Extensive peptide ligand exchange by surface class I major histocompatibility complex molecules independent of exogenous β2-microglobulin

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ABSTRACT Certain class I major histocompatibility complex molecules expressed on live cells have been shown to bind exogenous peptide ligands. However, it remains controversial whether this binding occurs by peptide exchange or to empty surface class I molecules. In this report we compare the surface binding and dissociation of two virus-derived ligands of the Ld class I molecule of the mouse. The peptide ligands were previously identified in immune responses to cytomegalovirus or lymphochoriomeningitis virus as immunodominant, optimally sized, and Ld restricted. Ligand dissociation was monitored on live cells indirectly by measuring the surface turnover of Ld–peptide complexes or directly by using labeled peptides. The cytomegalovirus-derived and lymphochoriomeningitis virus-derived peptides appeared to dissociate relatively rapidly; however, the cytomegalovirus-derived peptide had a more rapid off-rate than the lymphochoriomeningitis-derived peptide. Furthermore, these rates of dissociation appear to span that seen with endogenous Ld-associated peptides expressed by cells at 37°C. Exploiting the extraordinary accessibility of the surface Ld ligand binding site we developed an assay to quantitate peptide ligand exchange. Cells were precoated with saturating amounts of unlabeled peptide by overnight incubation and were then tested for secondary binding of labeled peptides in a 4-h assay. Our results unequivocally demonstrate the potential for surface class I molecules to undergo peptide exchange. Furthermore, peptide exchange was found to be largely independent of exogenous β2-microglobulin. This result implies that β2-microglobulin association and not β2-microglobulin exchange is the critical factor in peptide exchange by surface class I molecules. Because of the exquisite ability of T cells to discriminate different amounts of ligand bound to class I, the binding of exogenous peptides could play a critical role in normal or aberrant immune responses.

Class I major histocompatibility complex (MHC) molecules are membrane-bound, cell surface glycoproteins of Mr 45,000. These class I heavy chains associate with a non-MHC-encoded, non-membrane-bound light chain, β2-microglobulin (β2m). Heterodimers of class I with β2m bind immunogenic peptides and present them to cytotoxic T lymphocytes (CTLs). Presentation of peptides targets virus-infected cells for immune destruction by host CTLs. From studies using the drug brefeldin A (Bfa), it was shown that viral-derived peptides bind class I molecules in a pre-Golgi compartment, presumably the endoplasmic reticulum (1, 2). Several recent reports have suggested that once bound, peptides remain almost irreversibly associated with class I molecules. This notion was supported by the observations that peptides remained bound to the HLA class I molecules throughout their purification for crystallographic analysis (3). Furthermore, exogenous peptides were found to bind poorly to purified class I molecules, suggesting most remained peptide occupied (4). In contrast to these findings, there have been several recent reports of significant levels of ligand binding using live cells cultured with labeled peptides (5–9). These assays have employed cells with peptide-transport deficiencies (e.g., RMA-S) as well as nonselected cell types. Mechanistic analyses suggest that most of the binding of peptides by live cells occurs to surface class I molecules (10). However, it is unclear whether this binding involves empty or peptide-occupied class I molecules. The most extensive evidence for expression of empty class I molecules resulted from studies of RMA-S cells grown at 26°C (11). Class I molecules expressed at 26°C by RMA-S were found to be labile at 37°C unless provided exogenous peptide. This finding implied that peptide occupancy influences cell surface turnover of class I molecules. Extrapolating from these findings, recent studies of unselected cells grown at 37°C have speculated that peptide binding occurs to empty B27 (5) and Kd (8) class I molecules. However, this contention remains controversial and other mechanisms such as peptide exchange have not been adequately addressed. An additional mechanistic dilemma concerning the binding of peptide to surface class I is the role of exogenous β2m. Several reports using mouse class I molecules have suggested that β2m exchange plays an important role in ligand binding (12–14). If so, then peptide binding to mouse class I molecules could result from its propensity to exchange endogenous β2m for heterologous β2m in the culture medium. However, significant levels of ligand binding have been observed to mouse or human class I molecules on cells grown without serum (5, 6, 9, 10). Thus it remains unclear whether β2m exchange influences peptide ligand binding to surface class I molecules.

To probe the mechanism and functional significance of ligand binding to surface class I molecules we have studied the Ld molecule of the mouse. Exploiting the extraordinary ability of surface Ld molecules to be loaded with exogenous peptide, an assay to quantitate peptide exchange was developed. Furthermore, the propensity of Ld to dissociate self β2m for heterologous β2m allowed us to critically test the role of β2m exchange in peptide binding to surface class I molecules.

MATERIALS AND METHODS

Cell Lines. L-Ld cells were generated by introducing the Ld gene into murine Ltk–DAP-3 (H-2b) fibroblast cells (15). These cells were maintained at 37°C, 6.5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 10% fetal calf serum (HyClone), 2 mM L-glutamine, and 100 units of penicillin/streptomycin per ml (DMEM/fetal calf serum). In certain experiments, L-Ld cells were grown in serum-free

Abbreviations: Bfa, brefeldin A; CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; β2m, β2-microglobulin; hβ2m, human β2m; LCMV, lymphochoriomeningitis virus; MHC, major histocompatibility complex; SF, serum-free; mAb, monoclonal antibody.

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(SF) DMEM supplemented with 1% Nutridoma SP (Boehringer Mannheim) with or without purified human β2m (β2m; Calbiochem). In other assays cells were treated with 5 μg of Bfa per ml (Epigence Technologies, Madison, WI) to block new surface expression of class I molecules. In data not shown, this concentration of Bfa was found to be sufficient to completely block sialetion of Ld molecules.

**Monoclonal Antibodies (mAbs).** For detection of conformed Ld molecules, mAb 30-5-7 (a2 domain) was used (16). For detection of nonconformed Ld, molecules, mAb 64-3-7 (a1 domain) was used (17). Ld molecules have been shown to be non-ligand-associated Ld heavy chains (7). For detection of Kk molecules, mAb 11-4-1 was used (18). All three mAbs are of the IgG2 isotype.

**Flow Cytometry.** Flow cytometry was performed as described (7). Briefly, cells were incubated with a saturating concentration of mAb, washed, and incubated with a saturating concentration of fluorescein-conjugated F(ab')2, goat anti-mouse IgG, Fc specific (Cooper Biomedical). Labeled cells were analyzed using a FACScan (Becton Dickinson). Mean fluorescence values were converted from logarithmic amplification by linear regression analysis using the CONSORT 30 software.

**Peptide Synthesis and Labeling.** Peptides were synthesized using Merrifield’s solid-phase method (19) on a peptide synthesizer (model 431A; Applied Biosystems). Peptides were purified (>90%) by reverse-phase HPLC and subjected to purity assessment techniques as described (20). Peptides were iodinated using the Iodo-Beads (Pierce) method. Briefly, Iodo-Beads were washed twice with iodination buffer (100 mM sodium phosphate, pH 7.4). Two Iodo-Beads were mixed with 1–5 mCi of Na[125]I (New England Nuclear; 1 Ci = 37 GBq) in 200 μl of iodination buffer at room temperature for 5 min. A solution of 300 μl of peptide in iodination buffer was added to the Iodo-Beads reaction mixture. The iodination reaction was allowed to proceed for 15 min at room temperature and terminated by removing the reaction mixture from the Iodo-Beads. The radiolabeled peptide was recovered by transferring the reaction mixture to a 1-ml packed AG-1 × 8 ion-exchange resin column (Bio-Rad) to bind the free [125]I. After centrifugation (2500 × g, 5 min) the radiolabeled peptide-containing fluid passed through the column was collected and stored at 4°C before use. Peptides were labeled with specific activities between 0.2 and 1 × 10^16 cpm/mol.

**Peptide Binding Assay.** For measurements of peptide binding to live cells, 5 × 10^5 Ld cells were incubated for 4 h with 4.5–6 μM [125]I-labeled peptide. After incubation cells were washed four times with phosphate-buffered saline and lysed in 0.5% Nonidet P-40 with 0.2 mM phenylmethylsulfonyl fluoride. Class I–peptide complexes were precipitated by incubation with ascites fluid containing mAb for 30 min on ice. Precipitates were isolated using IgG sorb (The Enzyme Center, Malden, MA) and were assayed on a γ counter to quantify the amount of bound peptide.

**RESULTS AND DISCUSSION**

Previous studies have shown that a high percentage of surface Ld molecules have readily accessible ligand binding sites. Initially, this was shown by the observation that cells cultured with known Ld ligands dramatically increased their surface Ld expression (21). This induction was accounted for by the fact that the half-life of surface Ld was increased from 2 h to 6 h by culture with relevant peptides (10). These observations with Ld were in complete accordance with earlier studies of class I expression by RMA-S cells. An additional feature of RMA-S cells is that their surface class I expression is increased by culture at 25°C, and these cold-induced class I molecules are thermal labile at 37°C (11). To determine whether this property also extended to Ld, L-Ld cells were grown overnight at 25°C or 37°C. As shown in Fig. 1, culture at 25°C resulted in a 3-fold increase in Ld expression. To compare the thermal lability of Ld molecules expressed at 25°C versus 37°C, both groups of cells were cultured at 37°C and treated with Bfa to prevent new expression of class I molecules. Surface turnover of Ld was monitored by cytofluorometry at 0, 2, 4, and 6 h. As shown in Fig. 1A, Ld molecules induced at 25°C were extremely labile, as indicated by their decrease in surface expression to one-fourth the initial level by 2 h. These cold-induced Ld molecules were found to have a t1/2 of t~45 min when switched to 37°C (Fig. 1A Inset). In contrast to the initial precipitous loss of 25°C-induced Ld, the decay at later time points was comparable on cells previously cultured at either 25°C or 37°C (t1/2 t~2 h). For comparison, the endogenous Ld molecule Kk was also included in the above analysis. As shown in Fig. 1A, 25°C incubation resulted in a 50% increase in Kk expression and these induced molecules were also found to be thermal labile with a half-life significantly shorter than Kk molecules expressed at 37°C. Thus induction of labile class I molecules by reduced temperature extends to functional class I molecules expressed by nonelected cell types. The more pronounced effect of 25°C on Ld compared to other class I molecules represents another example of how Ld expression is strikingly similar to class I expression by RMA-S cells (11). Also similar to studies of RMA-S (11), exogenous peptide was found to stabilize the Ld molecules induced at 25°C (W.-R.L. and T.H.H., data not shown). These findings imply that the lability of cold-induced class I molecules results from the lack of, or a suboptimal, peptide ligand. In any case, the disparity between the turnover of Ld

![Fig. 1](image-url)
molecules expressed at 25°C versus 37°C implies that peptide occupancy of L^d is better at the physiological temperature. The aforementioned studies of RMA-S and L^d suggest that peptide occupancy prolongs cell surface turnover. To compare the ability of different ligands to stabilize L^d expression, L-L^d cells were cultured overnight at 37°C with two different viral-derived peptides, CMV-9mer or LCMV-9mer. In a fluorometric analysis the CMV-9mer and LCMV-9mer caused a 6-fold induction implying >83% of surface L^d molecules contained the fed peptide. To quantitate the duration of this induction, peptide-fed cells were washed to remove unbound peptide and were treated with Bfa. Cell surface turnover of L^d was then monitored by fluorometry at 0, 2, and 4 h. To directly compare L^d surface turnover rates between samples, the expression is presented in Fig. 1B as a percentage of the initial L^d molecules at time zero (coincident with Bfa addition). As a control, the turnover of L^d molecules on non-peptide-fed L-L^d cells was measured. This control should reflect the stability of L^d occupied by endogenous peptides. L^d molecules on cells fed the LCMV-9mer were significantly more stable than L^d on cells fed the CMV-9mer. For example, 56% of L^d remained on cells fed the LCMV-9mer after 4 h as compared to 17% for cells fed the CMV-9mer. This disparity presumably reflects differences in the respective dissociation of these peptides. The LCMV peptide was also found to have a much higher affinity than the CMV peptide, as determined in a lysate assay measuring de novo L^d folding (J.D.S. and T.H.H., data not shown). Interestingly, the turnover of L^d on non-fed L-L^d cells was found to be intermediate between the turnover of L^d on the cells fed viral peptides. This result suggests that these immunogenic viral peptides display affinities that span the spectrum of self endogenous peptides.

Another implication of the data presented above is that L^d turnover on cells pulsed with peptide (Fig. 1B) is significantly shorter than that previously observed on cells treated with continuous peptide (10). The t_1/2 of L^d molecules cultured in the continual presence of CMV peptide is ~6 h (10), whereas the t_1/2 of L^d from cells cultured with CMV peptide and washed is only 2 h (Fig. 1B). This difference implies that a single L^d molecule can rebind the fed peptide when available continuously in the culture medium. To directly test the hypothesis that peptide exchange occurs at the cell surface, cells were precoated with unlabeled peptides and then assayed for binding of labeled peptides. This is not a conventional competitive peptide binding experiment since the unlabeled and labeled peptides are added sequentially and not simultaneously. In an initial experiment (Fig. 2A) L-L^d cells were preincubated overnight at 37°C in the presence of saturating concentrations of unlabeled CMV-9mer, CMV-19mer, or LCMV-9mer or with no peptide as a control. Cells were then washed and aliquots of each sample were assayed for L^d expression by fluorometry. As shown in the fluorometric histograms in Fig. 2A, the cells fed the CMV-9mer or LCMV-9mer each showed a 5.8-fold induction of L^d, whereas the cells fed the CMV-19mer showed a 3-fold induction. In a parallel experiment, cells from each group were incubated with ^125I-CMV-9mer for 4 h at 37°C in the presence of Bfa. Lysates of cells were then immunoprecipitated with either mAb 30-5-7 to detect the amount of ligand bound to L^d or mAb 64-3-7 as a negative control. mAb 64-3-7 detects a non-peptide-associated L^d heavy chain (7). As shown in the upper portion of Fig. 2A, precoating led to a striking enhancement in the amount of ^125I-CMV binding during the 4-h assay. A comparison between the untreated control, CMV-19mer-precoated, and CMV-9mer-precoated

![Fig. 2. Precocating of cells with unlabeled peptide enhances subsequent binding of ^125I-labeled CMV-9mer (^125I-CMV-9mer) to L^d. (A) L-L^d cells were cultured overnight at 37°C in the presence of 250 μM CMV-9mer, CMV-19mer, or LCMV-9mer or with no peptide as indicated. The following morning cells were washed and L^d expression was measured on aliquots of each group of cells as indicated in the fluorescence-activated cell sorting histogram. In a parallel experiment 5 x 10^6 L-L^d cells from each group were treated with Bfa and assayed for binding of ^125I-CMV-9mer (4.5 μM) in a 4-h assay at 37°C. The amount of peptide specifically bound to L^d was assessed by precipitation using mAb 30-5-7, which detects a non-ligand-associated L^d heavy chain designated L^d-alt. Bkgd, background. (B) The protocol for this experiment was as in A except cells were precocated overnight with the indicated concentration of CMV-9mer or negative control NP-9mer peptide. Precoated cells were treated with Bfa and assayed for L^d expression (bottom) or ^125I-CMV-9mer binding (top). For the binding assay in this experiment, cells were incubated with mAb prior to lysis to ensure that only surface L^d molecules were detected. For details, see ref. 10.]
cells indicated a precise correlation between L<sup>d</sup> surface expression (Fig. 2A bottom) and the amount of labeled CMV-9mer bound (top). In contrast, the CMV-9mer and LCMV-9mer induced L<sup>d</sup> expression equally but did not result in an equivalent enhancement of labeled CMV peptide binding. This striking difference could be attributed to the aforementioned slower dissociation kinetics of the LCMV-9mer compared to the CMV-9mer (Fig. 1B). To confirm that the off-rate of the coated peptide is the critical factor determining the amount of peptide precoate enhancement, the same protocol as in Fig. 2A was employed substituting 125<sup>I</sup>-labeled LCMV (125<sup>I</sup>-LCMV) in the binding assay. Precoating cells with CMV enhanced secondary binding of 125<sup>I</sup>-LCMV 4.5-fold, whereas precoating with LCMV only enhanced binding 1.5-fold (J.D.S., W.-R.L., and T.H.H., data not shown). As before, this differential secondary binding of 125<sup>I</sup>-LCMV occurred in spite of the fact that the CMV- and LCMV-precoated peptides induced equal surface labeling (125<sup>I</sup>-LCMV; 125<sup>I</sup>-CMV) occurred in 1 ml of medium containing 100 μM 125<sup>I</sup>-CMV-9mer. Cells were washed, equally divided, and resuspended in 400 μl of medium supplemented with no peptide, 1 x 10<sup>-4</sup> M unlabeled CMV, 5 x 10<sup>-4</sup> M unlabeled CMV, 1 x 10<sup>-3</sup> unlabeled CMV, or 1 x 10<sup>-3</sup> unlabeled influenza nucleoprotein binding. These cells were incubated for 4 h at 37°C. Specific versus nonspecific binding to L<sup>d</sup> was monitored by immunoprecipitation using mAbs 30-5-7 and 64-3-7, respectively. In the experiment shown these respective mAbs were added after cell lysis. However, identical results were obtained by coating cells with mAb prior to lysis so as to only detect surface L<sup>d</sup> molecules. (Inset) Dissociation rate of 125<sup>I</sup>-LCMV-9mer from L<sup>d</sup>-cells incubated with 100 μM iodinated peptide, washed, resuspended, and cultured in the presence (c) or absence (e) of 1 mM unlabeled CMV inhibitor. To allow for direct comparison to the above experiments, these measurements were made in medium supplemented with 10% fetal calf serum. Peptide association with surface L<sup>d</sup> was monitored by immunoprecipitation at 0, 1, 2, and 4 h.

Tsomides et al. (22) reported a 3-fold difference of peptide dissociation from purified A2 molecules incubated in the presence or absence of excess unlabeled competing peptide. The difference in L<sup>d</sup>-peptide dissociation rates observed in the presence or absence of unlabeled inhibitor peptide can be explained by either of two models: (i) dissociated labeled peptide competes for rebinding with unlabeled peptide or, alternatively, (ii) competing unlabeled peptide may facilitate the dissociation of bound peptide. Indeed, the latter mechanism has been proposed in studies of peptide binding to class II MHC molecules (23). Regardless of the mechanism, the experiments shown in Fig. 3 establish that surface L<sup>d</sup> molecules exchange peptide ligand in an unexpectedly rapid, but specific, manner.

The ability to monitor peptide exchange using the above protocol allowed us to critically define the role of exogenous β<sub>m</sub> in surface class I ligand binding. To determine whether β<sub>m</sub> exchange facilitates peptide exchange, cells were initially grown under conditions to maximize β<sub>m</sub> association. It is necessary to maximize β<sub>m</sub> association, since our recent analyses of β<sub>m</sub> and β<sub>d</sub> chains indicates that β<sub>d</sub> chains preferentially bind exogenous ligand (10). Because L<sup>d</sup> molecules have a lower affinity for mouse β<sub>m</sub> compared to hβ<sub>m</sub>, L<sup>d</sup>-L<sup>d</sup> cells previously grown in SF medium were cultured overnight in the presence of purified hβ<sub>m</sub> with or without CMV-9mer (precoat, Fig. 4). The following day cells were washed and incubated with Bfa and 125<sup>I</sup>-CMV for 4 h at 37°C. The peptide binding assay was performed in the presence or absence of hβ<sub>m</sub> to assess the contribution of exogenous β<sub>m</sub> on peptide–ligand exchange. Specific ligand binding was assayed by immunoprecipitation on cells precoated with mAb prior to lysis. As shown in Fig. 4, cells incubated with CMV peptide plus hβ<sub>m</sub> showed a significant enhancement of secondary ligand binding in comparison with cells precoated with hβ<sub>m</sub> alone. As before, this enhancement correlated with increased surface L<sup>d</sup> expression (fluorescence-
molecules. Thus, although there may be allele-specific quantitative differences, we feel that peptide exchange is potentially important for all surface class I molecules. The basis for this assumption is that CTLs possess a remarkable ability to discriminate quantitative differences in the amount of class I ligand on the surface of antigen-presenting cells (27). Furthermore, higher levels of determinant density are required to initiate, as opposed to propagate, an immune response (27). Cell lysis resulting from various physiological phenomena such as virus or parasitic infection could release relatively high local concentrations of potential ligand. These peptides could then bind surface class I molecules, increase the determinant density, and thus initiate an aberrant immune response or influence an ongoing immune response.

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