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Processing of Exogenous Antigens for Presentation by Class I MHC Molecules Involves Post-Golgi Peptide Exchange Influenced by Peptide-MHC Complex Stability and Acidic pH

Peter J. Chefalo and Clifford V. Harding

Vacuolar alternate class I MHC (MHC-I) pathway involves processing of protein Ags expressed within the cell (endogenous Ags) for presentation of peptides derived from endogenous Ags by MHC-I molecules. However, it is well established that endogenous Ags can also be processed for presentation by MHC-I molecules via alternate MHC-I-ag processing mechanisms (1–9). Experimental evidence supports several different alternate MHC-I processing mechanisms.

Cytosolic alternate MHC-I processing mechanisms involve the egress of exogenous Ags from vacuolar (endocytic or phagocytic) compartments to enter the cytosol (10–13). These Ags merge with conventional MHC-I ag processing mechanisms. Their processing is dependent on proteasomes for proteolytic production of antigenic peptides and the TAP for transport of these peptides into the endoplasmic reticulum (ER). In this pathway, peptides bind to newly synthesized MHC-I molecules in the ER, and the resulting peptide-MHC-I complexes are transported from the ER through the Golgi complex and eventually to the cell surface. This pathway is blocked by deficits in TAP or treatment of cells with proteasome inhibitors or brefeldin A (an inhibitor of anterograde ER-Golgi transport) (11).

Vacuolar alternate MHC-I processing mechanisms involve the processing of exogenous Ag in vacuolar compartments without penetration of Ag into the cytosol. Vacuolar processing produces antigenic peptides that bind to MHC-I molecules that have already exited from the Golgi complex (i.e., MHC-I molecules in post-Golgi compartments). Hence, this pathway is relatively resistant to treatment with proteasome inhibitors or brefeldin A. However, the supply of peptide-receptive MHC-I molecules in post-Golgi compartments can be depleted by long incubations with brefeldin A or deficits in TAP function. In some experimental systems, vacuolar alternate MHC-I Ag processing is indirectly dependent on TAP and is partially inhibited by deficits in TAP, even though TAP does not transport the peptides derived from exogenous Ags in these systems (5, 14, 15). In other systems, vacuolar alternate MHC-I Ag processing is completely TAP independent (16–20). The site where peptides bind to MHC-I molecules during vacuolar alternate MHC-I Ag processing is still unclear, and binding may take place within intracellular processing compartments that contain MHC-I molecules (e.g., phagosomes) (21) or on the cell surface following recycling and regurgitation of peptides (22).

Early reports suggested that TAP-deficient cells express "empty" MHC-I molecules under certain conditions (e.g., at 26°C) (23). However, recent evidence indicates that such MHC-I molecules do contain peptides, but these peptide-MHC-I complexes are less stable than most complexes expressed on TAP-replete cells at 37°C (24). Decreased ER peptide supply in TAP-deficient cells may limit availability of high affinity peptides. Peptide binding may be required for MHC-I molecules to exit from the ER (25–29). Limited peptide supply in TAP-deficient cells may result in exit of...
peptide-MHC-I complexes that have relatively low affinity peptides, high dissociation rates, and decreased stability at 37°C (24). TAP-deficient cells have decreased levels of both total (30) and peptide-receptive (5, 14, 31) MHC-I on the cell surface (and possibly in post-Golgi vacuolar compartments that communicate with the cell surface), limiting the availability of peptide-receptive SVV-MHC-I molecules available for vacuolar alternate MHC-I Ag processing.

In this study, we analyze the source and nature of post-Golgi peptide-receptive MHC-I molecules that are used in alternate MHC-I processing. Because TAP contributes indirectly to vacuolar alternate MHC-I Ag processing without transporting peptide derived from exogenous Ag (5, 14), TAP-dependent loading of MHC-I with endogenous peptides may enhance exit of MHC-I molecules from the ER and their availability for subsequent binding and presentation of peptides derived from exogenous Ags. This model suggests that vacuolar alternate MHC-I Ag processing involves dissociation of one peptide and binding of peptide derived from exogenous Ag (i.e., peptide dissociation/exchange). Our experiments address the role of prior peptide occupancy in controlling the level of peptide-receptive MHC-I molecules that contribute to alternate MHC-I Ag processing. Macrophages from TAP1−/− mice provide a good system to address this question. Macrophages mediate alternate MHC-I processing of particulate Ag (2). TAP-deficient cells express MHC-I molecules that are not optimally loaded and can bind exogenous peptide (30) to generate a large cohort of homogenous peptide-MHC-I complexes. Such cohorts can be assessed as potential precursors for peptide-receptive MHC-I molecules that function in processing and presentation of exogenous Ag. Overnight incubation of TAP1−/− macrophages with a first (“stabilizing”) peptide stabilized Kα complexes and enhanced subsequent binding and presentation of a second (“read-out”) peptide by Kβ molecules. This enhancement was influenced by the stability of stabilizing peptide-Kα complexes and was increased by exposure of Kα molecules to acidic pH (similar to that of intracellular vacuolar compartments). These observations suggest that peptide exchange contributes to alternate MHC-I Ag processing and is influenced by the stability of peptide-MHC-I complexes and the pH of the processing environment.

**Materials and Methods**

**Animals and cells**

TAP1−/− mice (32) were backcrossed for six generations with C57BL/6 mice and were generously provided by Dr. L. Van Kaer (Vanderbilt University, Nashville, TN). TAP1−/− and C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were bred and housed under specific pathogen-free conditions at Case Western Reserve University (Cleveland, OH). Activated peritoneal macrophages were harvested 4 days after i.p. inoculation with Con A (100 μg/ml). The T hybridoma C8D08A1.3 (2) was used to detect SIINFEKL-Kβ complexes. The T hybridoma SVB1.6 was used to detect FAPGNYPAL-Kβ complexes. To obtain this hybridoma, C57BL/6 mice were infected intranasally with 10^3 PFU Sendai virus and sacrificed 19 days after infection. Spleen cells were stimulated in vitro with 1–5 mM Sendai virus nucleoprotein (324–332) (FAPGNYPAL) for 6 days, mixed 1:4 with BWS147 TCRαβ CD8+ cells (33), and fused with polyethylene glycol. Fusion products were screened and characterized, and SVB1.6 was selected for these studies. RMA-S cells are TAP-deficient (TAP2-mutant) Rauscher virus-transformed murine T cell lymphoma cells (H-2b) (30). Cells were cultured in standard medium consisting of DMEM (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 5 × 10^-5 M 2-ME, 1 mM sodium pyruvate, HEPES buffer, and penicillin-streptomycin (Life Technologies). Incubations were conducted in a 5% CO2 atmosphere at 37°C or 26°C.

**Ag preparations**

Peptides included SIINFEKL (OVA, 237–244), KVRFDKL (OVA, 58–65), FAPGNYPAL (Sendai virus nucleoprotein 324–332), RGYVYQGL (vesicular stomatitis virus nucleoprotein 52–59), ILKEPVHG (HIV reverse transcriptase 476–484), and DGSTDYGLQINSR (hen egg lysozyme 48–61). Peptides were synthesized (Princeton Biomolecules, Langhorne, PA, or Sigma, St. Louis, MO) with C-terminal 1 mM [35S]methionine and purified by reverse phase HPLC to final concentrations in standard medium. E. coli HB101.Crl-OVA constitutively expresses Crl-OVA, a fusion protein containing the SIINFEKL epitope (2). Viable HB101.Crl-OVA was used as a particulate Ag to assess alternate MHC-I Ag processing (1 × 10^5 HB101.Crl-OVA bacteria contain ~150 ng or 0.1 × M of Crl-OVA) (2). A negative control was provided by HB101.Crl- hen egg lysozyme, which expresses a related fusion protein that does not contain OVA sequence (34).

**Ag presentation protocols**

To obtain macrophages, Con A-elicited peritoneal exudate cells from TAP1−/− or C57BL/6 mice were incubated in 96-well plates (1 × 10^7) for 2 h and washed to remove nonadherent cells. In the first Ag incubation, macrophages were incubated for 18 h at 26°C in standard medium containing 5 μM stabilizing peptide (SIINFEKL, KVRFDKL, FAPGNYPAL, or RGYVYQGL). Cells were washed to remove stabilizing peptide and incubated for 110 min at 37°C in standard medium and an additional 10 min with brefeldin A (2 μg/ml) to inhibit transport of nascent MHC-I molecules through the Golgi complex and to the cell surface. In some experiments involving exposure to acidic pH, the medium was then replaced with citrate-buffere d saline at varying pH levels for 20 min at room temperature and subsequently washed three times in standard medium. In the second Ag incubation, readout peptide for HB101.Crl-OVA was added in the continued presence of brefeldin A for 2 h at 37°C. Cells were fixed in 1% paraformaldehyde and washed. T hybridoma cells (10^5/well) were added for 24 h. Culture supernatant (0.1 ml) was removed, frozen, thawed, and cultured with 5 × 10^3 IL-2-dependent CTL2 cells for 24 h (35), and 15 μl Alamar Blue (Alamar Biosciences, Sacramento CA) was then added for 18–24 h (36, 37). Alamar Blue is reduced metabolically active cells, shifting its relative absorbance at 550 and 595 nm (reduced and oxidized forms both have high absorbance at 550 nm, but only the oxidized form has high absorbance at 595 nm). CTL2-2 growth was assessed by subtracting OδS from Oδ0, using a plate spectrophotometer (Bio-Rad, Hercules, CA). This assay produced threshold sensitivity, plateau response, and IL-2 dose-response results similar to [3H]methylinthymidine incorporation CTL2-2-proliferation assays. Minimum response was observed with culture of CTL2-2 cells with 0.004–0.04 U/ml recombinant murine IL-2 (Roche, Indianapolis, IN; 2 U/μg); half-maximal response was seen with 0.04 U/ml IL-2; and a plateau response was produced by 0.4–4 U/ml IL-2. Ag processing assays were performed with quadruplicate wells. Each data point is expressed as mean ± SD.

**Flow cytometry**

RMA-S cells were incubated in six-well plates (2 × 10^6 cells/well) overnight at 37°C in standard medium containing 50 μM peptide. The cells were then washed and incubated in standard medium for various periods at 37°C in the presence of brefeldin A. The cells were washed three times in FACS buffer (PBS containing 0.1% rabbit serum and 0.1 mg/ml BSA), split into three wells of a round-bottom 96-well plate, incubated for 30 min at 4°C with 5 μg/ml biotinylated AF6-88.5 anti-Kβ Ab (BD PharMingen, San Diego, CA), washed, and incubated with 2 μg/ml CyChrome-conjugated streptavidin (BD PharMingen). Cells were fixed with paraformaldehyde and analyzed with a FACScan flow cytometer (BD Biosciences, San Jose, CA). Similar experiments were performed with macrophages (2 × 10^6 cells per 60-mm petri dish).

To determine the effects of stabilizing peptides on the subsequent formation of SIINFEKL-Kβ complexes, macrophages were prepared from TAP1−/− peritoneal exudate cells (2 × 10^7/100-mm petri dish), washed, and stimulated overnight in standard medium with 50 μM KVVRFDKL, FAPGNYPAL, or RGYVYQGL. The cells were washed three times in FACS buffer (PBS containing 0.1% rabbit serum and 0.1 mg/ml BSA), split into three wells of a round-bottom 96-well plate, incubated for 30 min at 4°C with 5 μg/ml biotinylated AF6-88.5 anti-Kβ Ab (BD PharMingen, San Diego, CA), washed, and incubated with 2 μg/ml CyChrome-conjugated streptavidin (BD PharMingen). Cells were fixed with paraformaldehyde and analyzed with a FACScan flow cytometer (BD Biosciences, San Jose, CA). Similar experiments were performed with macrophages (2 × 10^6 cells per 60-mm petri dish).

**Results**

**Initial incubation with stabilizing peptide enhances subsequent binding and presentation of readout peptide to T cells**

We explored the hypothesis that vacuolar alternate MHC-I Ag processing uses post-Golgi peptide-receptive MHC-I molecules that
derive from peptide-occupied MHC-I molecules via peptide dissocation/exchange as opposed to empty MHC-I molecules that have never bound peptide. Initial loading of MHC-I molecules may occur with an endogenous peptide in the ER, allowing export to post-Golgi compartments. Low affinity peptide-MHC-I complexes may subsequently participate in peptide dissociation/exchange, allowing MHC-I molecules to bind exogenous peptide or peptide derived from exogenous Ag by alternate MHC-I Ag processing.

In testing the role of peptide dissociation/exchange, it is difficult to manipulate the initial loading of MHC-I molecules with endogenous peptides in the ER. We overcame this difficulty by manipulating the loading of MHC-I molecules after their exit from the ER and studying their subsequent ability to bind and present new antigenic peptide. A large cohort of post-Golgi peptide-MHC-I complexes was generated with an exogenous (stabilizing) peptide to determine how these complexes affected or contributed to subsequent binding of a second exogenous (readout) peptide or binding of a peptide derived from alternate MHC-I processing of particulate Ag. TAP1−/− cells were used to achieve this goal, because many of their MHC-I molecules are poorly loaded (probably containing low affinity peptides). Thus, exogenous peptide can load a greater proportion of MHC-I molecules and have greater impact on their cell surface expression and potential contribution to peptide dissociation/exchange. Brefeldin A was added before addition of readout peptide to focus the experiment on the defined cohort of stabilizing peptide-MHC-I complexes in post-Golgi compartments and to exclude MHC-I molecules synthesized after the removal of stabilizing peptide. Thus, incubation of TAP1−/− macrophages with or without exogenous stabilizing peptide determined the presence or absence of a large cohort of post-Golgi peptide-MHC-I complexes that were potentially available for peptide dissociation/exchange.

These experiments assessed the effects of four different stabilizing peptides that bind Kb (SIINFEKL, KVVFDFKL, FAPGNYPAL, or RGYVYQQL) on the binding and presentation of Kβ-restricted readout peptides (SIINFEKL or FAPGNYPAL) to T hybridoma cells. Relative to incubation in standard medium with no stabilizing peptide, overnight incubation of TAP1−/− macrophages with RGYVYQQL or KVVFDFKL enhanced subsequent binding and presentation of FAPGNYPAL to SVB1.B6 T hybridoma cells (Fig. 1). As shown in Fig. 1B, the response to readout peptide was not altered by prior incubation with a peptide that does not bind to Kβ (HIV reverse transcriptase 476–484 peptide, ILKEPVHGβ, which binds HLA-A2). Addition of stabilizing peptide to wild-type C57BL/6 macrophages had little effect on production of readout peptide-Kβ complexes (data not shown and below), presumably because wild-type cells have a sufficient supply of endogenous stabilizing peptides. These results suggest that RGYVYQQL and KVVFDFKL bound and stabilized Kβ molecules, increasing expression of peptide-Kβ complexes that subsequently provided peptide-receptive Kβ molecules to bind and present the FAPGNYPAL readout peptide.

Enhancement of peptide-receptive MHC-I molecules by stabilizing peptide is influenced by the stability of stabilizing peptide-MHC-I complexes

In contrast to the results seen with RGYVYQQL or KVVFDFKL as stabilizing peptide, overnight incubation with SIINFEKL decreased subsequent binding and presentation of FAPGNYPAL (Fig. 1). We proposed that the stability of SIINFEKL-Kβ complexes might limit their dissociation and production of peptide-receptive Kβ molecules. Other studies have established the high affinity of SIINFEKL for Kβ ($K_d = 1.6–6.7 \times 10^{-9}$ M) (39–41) and high stability of SIINFEKL-Kβ complexes (39, 42). In addition, we studied the ability of the peptides used in this study to increase expression of thermostable Kβ molecules on TAP-deficient cells. This assay has been used by other groups as an indirect indication of relative peptide affinity and/or stability of peptide-MHC-I complexes, although this method does not allow calculation of true affinity (41, 43, 44). After overnight incubation with peptide at 26°C and incubation at 37°C for 2–4 h to down-regulate unstable MHC-I molecules, flow cytometry showed that SIINFEKL, KVVFDFKL, FAPGNYPAL, and RGYVYQQL all increased cell surface expression of Kβ on RMA-S cells, confirming that these peptides bound and stabilized Kβ (Fig. 2A). When DGSTDYGILQIN (which does not bind to Kβ) was used as the stabilizing peptide, enhancement of Kβ expression was not seen (data not shown). SIINFEKL enhanced Kβ expression substantially more than the other peptides, which appeared relatively similar in their ability to up-regulate Kβ. These data indicate that SIINFEKL formed a greater number of stable peptide-Kβ complexes than the other peptides under these conditions.

To directly assess the stability of peptide-Kβ complexes and to determine the half-life of different peptide-Kβ complexes, we studied the decay of specific peptide-induced enhancement of Kβ expression on RMA-S cells or TAP1−/− macrophages. Cells were incubated overnight with peptide to stabilize Kβ expression, washed, subjected to a chase incubation of varying length at 37°C to allow decay of specific peptide-Kβ complexes, and stained for determination of Kβ expression by flow cytometry. Under these conditions, the majority of Kβ molecules were loaded with the specific peptide (see medium controls in Figs. 2, A and C) so that the half-life of Kβ expression approximated the half-life of the specific peptide-MHC-I complex. On RMA-S cells, $t_{1/2}$ was determined to be 8.9 h for SIINFEKL-Kβ complexes, 3.4 h for FAPGNYPAL-Kβ complexes, 3.1 h for RGYVYQQL-Kβ complexes, and 1.6 h for KVVFDFKL-Kβ complexes (Fig. 2B). Similar results were obtained with TAP1−/− macrophages, with a $t_{1/2}$ of 7.2 h for SIINFEKL-Kβ complexes, 3.7 h for FAPGNYPAL-Kβ complexes, and 1.5 h for SIINFEKL-Kβ complexes.
incubation with each stabilizing peptide (t1/2) was calculated for incubations of RMA-S cells (2 × 10^6) with or without 50 μM stabilizing peptide, washed, incubated at 37°C for 2 h, and then stained for flow cytometry with biotinylated AF6-88.5 (anti-Kb) and CyChrome-conjugated streptavidin. Events were gated to include intact RMA-S cells. Results are expressed as specific MFV (MFV with AF6-88.5 – MFV with isotype control Ab). B, Stability of different stabilizing peptide-Kb complexes on RMA-S cells. RMA-S cells were incubated overnight at 26°C with or without stabilizing peptide, washed, subjected to a chase incubation of varying lengths at 37°C, and stained for Kb as described above. Data are from the same experiment as A. The log2 of peptide-specific MFV (defined as MFV with peptide – MFV without peptide) is plotted vs. time of chase incubation. Linear least squares regression was used to determine the best-fit line, and the slope (m) was used to calculate the half-life of Kβ expression after incubation with each stabilizing peptide (t1/2 = log2(2)/m = 1/m). C, Enhancement of Kβ expression on TAP1-/- macrophages by different stabilizing peptides. The experiment was performed as in A except that macrophages were incubated with 5 μM stabilizing peptide. D, Stability of different stabilizing peptide-Kb complexes on TAP1-/- macrophages. The experiment was performed as in B except that macrophages were incubated with 5 μM stabilizing peptide. Data are from the same experiment as C.

FIGURE 2. Flow cytometric assessment of stabilizing peptide-Kb complexes on TAP-deficient RMA-S cells. A, Enhancement of Kβ expression on RMA-S cells by different stabilizing peptides. RMA-S cells (2 × 10^6) were incubated overnight at 26°C with or without 50 μM stabilizing peptide, washed, incubated at 37°C for 2 h, and then stained for flow cytometry with biotinylated AF6-88.5 (anti-Kβ) and CyChrome-conjugated streptavidin. Events were gated to include intact RMA-S cells. Results are expressed as specific MFV (MFV with AF6-88.5 – MFV with isotype control Ab). B, Stability of different stabilizing peptide-Kb complexes on RMA-S cells. RMA-S cells were incubated overnight at 26°C with or without stabilizing peptide, washed, subjected to a chase incubation of varying lengths at 37°C, and stained for Kβ as described above. Data are from the same experiment as A. The log2 of peptide-specific MFV (defined as MFV with peptide – MFV without peptide) is plotted vs. time of chase incubation. Linear least squares regression was used to determine the best-fit line, and the slope (m) was used to calculate the half-life of Kβ expression after incubation with each stabilizing peptide (t1/2 = log2(2)/m = 1/m). C, Enhancement of Kβ expression on TAP1-/- macrophages by different stabilizing peptides. The experiment was performed as in A except that macrophages were incubated with 5 μM stabilizing peptide. D, Stability of different stabilizing peptide-Kb complexes on TAP1-/- macrophages. The experiment was performed as in B except that macrophages were incubated with 5 μM stabilizing peptide. Data are from the same experiment as C.

Stabilizing peptide enhances subsequent production of SIINFEKL-Kβ complexes by alternate MHC-I processing of HB101.Crl-OVA to a greater degree than production of these complexes from soluble SIINFEKL

As observed in previous studies (2, 5, 14, 22), macrophages processed HB101.Crl-OVA (E. coli expressing an OVA fusion protein) to produce SIINFEKL-Kβ complexes that were detected by CD80VA1.3 T hybridoma cells (Fig. 3). Incubation of macrophages with HB101.Crl-hen egg lysozyme, which expresses a different antigenic fusion protein, resulted in no detectable response by CD80VA1.3 T hybridoma cells (data not shown), confirming the antigenic specificity of this system.

Incubation of macrophages with stabilizing peptide (FAPGNYPAL, KVVRFDKL, or RGYVYQGL) produced substantial enhancement of subsequent alternate MHC-I processing of HB101.Crl-OVA to produce SIINFEKL-Kβ complexes (Fig. 3B). This implies that stabilization of MHC-I molecules as peptide-MHC-I complexes provides precursors to the peptide-receptive MHC-I molecules that are used in alternate MHC-I Ag processing. In addition, enhancement of SIINFEKL-Kβ complexes was more pronounced with addition of HB101.Crl-OVA (Fig. 3B), which requires intracellular processing, than with addition of exogenous SIINFEKL (Fig. 3A), which can bind to cell surface Kβ molecules (in some experiments the enhancement of exogenous SIINFEKL presentation was even less than in Fig. 3A). This suggests that the intracellular processing environment promotes peptide dissociation/exchange to a greater degree than the environment at the cell surface.

Effects of stabilizing peptide on subsequent binding of readout peptide as quantified by flow cytometry detection of SIINFEKL-Kβ complexes

Experiments using a T cell assay supported the hypothesis that stabilizing peptides generate peptide-MHC-I complexes that can subsequently dissociate or undergo peptide exchange to bind an additional readout peptide. A T cell assay is advantageous for this type of experiment because it allows detection of physiological levels of peptide-MHC-I complexes, as can be generated by alternate MHC-I Ag processing. However, T cell assays do not provide quantitative assessment of peptide-MHC complexes and are subject to some potential artifacts. The mAb 25-D1.16 is specific for complexes, 2.8 h for RGYVYQGL-Kβ complexes, and 1.6 h for KVVRFDKL-Kβ complexes (Fig. 2D). These figures are similar to those produced by other studies, e.g., that of Chen et al. (39), which showed a t1/2 of 8.3 h for SIINFEKL-Kβ complexes and 2.7 h for KVVRFDKL-Kβ complexes. Thus, SIINFEKL-Kβ complexes are more stable than the other peptide-Kβ complexes examined here. The high stability of SIINFEKL-Kβ complexes may limit their ability to serve as precursors for peptide-receptive MHC-I molecules that are used in alternate MHC-I Ag processing or for binding and presentation of exogenous peptide.

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SIINFEKL-Kb complexes (38) and can be used to directly quantify these complexes by flow cytometry, although the lower sensitivity of this assay makes it useful only for measuring the production of complexes with relatively high concentrations of exogenous peptide (the physiological level of SIINFEKL-Kb complexes generated by alternate MHC-I Ag processing, e.g., of HB101.Crl-OVA, is not detectable by flow cytometry using this Ab).

Fig. 4 shows 25-D1.16 staining of cell surface SIINFEKL-Kb complexes on TAP1−/− macrophages that were first incubated with or without a stabilizing peptide (FAPGNYPAL, KVVR FDKL, or RGYVVQGL) and then incubated with or without SIINFEKL readout peptide. In each case, stabilizing peptide increased the production of SIINFEKL-Kb complexes. SIINFEKL-specific mean fluorescence value (MFV) was defined as the MFV with SIINFEKL minus the MFV without SIINFEKL. Incubation with stabilizing peptide increased SIINFEKL-specific MFV by 3.5- to 4.5-fold (see Fig. 4 legend). Staining of macrophages with 25-D1.16 was increased after incubation with KVVRFDKL even without addition of SIINFEKL. This indicates that 25-D1.16 also recognizes KVVRFDKL-Kb complexes, probably due to the similar carboxyl-terminal sequences of KVVRFDKL and SIINFEKL (FDKL vs FEKL). Even against the background with KVVR FDKL, addition of SIINFEKL produced an increase in 25-D1.16 staining consistent with a level of SIINFEKL-Kb complexes similar to that achieved after incubation with other stabilizing peptides. In conclusion, flow cytometry quantitatively demonstrated that prior incubation with stabilizing peptide caused substantial increases in the generation of SIINFEKL-Kb complexes.

**Acidic pH increases subsequent binding and presentation of readout peptide**

Stabilizing peptide caused a greater increase in presentation of SIINFEKL derived from alternate MHC-I processing of HB101.Crl-OVA than presentation of exogenous SIINFEKL peptide (Fig. 3). This suggested the hypothesis that peptide dissociation/exchange is promoted more by the acidic environment of phagosomes and phagolysosomes than the neutral pH at the cell surface. To test the role of pH in controlling peptide dissociation/exchange, freshly isolated C57BL/6 macrophages were exposed to buffers of varying pH for 20 min, neutralized, incubated with SIINFEKL for 2 h in the presence of brefeldin A, and then fixed. The resulting level of SIINFEKL-Kb complexes was assessed using CD8OVA1.3 T hybridoma cells. Exposure of macrophages to acidic conditions (pH 5.0–6.5, similar to the pH of endocytic or phagocytic compartments) substantially increased subsequent binding and presentation of SIINFEKL peptide (Fig. 5). Macrophages exposed to pH 4.5 exhibited markedly decreased subsequent binding and presentation of SIINFEKL, presumably due to cell injury at this very acidic pH. Because C57BL/6 macrophages should be replete with diverse endogenous peptide-Kb complexes, these data suggest that acidic pH enhances dissociation of previously associated peptides (endogenous stabilizing peptides), thereby increasing the number of peptide-receptive MHC-I molecules.

To further test the roles of both acidic pH and stabilizing peptide on availability of peptide-receptive MHC-I molecules, C57BL/6 and TAP1−/− macrophages were incubated overnight at 26°C with FAPGNYPAL stabilizing peptide, subsequently exposed to acidic pH, and then incubated with SIINFEKL readout peptide. SIINFEKL-Kb complexes were detected with CD8OVA1.3 T hybridoma cells. With C57BL/6 macrophages, addition of FAPGNYPAL stabilizing peptide had little effect on production of SIINFEKL-Kb complexes, presumably because these TAP-replete cells have a sufficient supply of endogenous stabilizing peptides. Incubation of C57BL/6 macrophages at acidic pH markedly enhanced subsequent production of SIINFEKL-Kb complexes (Fig. 6A, consistent with Fig. 5), suggesting that dissociation of endogenous stabilizing peptides was enhanced at acidic pH. With TAP1−/− macrophages, both FAPGNYPAL stabilizing peptide and incubation at acidic pH promoted formation of SIINFEKL-Kb complexes, and a combination of FAPGNYPAL stabilizing peptide and incubation at acidic pH provided the highest presentation of SIINFEKL-Kb complexes.
To assess the relationship between the stability of stabilizing peptide-MHC-I complexes and the role of pH in promoting peptide dissociation/exchange, a similar experiment was performed with a switch in the stabilizing and readout peptides. TAPI$^{-/-}$ macrophages were incubated overnight at 26°C with SIINFEKL stabilizing peptide, subsequently exposed to acidic pH, and then incubated with FAPGNYPAL readout peptide. FAPGNYPAL-Kb complexes were detected with SVB1.B6 T hybridoma cells. Acidic pH markedly enhanced production of FAPGNYPAL-Kb complexes (Fig. 7). Incubation of TAPI$^{-/-}$ macrophages with SIINFEKL stabilizing peptide reduced subsequent production of FAPGNYPAL-Kb complexes (consistent with Fig. 1), and this effect was seen whether or not an acidic incubation was included. Following incubation with SIINFEKL stabilizing peptide, production of FAPGNYPAL-Kb complexes was increased by acidic pH but was still lower than that observed in the absence of SIINFEKL stabilizing peptide (with or without incubation at acidic pH), and it is not clear that it resulted from dissociation of SIINFEKL-Kb complexes (as opposed to other peptide-Kb complexes). These results suggest that some peptides (e.g., SIINFEKL) may generate peptide-MHC-I complexes that are too stable to contribute substantially to the pool of precursors for peptide-receptive MHC-I molecules, even if these complexes traffic through acidic processing compartments. Nonetheless, many stabilizing peptides of reasonably high affinity (e.g., FAPGNYPAL or RGYYVYQGL) generate complexes that do contribute to the pool of precursors for peptide-receptive MHC-I molecules, especially when these complexes are exposed to acidic pH.

**Discussion**

The results of this study establish that peptide dissociation/exchange contributes significantly to vacuolar alternate MHC-I Ag processing. Some previous models do not include a role for MHC-I peptide dissociation/exchange and indicate that cell surface or post-Golgi peptide-receptive MHC-I molecules are empty, having escaped from the ER without binding a peptide. These models predict that incubation of cells with stabilizing peptide should reduce both the number of empty peptide-receptive MHC-I molecules and subsequent binding of readout peptide by MHC-I. In contrast, our results demonstrate that binding of stabilizing peptides can substantially enhance subsequent binding and presentation of exogenous readout peptide or peptide derived from exogenous Ag via alternate MHC-I Ag processing, implying a process of peptide dissociation/exchange. We propose that the role of stabilizing peptide is to bind MHC-I molecules in a reversible manner (depending on peptide affinity), increase the egress of MHC-I molecules from the ER (in the case of endogenous stabilizing peptides), increase the half-life of MHC-I molecules in post-Golgi compartments, and thereby increase the supply of precursors to peptide-receptive MHC-I molecules. Dissociation of stabilizing peptide-MHC-I complexes may then allow the formation of readout peptide-MHC-I complexes.

Two independent assay systems were used to establish this point. A T hybridoma assay was used to detect presentation of the SIINFEKL and FAPGNYPAL readout peptides. The advantages of this assay include the availability of T hybridomas with different antigenic specificities and its sensitivity to physiological levels of peptide-MHC-I complexes that are generated by alternate MHC-I Ag processing. Disadvantages of the T cell assay include the difficulty of directly quantifying peptide-MHC-I complexes and the possibility that variations in the numerous aspects of T cell activation may complicate the experiment. To complement the T hybridoma assay, SIINFEKL-Kb complexes were also detected by flow cytometry and staining with the 25-D1.16 mAb (38). The advantages of this approach include a more direct and quantitative detection of peptide-MHC-I complexes, but its disadvantages include availability of only a single Ab specificity for this study, a much lower sensitivity relative to T hybridoma assays, and a consequent inability of flow cytometry to detect the levels of peptide-MHC-I complexes generated during alternate MHC-I Ag processing (14). Thus, data presented here for experiments involving processing of exogenous Ag (e.g., HB101.Crl-OVA) exclusively reflect T hybridoma assays, whereas both T hybridoma and flow cytometry assays were used in experiments involving the presentation of exogenous peptide. Protocols for these two types of experiments provided very different concentrations of exogenous Ag, but the trends were consistent with those obtained using the T hybridoma assay.

**FIGURE 6.** Stabilizing peptide and exposure to acidic pH can increase subsequent binding and presentation of readout peptide. C57BL/6 or TAPI$^{-/-}$ macrophages were incubated overnight at 26°C with or without 5 μM FAPGNYPAL stabilizing peptide and then processed as in Fig. 5. Results are expressed as means of quadruplicate wells ± SD.

**FIGURE 7.** Production of FAPGNYPAL-Kb complexes is increased by prior exposure to acidic pH but decreased by prior exposure to SIINFEKL stabilizing peptide. Macrophages were processed as described in Fig. 6 except the stabilizing peptide was SIINFEKL (5 μM), the readout peptide was FAPGNYPAL and Kb-FAPGNYPAL complexes were detected with SVB1.B6 T hybridoma cells. Results are expressed as means of quadruplicate wells ± SD. Where error bars are not visible, they are smaller than the symbol width.
peptides, and the addition of exogenous stabilizing peptide did not constitutively express higher levels of peptide-receptive MHC-I molecules. The presence of exogenous peptide by MHC-I without prior addition of stabilizing peptide (as shown in this study). Addition of exogenous peptide or $\beta_2$-microglobulin promotes stability of MHC-I at the cell surface, but these agents or MHC-I molecules from the cell surface can also be internalized into post-Golgi vacuolar compartments to participate in alternate MHC-I Ag processing. We propose that the role of TAP in vacuolar alternate MHC-I Ag processing is not to transport readout peptides processed from exogenous Ag, but rather to deliver stabilizing peptides to MHC-I molecules in the ER, promoting the egress of effective precursors to peptide-receptive MHC-I molecules to post-Golgi compartments.

The mechanism of peptide dissociation/exchange remains uncertain. Stabilizing peptides may dissociate to generate truly empty MHC-I molecules that undergo separate interactions to bind readout peptides. Empty MHC-I molecules would be relatively unstable, but their continuous production from endogenous stabilizing peptide-MHC-I complexes could provide a continuous source of peptide-receptive MHC-I for alternate MHC-I Ag-processing mechanisms. Stabilizing peptides would increase the lifetime of precursors to empty MHC-I molecules, which might sequentially bind several low affinity stabilizing peptides before binding a high affinity peptide to create a stable peptide-MHC-I complex (a process akin to the “peptide editing” proposed for class II MHC (MHC-II) molecules). Alternatively, binding of readout peptide may be mechanistically linked to dissociation of a stabilizing peptide, allowing stabilizing peptide to occupy and stabilize MHC-I molecules up to the moment of readout peptide binding.

Peptide dissociation/exchange depends on the dissociation rate of stabilizing peptide-MHC-I complexes. Stabilizing peptides with very high affinity may create stable peptide-MHC-I complexes with such low dissociation rates that they do not contribute to peptide exchange. Increasing the loading of MHC-I with one such stabilizing peptide (SIINFEKL) decreased subsequent binding and presentation of readout peptide (Figs. 1 and 7). Other studies have also indicated that peptide exchange is influenced by the stability of preexisting peptide-MHC-I complexes (47). The exact half-life of endogenous stabilizing peptide-MHC-I complexes that normally serve as precursors to peptide-receptive MHC-I molecules is not clear. Vacular alternate MHC-I Ag processing is maintained in the presence of brefeldin A (2, 5, 22), implicating a post-Golgi pool of stabilizing peptide-MHC-I complexes that are precursors to peptide-receptive MHC-I molecules. However, long incubations with brefeldin A inhibit alternate MHC-I Ag processing (5), suggesting that the subset of stabilizing peptide-MHC-I complexes that contribute to peptide dissociation/exchange eventually decays with a half-life shorter than that of high stability peptide-MHC-I complexes.

The enhancement of readout peptide presentation by stabilizing peptide was greater for presentation of SIINFEKL derived from HB101.Crl-OVA, which is processed in acidic phagolysosomal compartments, than for presentation of exogenous SIINFEKL, which can bind to K$\alpha$ at the cell surface at neutral pH. This suggested the hypothesis that peptide exchange is promoted by the acidic pH. Brief exposure of C57BL/6 or TAP$^{-/-}$ macrophages to acidic pH increased subsequent binding of readout peptide to surface MHC-I molecules, supporting this hypothesis. Other studies have also indicated that optimum peptide binding or exchange is obtained under acidic conditions (58–60). For example, Stryhn et al. reported that the dissociation of peptide-MHC-I complexes produced peptide-receptive MHC-I molecules that bound exogenous peptide (45, 46). Several studies from Hansen and colleagues have indicated that L$\beta$ can undergo extensive peptide exchange (42, 47–49). Empty MHC-I molecules (50) or free MHC-I H chains (51–55) have been detected on the cell surface, where they may arise by peptide dissociation, and some studies have demonstrated that these molecules reassemble with peptide and $\beta_2$-microglobulin to form peptide-MHC-I complexes. Thus, several populations of potentially peptide-receptive MHC-I molecules may arise via peptide dissociation/exchange.

Our model is consistent with studies that indicate a requirement for peptide loading to promote efficient egress of MHC-I molecules from the ER and other studies that demonstrate the existence of MHC-I peptide exchange in post-Golgi compartments (including the cell surface). MHC-I molecules are retained in the ER by a calnexin- and tapasin-dependent mechanism until they bind a peptide (25–29). Even the thermolabile MHC-I molecules expressed on the surface of TAP-deficient cells (previously considered empty) are actually loaded with peptides, most of which are presumably of relatively low affinity (24). Following exit from the ER, some peptide-MHC-I complexes may dissociate to participate in peptide exchange or exist as short-lived empty MHC-I molecules (MHC-I H chain plus $\beta_2$-microglobulin or free MHC-I H chains). Ojcius et al. reported that the dissociation of peptide-MHC-I complexes produced peptide-receptive MHC-I molecules that bound exogenous peptide (45, 46). Several studies from Hansen and colleagues have indicated that L$\beta$ can undergo extensive peptide exchange (42, 47–49). Empty MHC-I molecules (50) or free MHC-I H chains (51–55) have been detected on the cell surface, where they may arise by peptide dissociation, and some studies have demonstrated that these molecules reassemble with peptide and $\beta_2$-microglobulin to form peptide-MHC-I complexes. Thus, several populations of potentially peptide-receptive MHC-I molecules may arise via peptide dissociation/exchange.

The deficit of endogenous stabilizing peptide and consequently decreased levels of peptide-receptive MHC-I molecules explains the decreased efficiency of vacuolar alternate MHC-I Ag processing in TAP-deficient macrophages. The decrease in alternate MHC-I Ag processing associated with TAP deficit is reversed by conditions that stabilize poorly loaded MHC-I molecules, such as incubation at 26°C (5, 14) with $\beta_2$-microglobulin (5) or with stabilizing peptide (as shown in this study). Addition of exogenous peptide or $\beta_2$-microglobulin promotes stability of MHC-I at the cell surface, but these agents or MHC-I molecules from the cell surface can also be internalized into post-Golgi vacuolar compartments to participate in alternate MHC-I Ag processing. We propose that the role of TAP in vacuolar alternate MHC-I Ag processing is not to transport readout peptides processed from exogenous Ag, but rather to deliver stabilizing peptides to MHC-I molecules in the ER, promoting the egress of effective precursors to peptide-receptive MHC-I molecules to post-Golgi compartments.

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et al. (58) suggested that acid-dependent dissociation of peptide-MHC-I complexes results in peptide exchange that may contribute to the presentation of exogenous Ags by MHC-I molecules, although their studies also indicated that the effect of pH and the effective range of pH varied for different peptide-MHC-I combinations and with the presence of exogenous β2-microglobulin (these factors may explain some variation in the pH optima observed in different studies). Overall, these observations suggest that phagolysosomes or other acidified intracellular vacuolar compartments are sites where peptides derived from exogenous Ag bind to MHC-I molecules during alternate MHC-I Ag processing.

There are interesting parallels between the vacuolar alternate MHC-I and conventional MHC-II Ag processing pathways. Both MHC-I and MHC-II molecules require chaperones for stabilization before peptide binding and interaction with other proteins to promote proper peptide loading (e.g., tapasin and calnexin for MHC-I and invariant chain and HLA-DM for MHC-II). MHC-I and MHC-II molecules are unstable without bound peptide, and both are occupied by stabilizing peptides before binding of readout peptides (MHC-II uses invariant chain and invariant chain-derived peptides, whereas MHC-I uses stabilizing peptides as described in this study). Furthermore, two recent studies of MHC-II molecules (61, 62) illustrate some of the same principles that we have observed with MHC-I molecules. Elegant peptide binding studies demonstrated that binding of low affinity peptides to MHC-II maintains or promotes a MHC-II conformation that is “active” (61) or “peptide friendly” (62), i.e., peptide receptive. These studies indicate that the rate of peptide binding to empty MHC-II is much slower than peptide binding to MHC-II molecules that have previously bound a low affinity variant peptide, implying that stabilizing peptides promote a peptide-receptive MHC conformation. This parallels our observation that stabilizing peptide can promote the subsequent binding of readout peptide to MHC-I molecules. Furthermore, the studies of MHC-II showed that the enhancement of readout peptide binding was linked to the rate of stabilizing peptide dissociation, similar to our observation that highly stable complexes of stabilizing peptide with MHC-I did not enhance (or even decreased) subsequent readout peptide binding. Thus, both MHC-I and MHC-II molecules use stabilizing peptides to maintain a peptide-receptive state and enhance their subsequent ability to bind readout peptides. As peptide exchange occurs, both MHC-I and MHC-II molecules may undergo peptide editing until a very high affinity peptide generates a highly stable complex for presentation at the cell surface.

We conclude that peptide dissociation/exchange plays an important role in the genesis of peptide-receptive MHC-I molecules used for alternate MHC-I Ag processing. Endogenous stabilizing peptides may be transported by TAP to promote assembly of stabilizing peptide-MHC-I complexes in the ER, their exit to post-Golgi compartments, and their persistence as precursors to peptide-receptive MHC-I molecules. Alternate MHC-I Ag processing, then, involves the dissociation of stabilizing peptide and its replacement by readout peptide derived from exogenous Ag. This process is optimized at acidic pH, similar to conditions in post-Golgi vacuolar compartments that process exogenous Ags (e.g., phagolysosomes), suggesting that alternate MHC-I Ag processing involves binding of peptides derived from exogenous Ags to MHC-I molecules in these compartments.

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References


CORRECTIONS


An error was made in describing the protocol used for experiments shown in Figs. 5, 6, and 7. Text in the Materials and Methods and Results sections, along with figure legends for Figs. 5, 6, and 7 implied that antigenic readout peptide was only added subsequent to the incubation of macrophages in citrate-buffered saline at varying pH, but peptide was actually added at the indicated concentrations both during the incubation in citrate-buffered saline at varying pH and in the subsequent incubation for 2 h in normal medium. This correction does not change the conclusion of the paper, but is important for executing future studies.


In Fig. 1A, the last lane in the lower right panel was misplaced. It should have appeared as the first lane in the left side of the lower panel. The corrected figure is shown below.