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Ovalbumin-Derived Precursor Peptides Are Transferred Sequentially from gp96 and Calreticulin to MHC Class I in the Endoplasmic Reticulum

Laura E. Kropp,* Manish Garg,† and Robert J. Binder*  

Cellular peptides generated by proteasomal degradation of proteins in the cytosol and destined for presentation by MHC class I (MHC-I) are associated with several chaperones. Heat shock proteins 70, 90, and the TCP-1 ring complex have been implicated as important cytosolic players for chaperoning these peptides. In this study, we report that gp96 and calreticulin are essential for chaperoning peptides in the endoplasmic reticulum. Importantly, we demonstrate that cellular peptides are transferred sequentially from gp96 to calreticulin and then to MHC-I forming a relay line. Disruption of this relay line by removal of gp96 or calreticulin prevents the binding of peptides by MHC-I and hence presentation of the MHC-I–peptide complex on the cell surface. Our results are important for understanding how peptides are processed and trafficked within the endoplasmic reticulum before exiting in association with MHC-I H chains and β2-microglobulin as a trimolecular complex. The Journal of Immunology, 2010, 184: 000–000.

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ellular peptides undergo proteolysis in the cytosol by the proteasome and other aminopeptidases and some of the resulting peptides are transported into the endoplasmic reticulum (ER) by the TAP (1). Within the ER, the peptides are loaded onto MHC class I (MHC-I) with the aid of various proteins that constitute the peptide loading complex (PLC), whose prescribed role is to stabilize the empty MHC-I H chains prior to its association with peptide and β2-microglobulin (β2m) (1). Peptides are further trimmed by the endoplasmic reticulum-associated aminopeptidase (ERAAP) as they simultaneously associate with MHC-I (2, 3). One of the key unresolved questions in this pathway pertains to the manner in which peptides are channeled from the proteasome to TAP in the cytosol and, in the ER, from TAP to the MHC-I molecules. There is little evidence that the cell detectably harbors free peptides either in the cytosol or the ER; on the contrary a vigorous search for these free peptides has revealed none (4). In addition, hydrophobic side chains of amino acids in these short peptides cannot exist natively in the hydrophilic cytosol and ER because of poor solubility (5). Furthermore, peptides traveling in the cell by random diffusion cannot account for the calculated efficiency for Ag processing and presentation by MHC-I that ranges from one MHC peptide per 30 (6) to 1 per 1000 (7) proteins degraded. To address these observations, it was proposed that peptides exist in association with peptide-binding proteins (chaperones) in the cytosol and ER (8). As heat shock proteins (HSPs) are known to chaperone a wide array of molecules, including antigenic peptides destined for MHC-I presentation (9), the HSPs are prime candidates for forming a relay line to shuttle peptides within the compartments of the cell within which they reside (8). Hsp90, hsp70, and TAP-1 ring complex in the cytosol have been directly implicated, independently of one another, in the processing events for peptides destined to be presented by MHC-I (10–15).

How the peptides traffic from TAP to the peptide-binding groove of the MHC-I is not known. In a large number of studies, the ER chaperones, calreticulin and gp96, have been shown to associate with antigenic peptides (9). In this article, we have examined the relative contribution of gp96 and calreticulin to peptide binding and the transfer of these peptides, within the ER, to MHC-I. Our results show that 1), both gp96 and calreticulin bind the MHC-I antigenic (precursor) peptides, although with different efficiency, 2), the repertoire of MHC-I precursor peptides bound by the ER chaperones is dictated by the MHC expressed by the cell, and 3), that peptides entering the ER sequentially associate with gp96 and then calreticulin before being loaded onto MHC. These studies define an indispensable role for gp96 and calreticulin in the processing of Ags destined for MHC-I presentation and provide evidence for the existence of an ordered relay line consisting of HSP chaperones in the ER.

Materials and Methods

Cell lines and reagents

PIK23.2 cells have previously been described in detail (16). The Kb containing plasmid (pRSV5.new.Kb) was a kind gift from Dr. Ted Hansen and has been previously described (17). PIK23.2 cells were transfected using FuGENE with the Kb plasmid and cloned. PIK23.2 Kb cells were maintained in complete RPMI 1640 plus genetin and blasticidin. The conformation specific anti-Kb Ab (Y3) was purified from a hybridoma obtained from American Type Culture Collection (Manassas, VA). The β2m Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and was used for immunoprecipitation of total MHC-I from PIK23.2 cells. Calreticulin Abs were from Stressgen (Victoria, British Columbia, Canada) and Affinity Bioreagents (Golden, CO) and gp96 and calnexin Abs from Stressgen. Gp96 and calreticulin were purified according to published reports (18, 19) and were not contaminated with each other or with the

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Abbreviations used in this paper: b2m, β2-microglobulin; BFA, brefeldin A; ER, endoplasmic reticulum; ERAAP, endoplasmic reticulum-associated aminopeptidase; HSP, heat shock protein; MHC-I, MHC class I; PLC, peptide loading complex; siRNA, small interfering RNA.

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other ER chaperones ERP57, protein disulfide isomerase, or calnexin (data not shown). Briefly, cell pellets were homogenized in three times volume of hypotonic buffer (30 mM NaHCO₃, 1 mM PMSF, pH 7.4) and a 100,000 g supernatant was obtained. Solid ammonium sulfate was added to bring the solution to 50% saturation. This was centrifuged at 22,000 × g for 30 min. The precipitate was discarded and the supernatant was subjected to subsequent fractionation at 80% ammonium sulfate. After centrifugation at 22,000 × g for 30 min, the precipitate was solubilized in PBS containing 2 mM Ca²⁺ and 2 mM Mg²⁺. This was applied to a Con A-Sepharose column. The buffer of Con A unbound material was changed to 25 mM Na citrate buffer, pH 5.3, by PD-10 column (Sephadex G-25). It was then applied to the CM-Sephadex C-50 column. The buffer of unbound of CM-Sephadex was then changed to 19 mM Na-phosphate buffer, pH 6.1 by PD-10 columns. It was then applied to the DEAE-Sephacel column, and the con A column was eluted with a linear gradient of 0.15–0.5 M NaCl in 20 mM Na-phosphate buffer. Con A columns were eluted with methyl mannospyranoside and eluents were exchanged into a phosphate buffer with PD-10 gel exclusion columns and applied to DEAE anion exchange columns. Gp96 eluted from these columns as a purified protein. Proteins were analyzed by SDS-PAGE (silver stain) and immunoblotting (Supplemental Fig. 1A). All other gels were stained with Coomassie stain.

Preparation of cell lysates and microsomes
Cell lysate was obtained by Dounce-homogenization of cells in 30 mM sodium bicarbonate buffer pH 7.4. Microsomes were purified as previously described (20) By comparing the amount of KDEL immunoblotting proteins in the total cell lysate to that in the microsome preparations (starting with the same number of cells), we estimate a 40% loss of material during the purification process (data not shown). This is factored into the results shown in Figs. 1D and 2C. Microsomes were not contaminated with cytotoxic proteins (Fig. 1C). (Supplemental Fig. 1B shows raw data not accounting for microsome loss).

Peptide stripping
Cell lysate, microsomes, or HSPs were incubated at room temperature in 0.2% trifluoroacetic acid with gentle rocking. After 1 h, the pH was adjusted to ~7 and the peptides were used immediately or stored at ~20°C.

Small interfering RNA transfection
The 200,000 PIK23.2 K⁺ cells were plated per well in 2 ml complete RPMI-1640. FuGENE transfection reagent (Roche, Indianapolis, IN) was incubated in RPMI-1640 for 5 min at room temperature. Small interfering RNA (siRNA) was added to FuGENE at 3:2 (for a DNA amount of 1 μg) and incubated for 15 min. The siRNA-FuGENE reaction was added dropwise to each well containing cells. Cells were tested for HSP knockdown at various intervals thereafter by immunoblotting as described in Figs. 4 and 5. In some experiments cells were further treated with RSL1 for KOVAK induction as previously described (16). siRNA were obtained from Qiagen (Valencia, CA) (Flexitube gene solution for Calreticulin: GS12317; Flexitube gene solution for HSP9061: GS22027).

Immunoprecipitation
Cells were harvested, washed with PBS and lysed with an 0.5% NP40, 50 mM Tris lysis buffer with 0.5% octylglucoside. Lysate was precleared with BSA-coated Protein G beads. The respective Ab was added in excess to the precleared lysate for 2 h and then precipitated with Protein G beads. Beads were washed three times with the NP40 lysis buffer, twice with lysis buffer plus 1% SDS, and twice with lysis buffer plus 0.5 M NaCl. Beads were either boiled in sample buffer (for SDS-PAGE) or stripped for peptides.

B3Z Assay
Peptides stripped from various samples were pulsed onto 10,000 EL.4 cells for 1 h at room temperature. After washing with PBS, cells were incubated with 10,000 B3Z cells overnight (12 h) at 37°C in 96-well plates. LacZ assays have been described previously (12). In all experiments, the absorbance at 595 nm obtained with RSL1 uninduced cells was always <5% of the signal obtained with RSL1-induced cells and subtracted from the values obtained with the RSL1-induced cells to obtain the Specific Ab-sorbance. 595 nm. The stimulation of B3Z cells follows a linear relation over three orders of magnitude of peptide concentration (Supplemental Fig. 2). We use the B3Z cells in this linear range of detection in all experiments. In each experiment, we used EL.4 cells pulsed with 0.01 μM of OVA8 peptide as a positive control and the signal from this control was always from 2–5-fold higher than any group in any experiment. This determines that the B3Z response is not saturating nor is it limiting.

Brefeldin A treatment
The 30 million PIK23.2 and PIK23.2 K⁺ cells were treated as previously described (11). Brefeldin A (BFA) was present during the 18 h induction with RSL1. Cells were washed once with PBS and used immediately for either microsome preparation or for immunoprecipitation.

Results
An antigenic system to measure MHC-I precursor peptides is specific and titratable
As HSPs bind peptides without any apparent restrictions to peptide sequence or length (9), and as an overwhelming percentage of the chaperoned peptides are self-peptides, it was essential to use a system where the precise Ag could be liberated from all of its precursor entities and measured specifically. To quantify and track a specific epitope during its processing within the cell, we used the previously established, modified OVA construct, KOVAK, (12–14, 16). KOVAK was tagged with GFP and placed under the Rhesovirus ligand RSL1-inducible promoter and transfected into P815 cells to obtain PIK23.2 cells (H⁻²). KOVAK induction can be verified by flow cytometry (16). Flanked by two lysine residues, the modified OVA peptide (OVA; SIINFEHL) that is presented by MHC-I K⁺ can be liberated from any precursor peptide, regardless of its length or sequence by treatment of the protein product with a combination of trypsin and carboxypeptidase B. The liberated OVA peptide is then detected by pulsing it onto a K⁺-expressing cell line (EL.4) for presentation to the K⁺-SIINFEH/K(H)-specific T cell hybridoma B3Z. We tested whether OVA-specific peptides could be isolated from induced cells. Titrated numbers of PIK23.2 cells were induced with the indicated amounts of RSL1 (500, 250, or 125 nM) and the peptides were released from (the chaperones in) lysates using a combination of heat and acid. Peptides were presented by EL.4 cells to B3Z cells. Activated B3Z cells were developed with a colorimetric assay. The amount of OVA-specific peptides recovered from PIK23.2 cells was directly proportional to the amount of RSL1 used to induce the cells and the number of cells used for peptide preparation Fig. 1A. The signal obtained from uninduced PIK23.2 cells was used as the background and subtracted from the positive signals. In all the following experiments, cells were induced with 500 nM RSL1. To determine whether peptides in cells were free (unassociated) or exclusively bound to chaperones, we omitted the heat and acid treatment during the peptide isolation. When this treatment was omitted, no OVA-specific peptides could be detected, whereas a robust signal was obtained when cell lysates were treated with heat and acid (Fig. 1A). This result demonstrates that all the OVA (precursor) peptides in our experimental system are not free and therefore bound to chaperones.

Microsomes contain significant amount of the OVA-precursor peptides
The ER (microsomes) was purified and verified morphologically (Fig. 1B) and biochemically (Fig. 1C). Per protein amount the microsomes have a significant enrichment of ER resident proteins (calreticulin) and no cytotoxic proteins (represented by the absence of the abundant hsp70) (Fig. 1C). PIK23.2 cells were induced with RSL1 and we estimated the amount of OVA (precursor) peptides that exists at steady state levels in the ER. Microsomes were purified from 10 million cells or cell lysate was made from the same number of cells. Peptides were stripped from the microsomes or cell lysates and tested in a B3Z assay. Microsomes were determined to account for ~50% of the OVA (precursor) peptides seen in the...
whole cell lysate (Fig. 1C). We have not yet analyzed other cellular organelles for this peptide.

**Gp96 and calreticulin chaperone the majority of the antigenic peptides in the ER**

Because no free (unassociated) peptides were detected anywhere in the cell (Fig. 1Aii), we examined the ER peptide chaperones gp96 and calreticulin for the presence of OVA (precursor) peptides. By using semiquantitative immunoblotting (Fig. 2A, 2B), we were able to estimate the amounts of the abundant chaperones gp96 and calreticulin in PIK23.2 cells. Titrated quantities of cell lysate and purified protein (gp96 or calreticulin) were analyzed by SDS-PAGE and immunoblotted with Abs to cytosolic (hsp70) or ER (calreticulin) resident proteins. Microsomes or the equivalent microgram amount of cell lysate were analyzed by SDS-PAGE and immunoblotted with Abs to cytosolic (hsp70) or ER (calreticulin) resident proteins. Microsomes contain a significant portion of the antigenic (precursor) peptides of the cell. Peptides were extracted from 10 million RSL1-induced cell equivalents of microsomes or cell lysate as described in Materials and Methods. Peptides were analyzed in a B3Z assay. The signal obtained in the microsome fraction has been adjusted for the amount of material lost during its preparation. Each group was set up in duplicate and data are represented as mean ± SEM. One representative experiment of several is shown.

**ER HSP-chaperoned, KOVAK-derived peptide repertoires are dictated by MHC haplotype**

PIK23.2 cells do not express Kb that presents the final SIINFEHL epitope. To determine what effect the expression of Kb will have on peptides chaperoned by gp96, PIK23.2 cells were transfected with Kb. The cell surface Kb expression was confirmed by flow cytometry, which also shows the level of RSL1 induction of GFP-KOVAK was not affected by Kb expression (Fig. 3A). Peptides were stripped from lysates of equal number (two doses) of these two cell types and analyzed in a B3Z assay. No statistically significant differences were observed in the quantity of OVA (precursor) peptides retrieved from PIK23.2 and PIK23.2Kb cells, $p > 0.5$ (Fig. 3B), consistent with the equal GFP induction measured in Fig. 3A. Gp96 was then immunoprecipitated from equal numbers of PIK23.2 or PIK23.2Kb cells. Immunoblotting confirmed that comparable
FIGURE 2. A majority of peptides in the ER are associated with the HSPs gp96 and calreticulin. A and B, Estimation of the quantities of gp96 and calreticulin in cells. A, Tritrated amounts of purified gp96 or calreticulin or cell lysates were resolved by SDS-PAGE and immunoblotted with their respective Abs. Immunoblots were scanned and quantified and the relative OD units are indicated below the blots. B, Scatterplots were obtained from the intensities of the signal in the immunoblot of the purified protein. The amount of protein in cell lysate was obtained from these graphs (table). C, Peptides were extracted from 10 million cell equivalents of microsomes (adjusted for loss; Supplemental Fig. 1B for unadjusted graph), gp96, or calreticulin. Peptides were analyzed in a B3Z assay. Each group was set up in duplicate and data are represented as mean ± SEM. One representative experiment of three independent experiments is shown.

quantities of gp96 were being pulled down (Fig. 3C, inset). Peptides stripped from gp96 immunoprecipitates were then analyzed by the B3Z assay. The amount of OVA (precursor) peptides stripped from PIK23.2-derived gp96 was several times greater than the amount of these peptides stripped from PIK23.2Kb-derived gp96 (Fig. 3C). This difference suggests that these peptides that are no longer bound to gp96 are elsewhere, possibly with Kb. Peptides were extracted from immunoprecipitated calnexin or MHC-I (H-2k) as controls and analyzed in the B3Z assay. No signal was associated with these molecules.

We tested the idea that if OVA peptides from gp96 are being transferred to Kb, then these Kb molecules will exit to the cell surface, decreasing the amount of OVA (precursor) peptides in the ER. Despite the equal amount of Ag made in either cell type (Fig. 3A), the total amount of OVA (precursor) peptides in microsomes of PIK23.2 cells is significantly more than in PIK23.2Kb cells (Fig. 3D, no BFA bars). To examine if the Kb expression was providing an “outlet” for OVA peptides, PIK23.2 or PIK23.2Kb cells were treated with BFA that inhibits vesicular traffic from the ER/golgi to the cell surface thus retaining MHC molecules with their peptide cargo in the ER (21). BFA did not affect the amount of the total OVA-specific signal in cell lysate from either cell type (data not shown). Microsomes were then purified from untreated or BFA treated, RSL1-induced PIK23.2 or PIK23.2Kb cells, and peptides extracted and measured in the B3Z assay. BFA treatment of PIK23.2Kb cells retained more of the OVA-specific peptides within the microsomes leading to an increase in B3Z activation, to levels seen in PIK23.2 cells (Fig. 3D). No significant effect of BFA on PIK23.2 cells was observed compared with BFA-untreated cells. These results demonstrate that the OVA-peptides in the ER constitute a pool bound to chaperones and MHC-I; this pool is affected by the small proportion of peptides bound to MHC-I that are able to exit the ER.

The relay line of Ag presentation formed by gp96 and calreticulin

We hypothesized that there could be four scenarios as to how the peptides are trafficked in the ER in relation to these chaperones and MHC-I (Fig. 4A): i, peptides from TAP bind to gp96, are transferred to calreticulin and then to MHC I; ii, peptides from TAP bind to calreticulin, are transferred to gp96 and then to MHC I; iii, peptides from TAP bind to gp96 and calreticulin simultaneously and then the peptides are transferred to MHC-I independent of each other; or iv, peptides from TAP are directly transferred from TAP onto MHC-I independent of either calreticulin or gp96. Other ER proteins may be intermediates in each of these scenarios. We tested these four scenarios first by knocking down the expression of calreticulin using siRNA and monitoring the peptides bound to gp96 and Kb after treatment. Treatment of PIK23.2Kb cells for 3 d with calreticulin-specific siRNA led to ~65% knock down of expression of calreticulin compared with cells not treated with siRNA (Fig. 4B). Cells recover shortly thereafter (day 4–5) to near maximum expression. No compensation (alteration) in gp96 expression was observed at any time point tested (data not shown). Calreticulin-specific siRNA-treated PIK23.2Kb cells were then induced to express Ag for 18 h spanning day 3 or 4 as shown in the scheme (Fig. 4C). A group of cells were not treated with siRNA but induced with RSL1 for 18 h. Cells were lysed and immunoprecipitated with an Ab specific for gp96 or a conformationally dependent Ab (Y3) to peptide-bound Kb. Equal amounts of gp96 or Kb were immunoprecipitated in all groups; immunoprecipitated Kb molecules were probed with Abs specific to βm to confirm specific pull-down in PIK23.2Kb but not PIK23.2 cells (data not shown). Peptides obtained from gp96 immunoprecipitates (Fig. 4D) were tested in a B3Z assay and show that knock down of calreticulin lead to a robust increase (~10-fold) in the amount of OVA (precursor) peptides bound to gp96 compared with the untreated
group. This increase was specific to the treatment because the phenotype (increased gp96-peptide load) was significantly reversed when cells were allowed to recover calreticulin expression to normal levels. This result effectively ruled out scenario (ii) described in Fig. 4A because for this scenario a knock down of calreticulin should have caused a decrease in peptides associated with gp96. Peptides stripped from Kb immunoprecipitates showed a 3.5-fold decrease of SIINFEHL peptides after calreticulin knock down (Fig. 4E) suggesting that peptides from calreticulin are destined for Kb but occurs after gp96 (Fig. 4Ai). These data do not support scenarios (iii) or (iv) because, respectively, an increase in SIINFEHL peptides on gp96 should have resulted in a concomitant increase in SIINFEHL peptides on Kb or peptides on Kβ should have remained unchanged.

We next tested the four scenarios with respect to gp96 knockdown (Fig. 5A). PIK23.2Kβ cells were treated with gp96-specific siRNA and maximal knock down of gp96 (~75%) was obtained 2 d after (Fig. 5B). Cells recover significant amount of gp96 expression (~59% of untreated cells) on day 3. No compensation (alteration) in calreticulin expression was observed at any time point (data not shown). Gp96-specific siRNA-treated PIK23.2Kβ cells were then induced to express Ag for 18 h spanning day 2 or 3 as shown in the scheme (Fig. 5C). A group of cells were not treated with siRNA but induced with RSL1 for 18 h (untreated group). The siRNA untreated or treated cells were immunoprecipitated for calreticulin or Kb. The amount of calreticulin or Kb immunoprecipitated in each group was equal. Peptides were extracted from immunoprecipitated gp96, calnexin, or MHC-I and analyzed in the B3Z assay. D, Retention of peptides within the ER. PIK23.2 or PIK23.2Kβ cells were treated with BFA or left untreated and simultaneously induced with RSL1. Peptides in the purified microsomes were stripped and measured in a B3Z assay. For B and C, each group was set up in duplicate and data are represented as mean ± SEM. One representative experiment of two (A, B) or three (C) independent experiments is shown.
support scenario ii or iii because removing gp96 should have resulted in an increase or no change in SIINFEHL peptides on calreticulin. Also, these data do not support scenario iv because SIINFEHL peptides on Kb should have remained unchanged.

Gp96 and calreticulin are associated with N-terminal extended precursor peptides

The well-accepted model is that MHC-I precursor peptides that enter the ER have the correct C terminus for binding MHC-I but are extended on the N terminus (which is then trimmed by ERAAP) because the ER lacks C terminus trimming activity (1). We tested the nature of the extensions of the peptides bound to gp96 and calreticulin. Treatment of the precursor peptides in the KOV AK model with trypsin alone should release the exact epitope if the peptides have the correct C terminus and are extended only on the N terminus (13, 14). Gp96 and calreticulin were immunoprecipitated from RSL1-induced or uninduced PIK23.2 cells. Peptides were extracted from the immunoprecipitates and treated with trypsin and carboxypeptidase B or with trypsin alone or were left untreated. Peptides were tested in a B3Z assay. As shown in Fig. 6, B3Z signal was obtained when peptides were treated with trypsin alone indicating that these peptides had the correct C terminus for binding MHC-I. When peptides were additionally treated with carboxypeptidase B, an enhanced signal was obtained indicating that gp96 and calreticulin chaperone a quantity of peptides that are also extended on both the N and C termini. Fig. 6 shows that more N and C termini extended peptides were obtained from gp96 than calreticulin and no significant amount of exact peptides were obtained from either chaperone.

Discussion

We have examined the relative contribution of the ER chaperones gp96 and calreticulin to processing and presentation of a specific antigenic peptide. The main conclusions of our results are that 1) these two chaperones are essential for trafficking of peptides destined for presentation by MHC, 2) peptides are transferred in a relay line from gp96 to calreticulin, and not the reverse, and 3) the
peptides chaperoned by gp96 and calreticulin appear to consist of a pool of (KOVAK-derived) peptides, the repertoire of which is influenced by the haplotype of MHC-I that is expressed.

In the ER, a direct interaction between the MHC and TAP has been reported as part of the peptide-loading complex, and at first glance, precludes the necessity of peptide-binding chaperones in the ER

![Diagram of peptide processing](image)

**FIGURE 6.** Gp96 and calreticulin chaperone N-terminally extended MHC-I precursor peptides. Gp96 (A) or calreticulin (B) was immunoprecipitated from equal numbers of PIK23.2 cells induced with RSL1 for 18 h or left uninduced. Peptides were stripped and selectively digested with trypsin only, trypsin and carboxypeptidase B, or left undigested (none). Peptides were analyzed in a B3Z assay for the presence of the SIINFEHL peptide. One representative experiment of two independent experiments is shown.
(22). However, most of the peptides entering the ER through TAP are extended and require additional trimming by ERAAP and maybe other ER proteases (2, 3). Gp96 and calreticulin have now been shown to bind these peptides prior to their loading onto MHC-I. We observed that gp96 and calreticulin together bind slightly 
>50% of the OVA signal in the ER in the absence of K\(^+\). The remainder of the signal may represent epitope destruction or may be associated with other chaperones, such as protein disulfide isomerase (23–25). In light of the earlier demonstration that gp96 and calreticulin are the only ER chaperones that can prime immune responses specific to the peptides they chaperone (18, 19, 26), the participation of these other ER chaperones in the relay line of peptide transfer merits further investigation.

The ER has been suggested to be largely devoid of carboxyterminal trimming activities. Using an approach of selective enzymatic digestion (13, 14), we tested whether the peptides chaperoned by gp96 and calreticulin are elongated on the N or C terminus or both termini or unextended. Our results show that a significant portion of the peptides are N-terminally extended (with correct C terminus for binding MHC-I) and can therefore be converted to their exact size by ER proteases. Some of the peptides recovered from gp96 and calreticulin, however, were extended on both termini, though this fraction was smaller when recovered from calreticulin compared with gp96. This appears consistent with the arrangement of gp96, calreticulin and MHC-I in the ER relay line, where we propose chronological processing of extended peptides to the final size. The fate of the peptides extended on both termini may be for purposes other than the immunological. Surprisingly, we recovered no peptides of exact length (the presented peptide) from gp96 (10) or calreticulin.

In an earlier study, substantial knock down of gp96 did not reveal a loss of expression of MHC-I on the cell surface, which suggested no role for gp96 in Ag processing and presentation (27). However, the loss of MHC expression from the cell surface because of a lack of peptide binding to MHC in the ER requires a global abrogation of peptide transfer onto MHC-I. In this study, our analysis is restricted to a single Ag where the requirements (such as gp96 binding) are more specific and proximal to the peptide transfer event, and may not represent the average population of all peptides. An extension of our studies to other Ags is underway.

In different antigenic systems, a wide range of efficiency of Ag processing can be achieved (6, 7). Because the noncanonical signal sequence of OVA, residing in its first 137 residues, has been removed in the KOVAK construct we used, the protein is expressed exclusively in the cytosol (12). We observed that up to 50% of the signal of the expressed protein could be detected in the ER, representing the sum of the exact MHC-I peptides and those with various extensions (precursors). This is on the high end range of Ag processing efficiency. Possible explanations for this include association of TAP-independent peptides with gp96 (28), a higher rate and/or efficiency of degradation of (full length) KOVAK products or the production of a large number of defective ribosomal products (29). A further examination of the peptides stripped from gp96 and calreticulin should be able to reveal (based on their length and N/C terminus amino acids) whether these are truly TAP transported peptides and whether these can account for the total ER signal.

Based on our observation that KOVAK precursor peptides chaperoned by gp96 are affected by MHC-I type, one would predict that immunizing mice with gp96 purified from OVA-expressing cells of different haplotypes would elicit immune responses of disparate magnitudes. This has been experimentally tested and is not the case (30). However, those immunization experiments were not capable of detecting the 5- to 10-fold differences in specific peptides chaperoned by gp96 because of inherent amplification events during priming. Direct analysis of gp96-chaperoned peptides by mass spectrometry is under investigation and will shed further light on this issue. Similar to our observation, the feedback effect of the MHC-I type on Ag processing has been observed before (31).

Consistent with a role for gp96 and calreticulin in loading MHC-I molecules with peptides is the ability to communoprecipitate either chaperone with MHC-I (1, 32). The recent investigation of the dynamic interactions between Erp57 and tapasin has shed light on their role in the PLC at a molecular and structural level (33). With a similar analysis of other components of the PLC, these studies will inform on where and how calreticulin and gp96 fit into the PLC. Within the PLC calreticulin has been proposed to have a role in stabilizing empty MHC molecules by directly interacting with Erp57 and glycan residues of the MHC H chain (34). Thus, the studies on calreticulin knock down performed in this study may impact the stabilization and folding of MHC H chain assembly in the ER and thus indirectly affecting the recoverable peptides from the fully assembled MHC-I. At this point, we do not know the relative contribution of either function, although previous studies have shown that MHC H chains can still associate with peptides in the complete absence of calreticulin. The complete loss of KOVAK peptides bound to K\(^+\) after calreticulin knock down, seen in our experiments, may suggest a greater role for peptide transfer. We conclude that the “peptide editing” function of calreticulin proposed by Elliot and colleagues (35) may thus comprise of a dual function; peptide transfer and stabilization of MHC.

The linearity of the relay line of peptide transfer implies limited room for redundancy. Consistent with the indispensable role for HSPs in MHC-I peptide processing and presentation described in this study and in other studies (10–15), other steps in this pathway, including proteasomal processing (36), peptide transport by TAP (37) appear to have no apparent compensatory mechanisms. Finally, our observations can be extrapolated to the possible existence of a similar relay line in the cytosol involving hsp70, hsp90, TCP-1 ring complex, and TAP.

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Supplemental Figure 1. (A) Purity of gp96 and calreticulin preparations. Gp96 and calreticulin were purified from murine liver tissue as described in methods section. Apparently homogenous preparations of either protein was resolved by SDS-PAGE and stained with silver. (B) Microsomes contain a significant portion of the antigenic (precursor) peptides of the cell. Peptides were extracted from 10 million RSL1 induced cell equivalents of microsomes or cell lysate as described in Methods. Peptides were analyzed in a B3Z assay. The signal obtained in the microsome fraction has not been adjusted for the amount of material lost during its preparation. (Each group was set up in duplicate and data are represented as mean +/- SEM). One representative experiment of several is shown.

Supplemental Figure 2. Linear relationship between B3Z and peptide concentration. EL.4 cells were pulsed for one hour with titrated doses of SIINFEKL peptide or where pulsed with the equivalent amount of DMSO only. After washing with PBS, cells were incubated with 10,000 B3Z cells overnight (12 hours) at 37°C in 96 well plates. LacZ assays have been described previously (12) to obtain the Specific Absorbance 595nm.
Supplemental Fig. 1A

gp96  calreticulin

96kDa  55kDa
Supplemental Fig. 1B
Specific absorbance $A_{595}$

Concentration of SIINFEKL pulsed on EL.4 cells ($\mu$M)

- EL.4 + SIINFEKL
- EL.4 + DMSO

The graph shows the change in specific absorbance $A_{595}$ as the concentration of SIINFEKL pulsed on EL.4 cells decreases. The absorbance decreases significantly with increasing concentration, with two distinct lines representing EL.4 + SIINFEKL and EL.4 + DMSO.