluted peptides were analyzed after HPLC fractionation (Figures 5a–5c). Remarkably, the LK-SHL8 precursor was found in the material immunoprecipitated with a mixture of anti-$K^b$ monoclonal antibodies (Figure 5b) but not with antibodies specific for an epitope in the cytoplasmic tail of $K^b$ (Figure 5c). No antigenic activity was detected in the immunoprecipitates of LK-SHL8 incubated with B10.A(4R) microsomes (Figure 5a) despite the fact that two of the anti-$K^b$ monoclonals (5F1 and Y3) also crossreact with the $K^k$ MHC molecule expressed by these mice (Jones and Janeway, 1981; Sherman and Randolph, 1981). In subsequent experiments, we determined that the 5F1, EH144, and 28.8.6S (but not Y3 and AF6) monoclonals yielded the LK-SHL8 peptide in the immunoprecipitate (Figures 5d and 5e; data not shown), although all the tested monoclonals recognized the mature $K^b$ molecules on the cell surface and precipitated $K^b$ from lysates of metabolically labeled cells (data not shown). Direct evidence that both 5F1 and Y3 mAb were equivalent in their ability to immunoprecipitate the final SHL8/$K^b$ complex was obtained by comparing the $K^b$ bound peptides when the microsomes were incubated with the LK-SHL8 precursor without protease inhibitors at room temperature to allow the reactions to proceed to completion. In contrast to the differential recovery of the LK-SHL8 peptide observed above (Figures 5d and 5e), immunoprecipitates of B10.A(5R) microsomes with either 5F1 or Y3 yielded the SHL8 peptide (Figures 5f and 5g). Again, no activity was detected in the immunoprecipitates from B10.A(4R) mice, demonstrating that the SHL8 peptide was specifically bound to the $K^b$ MHC (Figures 5h and 5i).

It is interesting that 5F1 and EH144 but not Y3, AF6, or the anti-tail antiserum permitted the isolation of the LK-SHL8/$K^b$ complex. The epitopes recognized by Y3, 5F1, and EH144 have been mapped to opposite ends of the peptide binding groove containing the carboxy-termini (Y3) and the amino-termini (5F1, EH144) of the bound peptide, respectively (Ajitkumar et al., 1988). Because the LK-SHL8 peptide is likely to extend outside the $K^b$ peptide binding groove at its N terminus, it was surprising that the N terminus specific 5F1 mAb rather

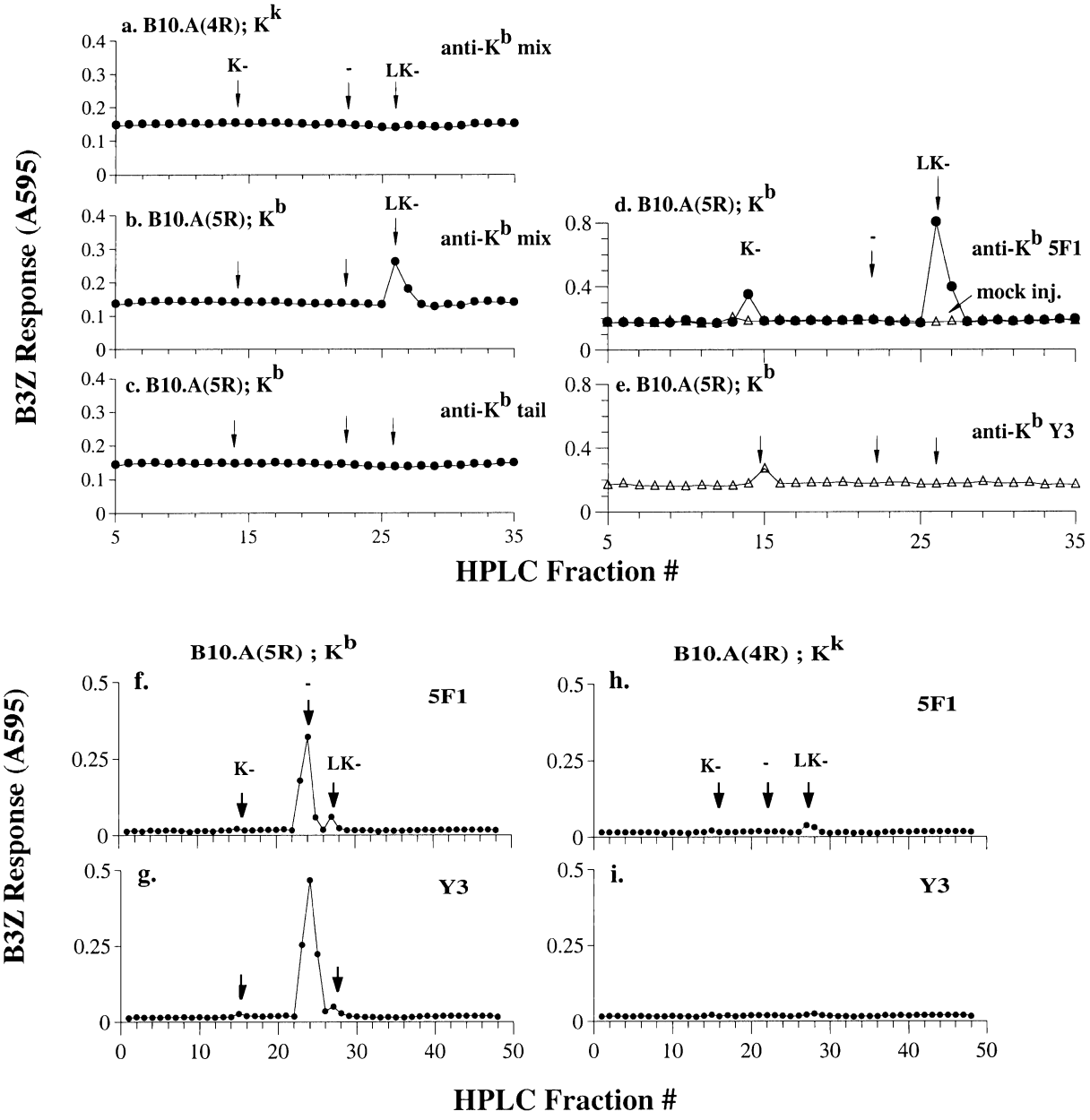


Figure 5. The LK-SHL8 (LK-) Precursor is Bound to $K^b$ MHC in a Distinct Conformation

B3Z T cell responses to HPLC fractionated peptides eluted from immunoprecipitates prepared from LK-SHL8 incubated with permeabilized (a) B10.A(4R) or (b–e) B10.A(5R) microsomes. (a and b) A mixture of anti-$K^b$ monoclonal antibodies, (c) an anti-$K^b$ tail specific antiserum, or (d and e) single anti-$K^b$ monoclonal antibodies indicated were used for the immunoprecipitation. HPLC fractions were analyzed as in Figure 2. Both 5F1 and Y3 can immunoprecipitate the SHL8/$K^b$ complex from (f and g) B10.A(5R), but not (h and i) B10.A(4R) microsomes that were incubated with the LK-SHL8 precursor in the absence of inhibitors. The immunoprecipitates were extracted, fractionated by HPLC, and analyzed as in Figure 2. The arrows indicate the peak elution times of the indicated peptides in the top panels.

than the C terminus specific Y3 mAb should react with the LK-SHL8/$K^b$ complex. However, because LK-SHL8 is bound relatively weakly to the $K^b$ molecules on the cell surface (data not shown), it is possible that only the N terminus-specific antibodies (5F1 and EH144) can stabilize this complex sufficiently to allow its isolation. This would also explain why the anti-$K^b$ tail antibody failed to immunoprecipitate the LK-SHL8/$K^b$ complex. Alternatively, it is possible that the tail epitope of LK-SHL8 bound $K^b$ MHC may have been simply inaccessible to the antibodies as was observed earlier for nascent $K^b$ in pulse-chase analysis of intact cells (Song et al., 1994). We conclude that the LK-SHL8 precursor peptide was specifically bound to $K^b$ MHC in the microsomes in a conformation distinct from that of mature $K^b$ containing the optimally cleaved SHL8 product.

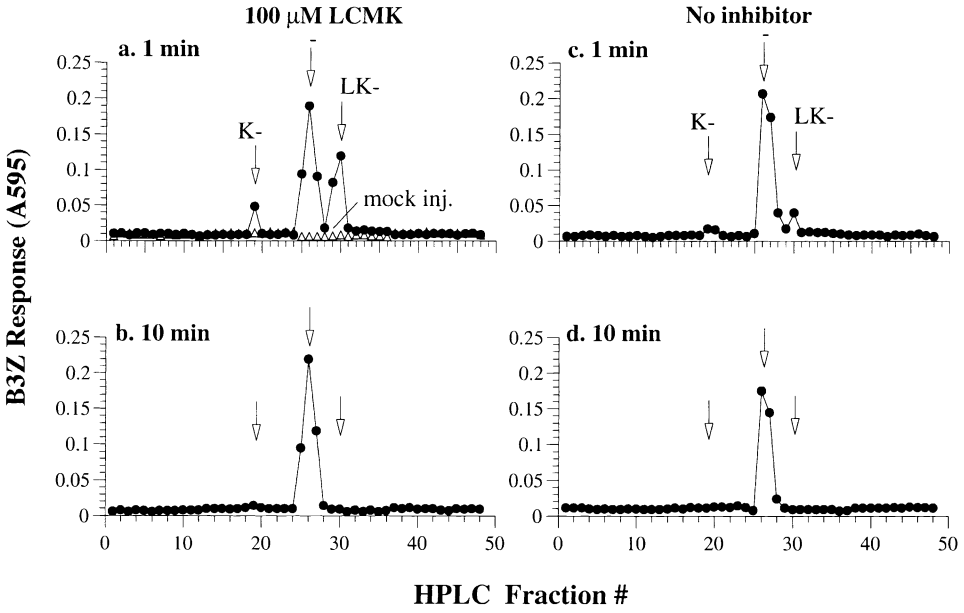


Figure 6. The LK-SHL8/K$^b$ Complex is Transient and is Trimmed to the SHL8/K$^b$ Product

B6 microsomes were incubated with the LK-SHL8 precursor in the (a and b) presence of suboptimal 100 μM LCMK concentration or (c and d) absence of the inhibitor for either 1 or 10 min. The material immunoprecipitated with 5F1 anti-K$^b$ monoclonal was extracted and analyzed after HPLC fractionation as in Figure 2. The arrows indicate the peak elution times of the peptides indicated in the top panels.

### K$^b$ Bound LK-SHL8 Precursor Is Trimmed to the SHL8 Product

To determine if the LK-SHL8 precursor peptide bound to K$^b$ MHC served as an intermediate for generation of the final SHL8 peptide/K$^b$ complex, we carried out the peptide trimming reactions in the presence of suboptimal inhibitor concentration. Microsomes from B6 mice were lysed with detergent and incubated with the LK-SHL8 precursor peptide in the presence of 100 μM LCMK inhibitor, and the K$^b$ bound peptides were analyzed after immunoprecipitation with the anti-K$^b$ mAb, 5F1 (Figure 6). In contrast to the K$^b$ immunoprecipitated from microsomes in presence of 1 mM LCMK (Figure 5d), these K$^b$ molecules contained both the LK-SHL8 precursor as well as a substantial amount of the SHL8 product peptides consistent with partial inhibition of the trimming reaction (Figure 6a). However, after 10 min the anti-K$^b$ immunoprecipitates contained negligible amount of LK-SHL8 and only the SHL8 product was detectable in the extract (Figure 6b). Again, in the absence of the LCMK inhibitor, only the SHL8 product (but no LK-SHL8) was detected in the anti-K$^b$ immunoprecipitates (Figures 6c and 6d). This result argues strongly against the possibility that the LK-SHL8/K$^b$ complex was a "sink" for the LK-SHL8 peptide, and provides further evidence that the LK-SHL8/K$^b$ complex can serve as a substrate for the aminopeptidase that yielded the final SHL8/K$^b$ complex.

### Peptide Trimming but Not MHC Binding Is Inhibited in Depleted Microsomes

To further characterize the proteolytic activity responsible for the removal of the N-terminal flanking residues, we depleted microsomes of their lumenal content by treatment at high pH (Hamman et al., 1998) and tested them for their ability to trim the LK-SHL8 peptide. In contrast to the undepleted microsomes where the LK-SHL8 peptide was readily trimmed to its K-SHL8 and SHL8 products, only the intact LK-SHL8 peptide was recovered from the depleted microsomes (Figures 7a and 7b). Western blot analysis confirmed that depletion of the lumenal proteins in the microsomes caused a dramatic reduction in the soluble gp96 but not in the membrane-bound K$^b$ or calnexin proteins (Figure 7c). The K$^b$ molecules in the depleted microsomes were nevertheless capable of binding peptides as judged by the presence of antigenic peptide in the anti-K$^b$ immunoprecipitates (Figures 7d and 7e). Again, this activity was recovered only from the K$^b$-expressing B10.A(5R) microsomes and not those from B10.A(4R) microsomes lacking K$^b$ MHC, ruling out nonspecific associations with other microsomal components. We conclude that depletion of the lumenal content of the microsomes abrogates the activity of protease(s) responsible for trimming the N-terminal flanking residues of the antigenic precursor peptide. The data, however, do not allow us to distinguish whether the loss of trimming activity was due to depletion of a soluble protease, an essential cofactor, or was due to denaturation of a membrane-bound molecule under these conditions.

The generation of all known peptides presented by MHC class I molecules requires the removal of both N- and C-terminal flanking residues from their precursor polypeptides (Rammensee et al., 1995). Some of these peptides may be generated in the cytosol itself by the proteasome in concert with two recently described cytoplasmic proteases (Stoltze et al., 2000). There is, however, compelling evidence that the C termini of most antigenic peptides are generated in the cytosol by the proteasome (Craiu et al., 1997; Rock and Goldberg, 1999; Serwold and Shastri, 1999), and these N-terminally

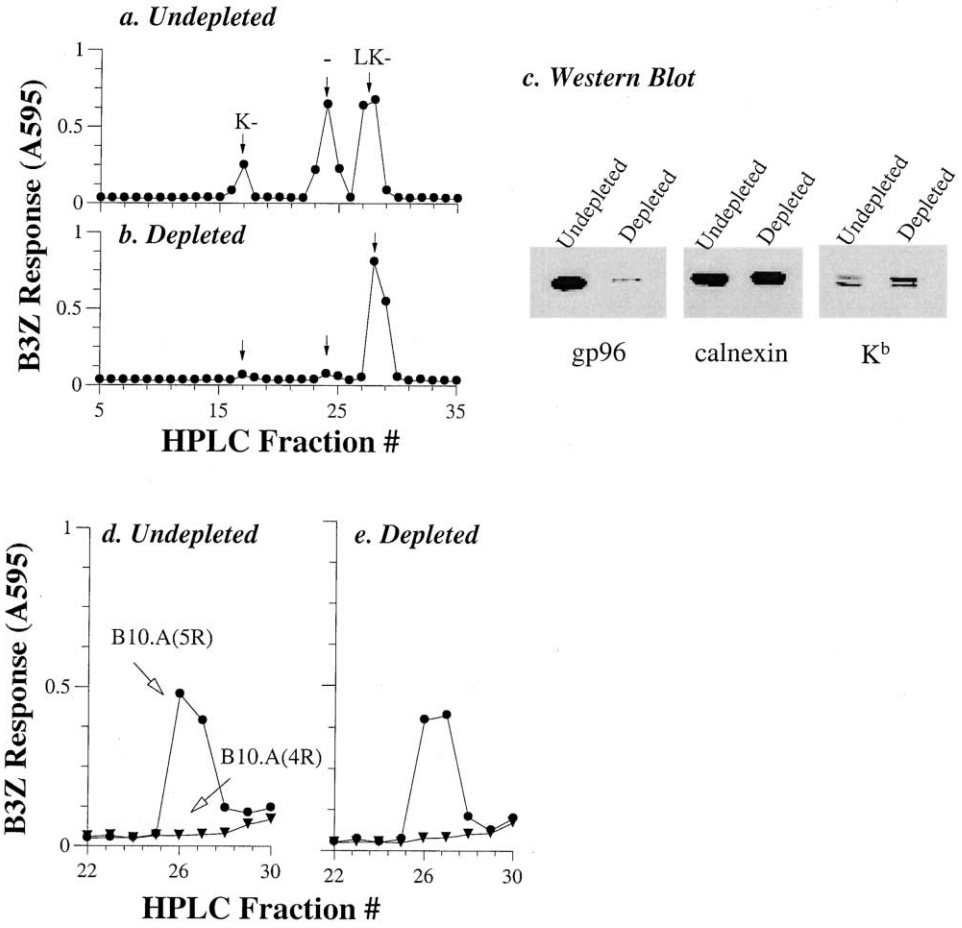


Figure 7. Cleavage of LK-SHL8 to the K-SHL8 (K-) and SHL8 (-) Products is Blocked by Depletion of Lumenal Content of the Microsomes

B3Z T cell responses to HPLC fractionated peptide extracts recovered from LK-SHL8 incubated for 5 min at RT with permeabilized C57BL/6 microsomes which were either used (a) undepleted or (b) after depletion of their lumenal content as described in Experimental Procedures. (c) Depletion of lumenal content of microsomes correlates with dramatic reduction in the amount of soluble gp96 protein relative to the membrane-bound calnexin and $K^b$ proteins detected by Western blotting. (d and e) B3Z T cell responses to HPLC fractionated peptides eluted from anti-$K^b$ immunoprecipitates prepared from SHL8 incubated with microsomes that were (d) undepleted or (e) depleted of their lumenal content. Microsomes were from B10.A(5R) (J) or B10.A(4R) (P) mice. HPLC fractions were analyzed as in legend to Figure 2.

extended peptides are selectively transported by TAP into the ER (Heemels and Ploegh, 1995; Neefjes et al., 1995; Van Endert et al., 1995; Androlowicz and Cresswell, 1996; Paz et al., 1999), where there is little, if any, carboxypeptidase activity (Powis et al., 1996; Craiu et al., 1997; Yellen-Shaw et al., 1997; Serwold and Shastri, 1999). Our finding that N-terminal flanking residues can be removed in the ER—in an MHC-dependent manner—allows the MHC molecules to "customize" the available peptide pool. We suggest that the ER trimming mechanism involves intermediate complexes of N-terminally extended peptides bound to the MHC molecule, which serve as substrates for a microsomal protease(s). The transient nature of this complex, the rapid rate of trimming, and the lack of sensitive assays for detecting extended versions of MHC bound antigenic peptides explain why this complex was not detected earlier. Our data strongly support the earlier hypotheses (Falk et al., 1990; Malarkannan et al., 1995; Paz et al., 1999) that MHC class I molecules serve as templates for guiding a yet unknown ER aminopeptidase(s) to generate the optimal MHC class I binding epitopes. Most importantly, they can explain how the antigen-processing pathway efficiently generates thousands of different peptides that fit exactly within the antigen binding grooves of the MHC class I molecules.

## Experimental Procedures

### Cells, Antibodies, Peptides, and T Cell Activation Assays

The SHL8/$K^b$-specific, lacZ-inducible, B3Z T cell hybridoma K89B7 ($K^b$ and B7.2-expressing L cells); synthetic peptides LK-SIINFEHL (LK-SHL8), K-SHL8, and SHL8; and T cell activation assays to analyze antigenic activity in HPLC fractionated cell extracts have been described (Karttunen et al., 1992; Shastri and Gonzalez, 1993; Paz et al., 1999). Monoclonal anti-$K^b$ antibodies EH.144, Y3, 5F1.2.14, 28.8.6S, and AF6.88.5.3 were a kind gift of Dr. S. Nathenson or were obtained from ATCC. Polyclonal rabbit anti-$K^b$ tail antiserum was a kind gift of Dr. E. Song (Song et al., 1994). Western blots were performed according to standard procedures using anti-gp96, anti-$K^b$ tail antiserum, and anti-calnexin antibodies (Stressgen).

### Preparation of Microsomes

Microsomes were prepared from C57BL/6 (H-$2^b$), B6.129$^{-Tap1tm1Arp}$ (TAP1$^{-/-}$), B10.A(5R) ($K^b$, $D^d$, $L^d$), B10.A(4R) ($K^k$, $D^b$), and B10.D2 (H-$2^d$)

mice obtained from the Jackson Laboratories according to earlier protocols (Walter and Blobel, 1983), with the modifications described by Shepherd (Shepherd et al., 1993). Briefly, mice were injected intraperitoneally with 0.2 mg polyinosinic-polycytidylic acid (Sigma) 36 hr before sacrifice. Spleens and livers were removed and collected in a beaker on ice. The organs were washed with ice-cold buffer A (50 mM triethanolamine, 50 mM KOAc [pH 7.5], 250 mM sucrose, 6 mM MgOAc, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.027 U/ml aprotinin, 1mM EDTA [pH 7.5]) to remove blood. All subsequent steps were performed on ice or at 4°C. The organs were weighed and 3–4 ml of buffer A was added per gram of tissue. The tissue was minced and homogenized using a motor-driven Potter-Elvehjem teflon tissue grinder (chamber clearance 0.1 mm) at half maximal speed with five passes up and down of 10 s each. The homogenate was subjected to differential centrifugation steps as described. The homogenate was centrifuged at 1000 × g for 10 min to pellet nuclei. The floating fatty material was removed by aspiration. The homogenate was then spun for 10 min at 10,000 × g to pellet the mitochondrial fraction. The supernatant was collected and loaded onto a sucrose-cushion (1.3 M sucrose in buffer A) and centrifuged at 140,000 × g for 2 hr. The pellet was resuspended in buffer B (50 mM HEPES, 250 mM sucrose, 1 mM dithiothreitol [pH 7.3]). Crude microsomes were snap-frozen in 25 μl aliquots and stored at −70°C. Aliquots were used only once after thawing.

### In Vitro Peptide Translocation Assay

In vitro peptide translocation assays were performed in 100 μl reactions in transport buffer (50 mM HEPES [pH 7.5], 150 mM KOAc, 5 mM MgOAc, 250 mM sucrose, 1 mM DTT) with or without ATP (50 μM) and ATP regenerating system (2.5 mM phosphocreatine [Sigma] and 0.05 U/ml of creatine-phosphokinase) (Shepherd et al., 1993; Paz et al., 1999). Synthetic peptide (50–100 fmol) was added followed by the microsomes (5 μl, 10–50 μg of protein). The reactions were carefully mixed and incubated at RT for 10 min. When proteinase K (Sigma) digestion was performed, the enzyme (100 μg/ml) was added directly after peptide translocation on ice for 30 min. The reaction was stopped by the addition of 1 mM PMSF. Reactions were spun over a sucrose-cushion (0.5 M sucrose in 1 M KOAc, 50 mM HEPES) spiked with 10 μM irrelevant peptide for 15 min at 140,000 × g. The pellets were extracted with 10% formic acid supplemented with 10 μM irrelevant peptide and heated to 95°C for 5 min. After removing particulate debris by centrifugation, the supernatants were either dried in 96-well plates for direct analysis or passed through a 10 kDa cut-off filter for HPLC analysis.

### HPLC Analysis

Extracts were passed through 10 kDa cut-off filters before injection into the HPLC. Extracts 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 $H_2O$ (buffer A) and 0.1% TFA in acetonitrile (buffer B): 0–5 min, 23% B; 5–19 min, 23%–30% B; 19–22 min, 30%–77% B at a flow rate of 0.25 ml/min. Five drop fractions were collected from 5–29 min in flat-bottom 96-well plates and dried in a vacuum centrifuge. The fractions were digested with trypsin and tested for antigenic activity as described below. HPLC runs from mock injections were routinely analyzed to rule out crosscontamination among samples.

### In Vitro Peptide Cleavage Assay

Five microliters of microsomes (corresponding to ~50 μg protein) were permeabilized in 5 μl PBS-0.5% NP-40 with 1 mM PMSF and 0.2 U/ml aprotinin for 30 min at 4°C. Fifty femtomoles of precursor LK-SHL8 (LKSIINFEHL) in 2.5 μl was added and incubated with the "open" microsomes at RT for 10 min. The reaction products were extracted with 500 μl of 10% formic acid containing 2–10 μM irrelevant carrier peptide, heated to 95°C for 5 min, and passed through 10 kDa cut-off filter. Where indicated, the peptide incubations with "open" microsomes included the protease-inhibitors Leucine chloromethylketone (LCMK, 1 mM [Sigma]) or bestatin (250 μM [Sigma]) (Umezawa, 1982). For competition experiments, 50 pmol $D^b$ binding peptide NP (ASNENMETM) or the $K^b$ binding peptide SFV8 (SSWDFITV) was added to the "open" microsomes before addition of LK-SHL8 peptide. The reaction was incubated at RT for 5 min and analyzed as above after extraction.

### Immunoprecipitations

Fifty microliters of permeabilized microsomes was incubated on ice with 500 fmoles of LK-SHL8 precursor and the protease inhibitors LCMK (100 μM or 1 mM) and bestatin (0.6 mM) for 1 min or at RT for indicated time. The reaction was diluted by the addition of anti-$K^b$ antibodies bound to protein A/G-sepharose in TBS-0.25% NP40. After 1 hr at 4°C, the beads were washed four times with TBS-0.05% NP-40 with or without the inhibitors. Peptides associated with the immunoprecipitated material were extracted at 95°C with 500 μl 10% formic acid containing 2–10 μM of an irrelevant peptide as a carrier and analyzed after HPLC fractionation and trypsin treatment of the fractions as described (Paz et al., 1999).

### Depletion of Lumenal Proteins from Microsomes

Lumenal proteins were depleted from the microsomes as described (Hamman et al., 1998). Briefly, 50 μl microsomes were diluted with 50 μl 0.1 M triethanolamine (pH 7.5), 400 mM sucrose, and 2 mM DTT on ice. One hundred microliters of 0.5 M HEPES, 0.5 M CAPS (pH 10.5) was then added, followed by 800 μl ice-cold $H_2O$. The dilute microsome suspension was incubated for 20 min and overlaid onto a 200 μl cushion of 0.5 M sucrose in 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, and 40 mM KOAc. Membranes were collected by centrifugation in a TLA100.2 rotor (Beckman) for 20 min at 60,000 rpm. Lumen-extracted microsomes were resuspended in 50 μl of 50 mM HEPES (pH 7.5), 200 mM sucrose, and 1 mM DTT. Ten microliters of these extracted microsomes were permeabilized in 10 μl PBS-0.5% NP-40 for the in vitro cleavage reactions. To compensate for any loss, 100 ng of recombinant β2-microglobulin was added to the depleted "open" microsomal preparations.

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