Disparate Interaction of Peptide Ligand with Nascent Versus Mature Class I Major Histocompatibility Complex Molecules: Comparisons of Peptide Binding to Alternative Forms of L^d in Cell Lysates and the Cell Surface

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Summary

To determine the mechanism and structural consequences of peptide binding to class I molecules, we have studied the L^d molecule of the mouse. Previous studies have shown that a significant proportion of surface and intracellular L^d molecules can be detected in an alternative conformation designated L^dalt. L^dalt molecules are non-ligand associated and show weak if any β_2-microglobulin (β_2m) association. We report here that L^d molecules have a relatively rapid surface turnover compared with other class I molecules and that exogenous peptide dramatically prolongs L^d surface half-life. By contrast, L^dalt molecules are stably expressed on the surface and their half-life is unaffected by exogenous peptide. To study the surface interaction of peptide with L^d, live cells were incubated with iodinated peptides and L^d molecules were precipitated from cells precoated with monoclonal antibody before lysis. Using this assay, peptide binding to surface L^d molecules was found not to depend upon exchange with exogenous β_2m, but did correlate with the level of β_2m association. To study the intracellular interaction of peptide with L^d, cell lysates were used. In cell lysates, peptide was found to convert L^dalt molecules to properly folded L^d. This peptide-induced folding was almost complete at earlier but not later time points in pulse-chase analyses. Furthermore, conversion of L^dalt to L^d was found to affect almost exclusively immature (Endo H^s) class I molecules. Thus intrinsic properties of immature L^dalt molecules or their associated chaperonins are maintained in cell lysates that allow them to undergo de novo folding in vitro. These combined results demonstrate that immature L^dalt molecules are precursors awaiting constituents such as peptide and β_2m that influence folding, whereas surface L^dalt molecules appear refractory to association with peptide, β_2m, and consequent folding.

Class I MHC molecules are membrane-bound glycoproteins that function as receptors for peptide ligands which are presented to CTL. For example, after infection, cells process virus-derived proteins into short peptides of eight to nine amino acids that bind self class I molecules (1–3). When expressed on the cell surface, peptide–class I complexes can identify virally infected cells for destruction by host immune CTL. Studies using the drug brefeldin A (Bfa)[1] suggest that the initial binding of peptide by class I occurs in a pre-Golgi compartment, presumably the endoplasmic reticulum (ER) (4, 5). Although present dogma dictates that the initial interaction of peptide with class I is physiologically the most important, recent studies have shown that considerable peptide binding can occur at the cell surface for at least certain class I molecules (6–10). In spite of recent progress delineating the consequences of the intracellular versus extracellular binding of peptide by class I, it remains unclear whether they are mechanistically equivalent. Furthermore, peptide interactions have only been studied for a limited number of different class I molecules and already several important locus-specific distinctions have been noted. Thus, even though there is general agreement concerning certain principles governing peptide–class I association, there is also considerable uncertainty or controversy.

[1] *Abbreviations used in this paper:* Bfa, brefeldin A; Endo H, endoglycosidase H; ER, endoplasmic reticulum; hβ_2m, human β_2-microglobulin; LCMV, lymphochoriomeningitis virus; NMS, normal mouse serum; PAS, protein A Sepharose; SF, serum-free.

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In regard to the intracellular interactions of peptide with class I, recent studies suggest that specific ligands play an integral role in the folding of the nascent class I molecule. The initial support for this hypothesis came from studies of the RMA.S cell line, a mutagenized cell line immunoselected to be class I deficient (11). Indeed, there is considerable indirect evidence indicating that RMA.S has a defect in the transport of intracellular peptides (6, 12). At 37°C most of the RMA.S class I molecules are sensitive to endoglycosidase H (Endo H), implying that they are ER retained, and most cannot be detected by conformation-dependent (i.e., $\alpha1/\alpha2$ domain) antibodies, implying that they are misfolded or unassembled. Townsend et al. (13) reported that when specific peptide ligands were added to RMA.S cell lysates, properly conformed and assembled class I molecules were detected. In agreement with this observation, peptide ligands were found to specifically facilitate the folding of HLA.B27 molecules produced in a cell-free translation system (14). Even though these studies provide strong evidence that peptide influences de novo folding of class I, other findings suggest that class I molecules can attain functional conformation in the absence of peptide. For example, untreated lysates of RMA.S cells grown at 37°C contain significant amounts of conformed class I molecules and RMA.S cells grown at 25°C express high levels of class I molecules recognized by conformation-dependent mAb. Thus, peptide may not be an absolute requirement for de novo folding. Besides peptide ligand, the other known participant in class I folding is β_2-microglobulin (β_2m). Studies of the β_2m-deficient cell lines DAUDI (15) and RIE (16) suggested that intracellular β_2m is a requisite for folding and transport of most class I heavy chains. An exception to this rule came from studies of RIE.D^b, a β_2m^- cell line transfected with the D^b gene under a strong viral promoter (17). Surface D^b molecules are expressed by RIE.D^b cells but remain unassembled and are not recognized by conformation-dependent antibodies. This observation suggests that β_2m association is not an absolute requirement for intracellular transport, a conclusion also supported by recent studies of L^d (18) and D^{dm6} (19) molecules. However, studies of RIE.D^b would suggest that intracellular β_2m is required for proper folding. In apparent contradiction of this conclusion, Townsend and colleagues (20) reported that the addition of either peptide ligand or β_2m to an RIE.D^b lysate resulted in the detection of conformed D^b molecules. Thus, even though both peptide and β_2m clearly influence the folding of class I molecules, their separate contributions under physiological conditions have yet to be defined and very well may differ significantly among class I molecules.

In regard to the consequences and mechanisms of peptide binding to surface class I molecules, studies of RMA.S indicate that ligand association is required for stable class I expression. RMA.S cells grown at 25°C express what appears to be empty class I molecules that, when switched to 37°C, rapidly become undetected by conformation-dependent mAbs (6, 12). Furthermore, either specific peptide (11) or mAb (21) was found to dramatically and specifically increase the half-life of class I molecules expressed by RMA.S. In studies of unselected live cells cultured at 37°C with labeled peptide ligands, specific binding was observed to L^d (9), K^d (10), B27 (7), and D^b (8) molecules. These findings were interpreted as evidence that a significant proportion of these class I molecules are expressed at 37°C with empty ligand binding sites (7, 10). In murine systems evidence has been provided that β_2m exchange may influence peptide binding at the cell surface. Clearly, the addition of human β_2m can facilitate peptide binding to murine class I molecules (22–24). However, certain mouse class I molecules show a strong propensity to exchange their own β_2m for either human or bovine β_2m, and this exchange may facilitate peptide binding at the surface. It is therefore unclear whether peptide binds to surface class I molecules previously conformed by intracellular peptide, to open folded class I molecules that have not yet encountered peptide, or to unfolded class I molecules that can fold at the cell surface. Thus, the mechanism and physiological significance of peptide binding to surface class I is unclear and again may differ significantly among class I molecules.

Our approach to investigating the structural and functional consequences of the interaction of peptide with class I has focused on the L^d molecule. The uncharacteristically low level of surface expression of L^d relative to K^d or D^d was found to the dramatically and specifically increased by culturing H-2^d haplotype cells with known L^d ligands (25). This result indicated that a high percentage of L^d molecules have readily accessible binding sites. Exploiting this feature of L^d, a binding assay was developed whereby live cells were grown with ^{125}I-labeled peptides (9). In these assays, we discovered a unique mAb, designated 64-3-7, that detects a non–peptide associated conformational variant of L^d. This alternative form of L^d, designated L^dalt, showed weak if any β_2m association and was detected both intracellularly and at the cell surface (9). In this communication we present data indicating that when cells are cultured with peptide, binding occurs to properly folded surface L^d molecules and significantly prolongs their half-life. By contrast, when cell lysates are incubated with peptide, binding occurs to immature or nascent L^dalt molecules and facilitates their conversion to proper conformation. These distinctions between the effect of peptide on immature vs. surface L^d molecules are discussed in the context of other recent studies of class I–ligand interaction.

Materials and Methods

Cell Lines. L-L^d cells were generated by introducing the L^d gene into murine Ltk$^-$DAP-3 (H-2^k) fibroblast cells. All of the cell lines used were maintained at 37°C, 6.5% CO_2 in DME (Gibco Laboratories, Grand Island, NY) containing 10% FCS (Hyclone Laboratories Inc., Logan, UT), 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin (DME-FCS). In certain experiments, L-L^d cells were grown in serum-free DME medium supplemented with 1% Nutridoma SP (Boehringer Mannheim Corp., Indianapolis, IN) for 3 d before the assays. In other assays cells were treated with Bfa (Epicentre Technologies, Madison, WI) to block new surface expression of class I molecules. Concentrations between 1 and 5 μg/ml were used. In data not shown, 1 μg/ml of Bfa was found to be sufficient to completely block sialation of L^d molecules.

mAb For detection of L^d molecules mAb 30-5-7 ($\alpha2$ domain)

(26), 64-3-7 (α1 domain) (27), or 28-14-8 (α3 domain) (26) were used. As control antibodies, 3-83 (K^k, D^k) (28), 11-4-1 (K^k) (29), and 34-5-8 (D^d) (30) were used. All mAbs are of the IgG2 isotype.

Immunoprecipitation. Immunoprecipitation of class I molecules with specific mAbs was performed as previously described (31). To monitor L^d antigenic conformation in biosynthetic lysates cells were labeled with [^{35}S]methionine and then lysed with 0.5% NP-40 in Tris-buffered saline, pH 7.0, containing freshly added PMSF (0.2 mM; Sigma Chemical Co., St. Louis, MO) and supplemented plus or minus β_2m and/or peptide for 30 min on ice. Cell lysates were incubated overnight at 4°C. After overnight incubation, lysates were centrifuged at 100,000 *g* for 1 h and supernatants were precleared with an equal volume of 10% (vol/vol) IgGsorb (The Enzyme Center, Malden, MA). Samples of these glycoproteins were next incubated with 0.1 vol of individual mAb ascites for 30 min on ice, followed by incubation with an equal volume of Protein A–Sepharose (PAS) beads for 30 min on ice. The IgGsorb-mAb-antigen complexes were washed three times in cold Tris-buffered saline containing 1% NP-40 and 0.2 mM PMSF. The mAb-Ag complexes were eluted by incubation of the samples in 0.125 M Tris-HCl, pH 6.8, 12.5% glycerol, 2% SDS, and 1% 2-ME (SDS-PAGE sample buffer) at 90°C for 2 min, followed by centrifugation to remove PAS. SDS-PAGE was performed on a 10–15% linear polyacrylamide gradient Laemmli buffer system (32). Prestained molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD) were included on every gel. The gels were then treated with EN^{3}HANCE (NEN-Dupont, Boston, MA), dried, and exposed to X-Omat AR film at −70°C for 1–10 d.

For measurements of surface binding of radioiodinated peptide, L-L^d cells were incubated for 4 h at 37°C with ^{125}I-labeled peptide. Cells were harvested and washed five times with DME (unsupplemented). Each cell sample was resuspended in 500 μl PBS and incubated 30 min on ice with 0.1 vol antibody. After the incubation cells were washed three times with DME (unsupplemented) and lysed in 0.5% NP-40/0.2 mM PMSF. Peptide–class I complexes were precipitated with an equal volume of 10% IgGsorb.

In lysate binding experiments, L-L^d cells were detergent lysed in 0.5% NP-40/0.2 mM PMSF supplemented plus or minus radiolabeled peptide ligand. Lysates were clarified by 1 h, 100,000 *g*, 4°C centrifugation and incubated overnight at 4°C. Glycoproteins from these lysates were purified by lentil lectin Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) affinity chromatography using 0.5 M α-methylmannoside for elution. Samples were incubated with 0.1 vol mAb for 30 min on ice. IgGsorb was used to precipitate class I complexes.

Flow Cytometry. Flow cytometry was performed as previously described (9). Briefly, 2–4 × 10^5 cells were placed in the wells of round-bottomed microtiter plates, washed once with HBSS (lacking phenol red) containing 0.2% BSA/0.1% sodium azide (FACS® medium), and incubated with a saturating concentration of mAb or with FACS® medium alone for 30 min at 4°C. The cells were washed three times with FACS® medium and then incubated with a saturating concentration of fluorescein-conjugated $F(ab')_2$ fragment of goat anti–mouse IgG, Fc-specific (Organon-Teknika-Cappel, Durham, NC) for 30 min at 4°C, washed with FACS® medium three times, and finally resuspended in FACS® medium containing 10 μg/ml propidium iodide. Fluorescein-labeled cells were analyzed using a FACScan® (Becton Dickinson & Co., Mountain View, CA) equipped with an argon laser tuned to 488 nm and operating at 150 mW of power. Fluorescence histograms were generated with logarithmic amplification of fluorescence emitted by single viable cells. Each sample analyzed comprised a minimum of 1 × 10^4 cells. Mean fluorescence values were converted from logarithmic amplification of fluorescence intensity by linear regression analysis using the CONSORT 30 computer software.

Peptide Synthesis. Peptides were synthesized using Merrifield's solid-phase method (33) on a peptide synthesizer (model 431A; Applied Biosystems, Inc., Foster City, CA). All peptide synthesis reagents were of high purity (>99%) and supplied by Applied Biosystems, Inc. The resin used for peptide synthesis was phenylacetamidomethyl and was preloaded with 0.5 mM of required amino acid. All amino acids used were t-Boc protected at the NH_2 terminus and their reactive side chains were protected with the standard groups recommended by Applied Biosystems, Inc. for t-Boc synthesis, with the exception of histidine. The histidine derivative used was *N*-α-*Boc*-*N*-π-benzyloxymethyl-L-histidine supplied by Bachem (Torrance, CA). The Boc-amino acids were coupled using carbodiimide-hydroxybenzotriazole coupling cycles as recommended by the manufacturer. The peptides were simultaneously deprotected and cleaved from the resin by treatment with anhydrous hydrogen fluoride/anisole/dimethyl sulfide 10:1:1 (vol/vol/vol) for 50 min at 0°C. The cleaved peptide was washed with diethylether to remove organic by-products generated during hydrogen fluoride cleavage. The peptide was then extracted from the resin with 30% acetic acid. The acetic acid was removed by rotary evaporation, and the remaining aqueous peptide solution was diluted fourfold with H_2O, shell frozen, and lyophilized. Peptides were purified (≥90%) by reverse-phase HPLC and subjected to purity assessment techniques as previously described (34).

Peptide Labeling. Peptides were iodinated using the Iodo-Beads (Pierce Chemical Co., Rockford, IL) 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, Boston, MA) in 200 μl 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 AG1-x8 ion exchange resin (Bio-Rad Laboratories, Richmond, CA) column to bind the free ^{125}I. After centrifugation (2,500 *g*, 5 min) the radiolabeled peptide-containing fluid forced 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.

Results

To define the parameters involved in the association of ligand with class I MHC molecules, we have studied the L^d molecule of the mouse. Previous studies of peptide-induced surface expression of L^d identified a unique mAb designated 64-3-7. Whereas expression of other epitopes was increased two- to fivefold after treatment with different known peptide ligands, the epitope recognized by 64-3-7 was unaffected (9). Immunoprecipitation studies showed that mAb 64-3-7 defined an alternative form of L^d (L^dalt) as compared with other mAbs to L^d (e.g., 30-5-7). L^dalt molecules were distinguished by their minimal, if any, β_2m association and their slower rate of oligosaccharide maturation. The intracellular ratio of 64-3-7$^+$ to 30-5-7$^+$ L^d molecules was found to be variable and generally higher in cells that overexpress L^d, such as the L-L^d transfected cell line. In an earlier report (9) we measured ligand binding to L^d by culturing L-L^d cells or

P815 cells with ^{125}I-labeled murine CMV peptide (pp89, 168-176) (35). After 4–6 h culture, cells were lysed in NP-40 and class I molecules were immunoprecipitated. Whereas 30-5-7 precipitates contained high levels of labeled CMV peptide, 64-3-7 precipitates contained no specific radioactivity (9). This result indicated that L^dalt molecules are not ligand associated, in contrast to 30-5-7$^+$ L^d molecules that demonstrated considerable ligand association. To quantitate what proportion of the binding in this assay occurred to surface

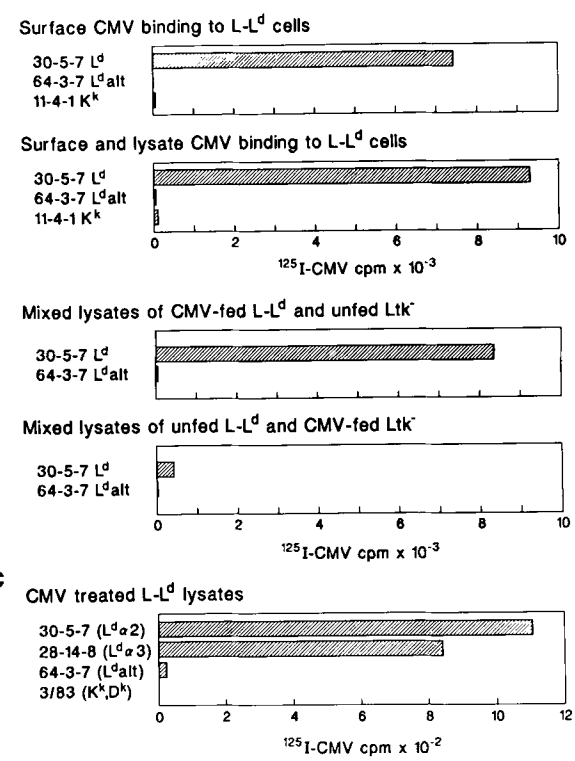
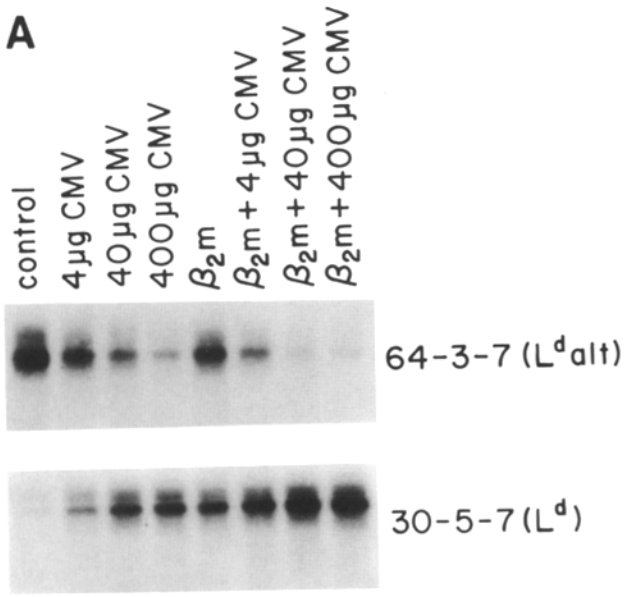
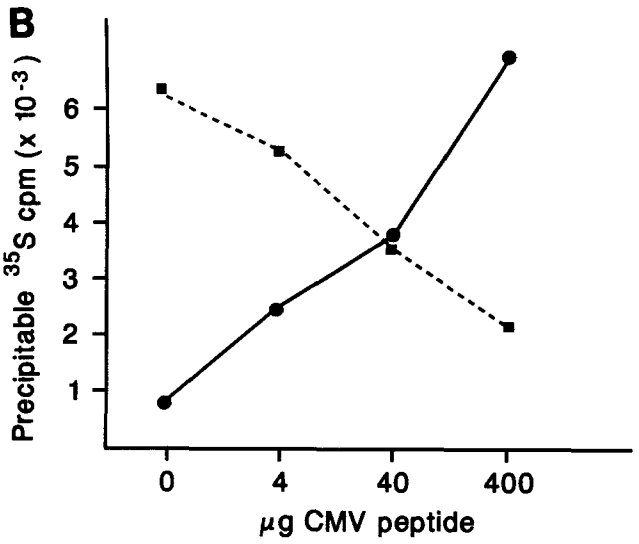


Figure 1. Comparisons of the binding of CMV peptide to alternative forms of L^d at the surface versus in cell lysates. (*A*) Quantitation of the amount of peptide binding at the surface versus the surface and cell lysate. To measure peptide binding to surface L^d molecules (*upper panel*), 5×10^7 L-L^d cells were incubated with ^{125}I-labeled CMV peptide (1×10^{-5} M) at 37°C for 4 h. Cells were then washed three times to remove unbound peptide and divided into three groups, and each group was treated with excess (100 μl ascites fluid) mAb 30-5-7, 64-3-7, or 11-4-1 as indicated along the left of the figure. After 30 min incubation with antibody on ice, cells were washed three times and precipitates were obtained using IgGsorb. Pellets were counted to quantitate the amount of peptide bound to L^d, L^dalt, and K^k molecules, respectively. To measure surface and lysate CMV binding to L-L^d cells (*A*, *lower panel*) the protocol was the same as above except that the respective mAbs (100 μl ascites) were also added after cell lysis. (*B*) CMV peptide binding in mixed lysates. 10×10^6 Ltk$^-$ DAP3 cells were incubated with 150 μg radiolabeled CMV peptide at 37°C for 4 h, washed extensively, lysed, and mixed with an equal number of mock-treated L-L^d cells. The lysate (500 μl, 0.5% NP-40) was incubated overnight at 4°C. Peptide-associated counts were precipitated with antibodies to class I and IgGsorb (*bottom panel*). The reciprocal experiment was similarly performed (*top panel*). (*C*) CMV peptide binding in cell lysates. 4×10^7 L-L^d cells grown at 37°C were lysed in 2 ml 0.5% NP-40 containing 160 μg of labeled CMV peptide. The lysate was incubated overnight at 4°C and then divided into four equal parts. Each aliquot was treated with a saturating concentration of mAb 30-5-7 (α2L^d), 28-14-8 (α3L^d), 64-3-7 (L^{d}alt), or 3/83(K^{k}D^k) and IgGsorb to precipitate class I molecules.

L^d molecules, the experiment shown in Fig. 1 *A* was performed. L-L^d cells were incubated with ^{125}I-CMV peptide for 4 h, after which time they were treated with an excess amount of mAb 30-5-7 (anti-L^d), 64-3-7 (anti-L^{d}alt), or 11-4-1 (anti-K^k). After extensive washing, half of each of these mAb-coated cells were lysed in NP-40 and Ab-class I complexes were precipitated. Precipitates were then counted to determine the amount of peptide bound to surface class I molecules. Under these conditions no Ab/L^d/peptide dissociation was observed in the lysate as determined by using excess competitors (data not shown). As shown in the upper panel of Fig. 1 *A*, of the three mAbs only 30-5-7 precipitated significant peptide-specific counts. The other half of the antibody-coated L-L^d cells were lysed and each lysate was treated again with the same respective mAb. Thus, precipitates from these samples should include surface and intracellular class I molecules with bound peptide. As shown in the lower panel of Fig. 1 *A*, only 30-5-7 precipitates again contained peptide-specific counts. Comparisons of the amount of peptide binding observed by surface versus surface plus lysate precipitate indicate that 80% of the peptide binding can be accounted for by surface 30-5-7$^+$ L^d molecules. The remaining 20% of the peptide binding could either have occurred intracellularly or in the lysate. To quantitate the amount of binding that can occur in a lysate, the mixed-lysate experiment shown in Fig. 1 *B* was performed. As a positive control a lysate from L-L^d cells that were fed ^{125}I-peptide was mixed with a lysate of Ltk$^-$ cells. As expected, significant specific peptide binding to 30-5-7$^+$ L^d was observed (Fig. 1 *B*, *upper panel*). In a reciprocal experiment, a lysate of unfed L-L^d cells was mixed with a lysate of CMV-fed Ltk$^-$ cells. In this mixture, binding to L^d could only occur in the lysate because Ltk$^-$ cell are L^d negative. As shown in the lower panel of Fig. 1 *B*, a small but specific amount of lysate binding to L^d was detected. Comparison of the amount of peptide binding measured in these reciprocal experiments (Fig. 1 *B*) indicates that only 5% of the binding to L^d occurred in the lysate when L-L^d cells was cultured with labeled peptide. Thus, using this assay system, most of the binding clearly occurs to surface L^d molecules before cell lysis.

Although the above experiments indicate that culturing cells with peptide results in binding to predominantly surface L^d molecules, they also suggest that binding to L^d can occur in cell lysates (Fig. 1 *B*). To better characterize this latter binding, a lysate of L-L^d cells was incubated with ^{125}I-labeled CMV peptide. As shown in Fig. 1 *C*, anti-L^d mAbs 30-5-7 and 28-14-8 precipitated significant peptide-bound molecules in contrast to 64-3-7 precipitates that contained no counts above background. In data not shown, comparable results were obtained with another known ligand for L^d derived from the lymphochoriomeningitis virus (LCMV) nucleoprotein (NP 118-126) (36). To define the relationship between alternative forms of L^d, various concentrations of peptide ligand plus or minus human β_2m (hβ_2m) were incubated with aliquots of biosynthetically labeled L-L^d lysates. Peptide treated lysates exhibited a dramatic, dose-dependent increase in 30-5-7$^+$ L^d molecules in conjunction with a proportional decrease in 64-3-7$^+$ L^d molecules (Fig. 2 *A*).





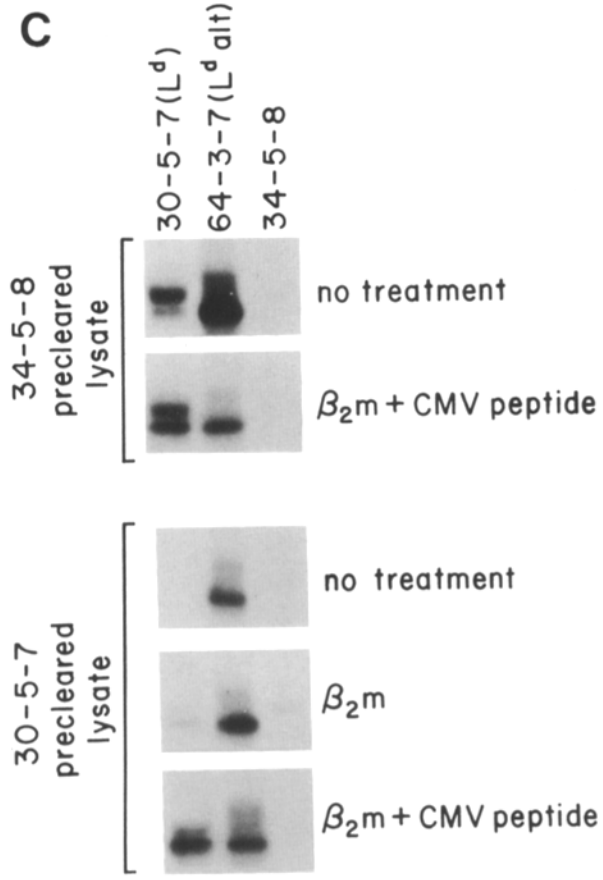


Figure 2. Treatment of L-L^d cell lysates with CMV peptide and/or hβ_2m converts 64-3-7$^+$ L^d to 30-5-7$^+$ L^d. (*A*) Dose dependency of the effect of peptide on the 64-3-7$^+$ and 30-5-7$^+$ forms of L^d. 1.6 × 10^8 L-L^d cells were labeled with [^{35}S]methionine for 3 h, solubilized in 0.5% NP-40, and divided into eight aliquots of 2 × 10^7 cells. Each aliquot (500 μl, 0.5% NP-40) was incubated at 4°C overnight with 3 μM hβ_2m (Sigma Chemical Co.), 3 μM hβ_2m and CMV peptide, or CMV peptide alone (peptide concentrations indicated along top of figure). L^d molecules were precipitated with saturating amounts of mAb 64-3-7 (*top row*) or 30-5-7 (*bottom row*) and analyzed by SDS-PAGE. Only class I heavy chains are shown. (*B*) Quantitative comparisons of the reciprocal effect of peptide on 64-3-7$^+$ L^d (■) versus 30-5-7$^+$ L^d (●). The precipitable counts shown were obtained from the samples analyzed in the first four lanes of *A*. (*C*) Peptide induction of 30-5-7$^+$ L^d in lysates precleared with mAb 30-5-7. Aliquots of an L-L^d lysate were precleared two times using PAS beads coated with either 30-5-7 (*bottom panel*) or 34-5-8 (*top panel*) used as an irrelevant control. Precleared lysates were aliquoted (2 × 10^7 L-L^d, 500 μl, 0.5% NP-40) and incubated overnight at 4°C with hβ_2m (3 μM) or CMV peptide (400 μg) or left untreated (as indicated along the right side of figure). Samples were precipitated with saturating amounts of mAb 30-5-7, 64-3-7, or 34-5-8 (as indicated along the top), along with PAS. Precipitates were resolved by SDS-PAGE and only class I heavy chains are shown.

This crisscross relationship between 64-3-7$^+$ L^d and 30-5-7$^+$ L^d molecules is graphically represented in Fig. 2 *B*. In the same experiment, addition of purified hβ_2m alone or together with peptide was found to shift the ratio of the two forms toward 30-5-7$^+$ L^d (Fig. 2 *A*). The addition of the LCMV peptide to an L-L^d cell lysate was also found to increase 30-5-7$^+$ L^d and decrease 64-3-7$^+$ L^d, whereas an irrelevant peptide had no effect (data not shown). To directly test whether 64-3-7$^+$ L^d molecules could be converted to 30-5-7$^+$ L^d molecules, a lysate was selectively precleared of all 30-5-7$^+$ L^d molecules and subsequently treated with hβ_2m/peptide. New 30-5-7$^+$ L^d molecules were detected in hβ_2m/peptide-treated lysates but not untreated controls (Fig. 2 *C*). This experiment formally established the

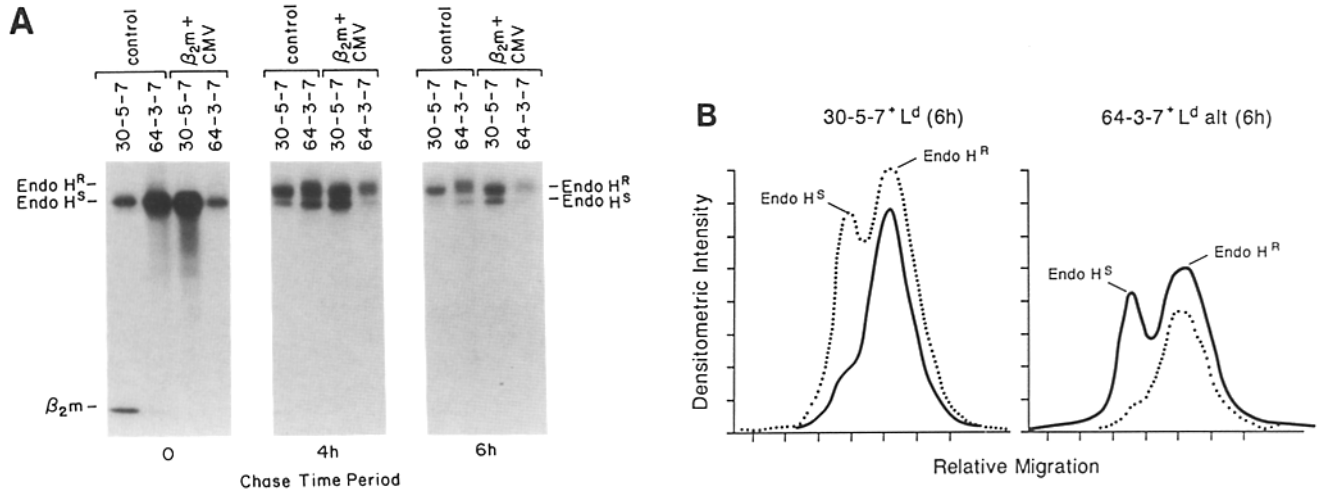


Figure 3. Pulse-chase experiment demonstrating that predominantly immature (Endo H^s) forms of L^d glycoproteins are susceptible to peptide-induced conformational change. (*A*) Biosynthetically labeled L-L^d cells were pulsed for 15 m and chased for 0, 4, or 6 h as indicated along the bottom of the figure. Lysates (2 × 10^7 L-L^d, 500 μl, 0.5% NP-40) from each chase period were incubated overnight (4°C) with or without hβ_2m (3 μM) and CMV peptide (40 μg). Class I molecules were precipitated with mAbs 64-3-7 and 30-5-7 and resolved by SDS-PAGE. Using this gel system, mature (Endo H^R) and immature (Endo H^s) class I molecules resolve into two separate bands as indicated. (*B*) Densitometric tracing of the class I precipitin bands from the 6-h chase period shown in Fig. 3 *A*. For comparison, the profiles of the control (——) and hβ_2m/peptide-treated (· · · ·) are superimposed.

precursor–product relationship between 64-3-7$^+$ L^d and 30-5-7$^+$ L^d. In conjunction with the aforementioned properties of L^{d}alt, these findings demonstrate that 64-3-7 uniquely detects nonconformed L^d molecules capable of binding peptide ligand. To better characterize the L^d molecules susceptible to antigenic conversion, pulse/chase experiments were performed. Even though 64-3-7$^+$ L^{d}alt molecules are transported more slowly than 30-5-7$^+$ L^d molecules, after a 4-h chase time mature (Endo H^R) forms of both are detected. As shown in Fig. 3 *A*, L^d molecules were decreasingly susceptible to peptide/hβ_2m-induced conversion with increased time of chase. At the 0 time point >80% of the 64-3-7$^+$ L^d molecules were convertible to 30-5-7$^+$ L^d; after 4 and 6 h that percentage dropped to 50 and 40%, respectively. Furthermore, Endo H^s forms were preferentially affected by peptide/hβ_2m treatment as observed in both the loss of im-

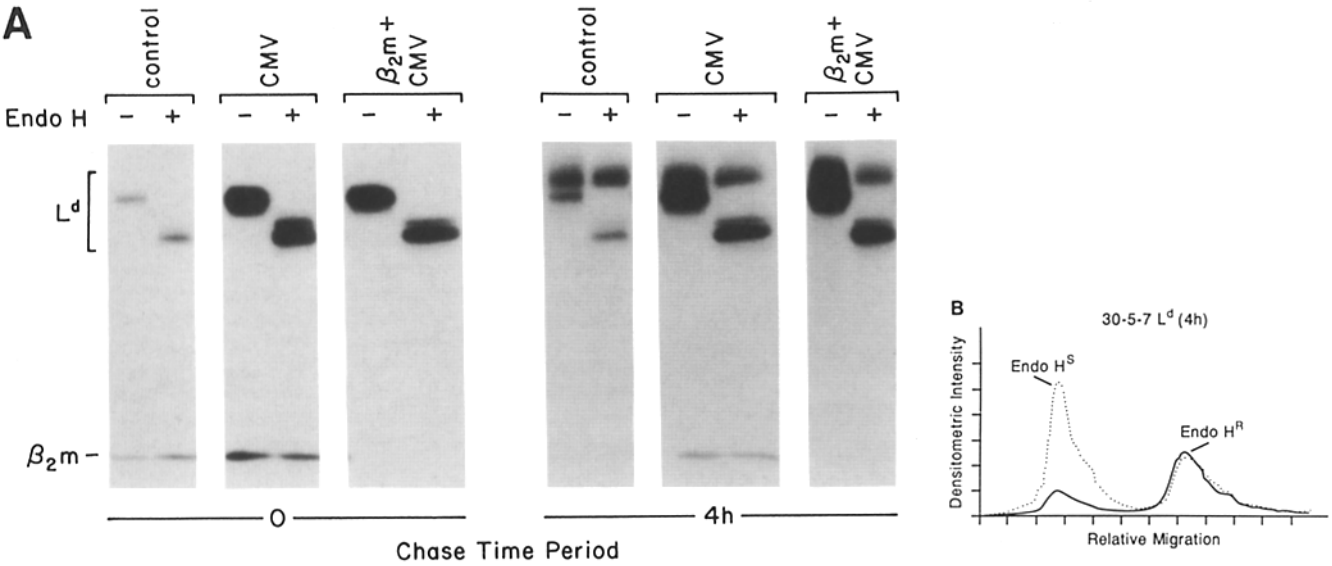


Figure 4. Pulse-chase experiment demonstrating that peptide alone is as effective as peptide and hβ_2m in inducing Endo H^s forms of 30-5-7$^+$ L^d. (*A*) Biosynthetically labeled L-L^d cells were pulsed for 15 m and chased for 0 or 4 h as indicated. Respective lysates (1.25 × 10^7 L-L^d, 500 μl, 0.5% NP-40) were divided and treated overnight with CMV peptide (40 μg), hβ_2m (3 μM) + CMV (40 μg) peptide, or untreated control. L^d molecules were precipitated with mAb 30-5-7 and half of each precipitate was digested with Endo H (+) (1 mU/ml; ICN ImmunoBiologicals, Lisle, IL), whereas the other half was mock digested (−). Precipitates were resolved by SDS-PAGE. (*B*) Densitometric tracing of the precipitin bands from the 4-h chase period shown in *A*. For comparison, the profiles of the control (——) and peptide-treated (· · · ·) are superimposed.

mature 64-3-7$^+$ L^{d}alt and the gain of immature 30-5-7$^+$ L^d (Fig. 3 *B*). A second important feature of this experiment is that nearly all 30-5-7$^+$ L^d molecules exchange mouse for hβ_2m. Compared with control lysates, hβ_2m/CMV-treated lysates undergo significant or complete exchange of exogenous cold hβ_2m for endogenous-labeled β_2m (Fig. 3 *A*). This result was also obtained with hβ_2m treatment alone (data not shown). To substantiate the observation that immature 64-3-7$^+$ L^d molecules are preferentially affected by peptide/hβ_2m treatment, a pulse-chase experiment was performed using Endo H digestion to formally define immature molecules (Fig. 4 *A*). In this experiment peptide or peptide/hβ_2m resulted in the comparable induction of 30-5-7$^+$ L^d molecules (a 90% increase at time 0 versus a 50% increase at time 4 h), implying that hβ_2m exchange is not required for peptide-induced folding of L^d in cell lysates. Furthermore, the affected molecules are clearly sensitive to Endo H digestion. As shown in Fig. 4 *B* densitometric tracings of 30-5-7 precipitates from the 4-h time point indicate that the peptide-induced 30-5-7$^+$ L^d molecules were exclusively immature (Endo H^S) forms. Thus, this in vitro antigenic conversion of L^d molecules accurately mimics in vivo folding of nascent L^d molecules, demonstrating that intrinsic properties of immature class I molecules or their associated chaperonins are maintained in vitro in cell lysates.

To determine the effect of peptide on expression and surface turnover of L^d forms, L-L^d cells were grown with peptide and/or Bfa (a drug that prevents new surface expression of class I). The expression of 30-5-7$^+$ L^d was dramatically increased by peptide treatment alone in that a threefold increase was observed during the 6-h incubation (Fig. 5 *A*). The maintenance of the high level of expression of peptide-induced L^d was dependent upon new arrival of L^d molecules at the cell surface as shown by comparing cells treated with peptide alone versus Bfa plus peptide (Fig. 5 *A*). However, it can also be seen that peptide has a substantial effect on L^d surface stability by comparing cells treated with Bfa versus Bfa plus peptide. 30-5-7$^+$ L^d molecules were rapidly turned over ($t_{1/2}$ ~2 h), but peptide greatly extended their half-life ($t_{1/2}$ >6 h). The rapidity with which L^d is turned over is in contrast to other class I molecules tested such as K^k (Fig. 5 *C*) or D^d (data not shown) that have half-lives >6 h. Unexpectedly, 64-3-7$^+$ L^{d}alt molecules were found to be stably expressed on the cell surface ($t_{1/2}$ ~6 h) (Fig. 5 *B*). In the assay shown in Fig. 5, peptide decreased the 64-3-7 expression. As previously reported, the magnitude of the peptide-induced decrease in 64-3-7$^+$ L^d varies considerably among assays or cell types with certain assays showing no decrease in 64-3-7$^+$ L^d in spite of considerable increases in 30-5-7$^+$ L^d expression (9). Although the nature of these disparities in 64-3-7$^+$ L^d expression with peptide are unclear, what is apparent is that the turnover of 64-3-7$^+$ L^d is largely unaffected by peptide. This conclusion is supported by data shown by the comparisons of Bfa versus Bfa + CMV peptide-treated cells (Fig. 5 *B*). This result suggests that peptide does not induce a significant proportion of 64-3-7$^+$ L^d molecules to convert to 30-5-7$^+$ L^d at the surface; nor does peptide appear to prevent surface 30-5-7$^+$ L^d molecules from

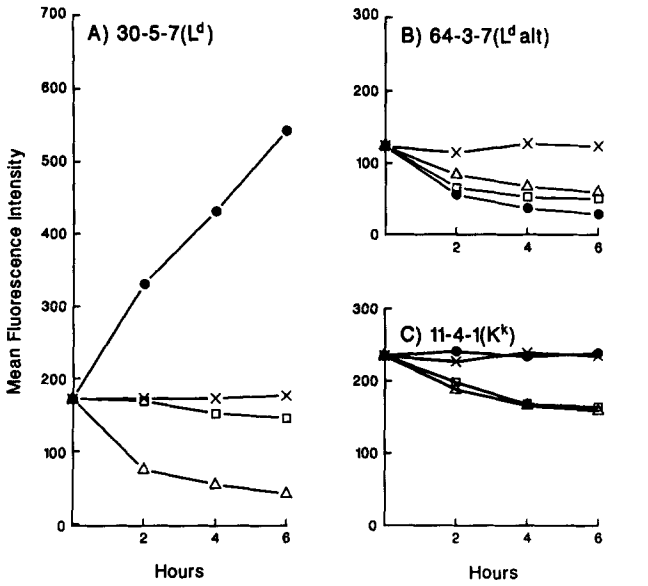


Figure 5. Comparison of the peptide inducibility and surface turnover of alternative forms of L^d on L-L^d cells. L-L^d cells (5 × 10^5/ml) were incubated with 10^{-4} M CMV peptide (●), medium (X), Bfa (△), or Bfa plus 10^{-4} M CMV peptide (□) for a period of 0, 2, 4, or 6 h. After peptide and/or Bfa treatment L-L^d cells were stained with mAb against L^d (30-5-7) (*A*), L^{d}alt (64-3-7) (*B*), or K^k(11-4-1) (*C*), respectively. Quantification of class I surface expression on these cells was analyzed by cytofluorometry on a FACScan®. Mean fluorescence values were converted from logarithmic amplification of fluorescence intensity by linear regression analysis.

"melting" to 64-3-7$^+$ L^d. Either of these above scenarios would have predicted that in the presence of Bfa, peptide would cause a significant decrease in 64-3-7$^+$ L^d expression. By contrast, peptide dramatically increased expression of 30-5-7$^+$ L^d in the presence of Bfa (Fig. 5 *A*). Thus, in spite of the near-complete conversion of 64-3-7$^+$ L$^+$ to 30-5-7$^+$ L^d in cell lysates, no measurable conversion was detected on the cell surface. The failure to detect conversion of cell surface 64-3-7$^+$ L^d was also noted in experiments where L-L^d were surface iodinated, detergent lysed, and incubated overnight with peptide (data not shown). Thus, the distinction between alternative forms of L^d appears to be fixed intracellularly and is relatively irreversible at the cell surface.

To better quantify the factors controlling the extracellular binding of peptide, direct binding of ^{125}I-labeled CMV to surface L^d was measured after cells were cultured under various conditions. In the experiment shown in Fig. 6 *A*, L-L^d cells were tested in a 4-h assay for their ability to bind peptide in media containing FCS, FCS + Bfa, serum free (SF), or SF + hβ_2m. Specific binding to surface L^d was determined by precoating cells with mAb before lysis (method detailed in the legend of Fig. 1). Precipitates of 30-5-7$^+$ L^d from cells grown with Bfa were found to contain about half as many peptide-specific counts as precipitates from cells grown in FCS. This reduced binding could clearly be accounted for by the reduced L^d expression after 4 h in Bfa (data not shown). Thus, peptide binding to L^d is not dependent upon new arrival of L^d molecules to the cell surface. When cells

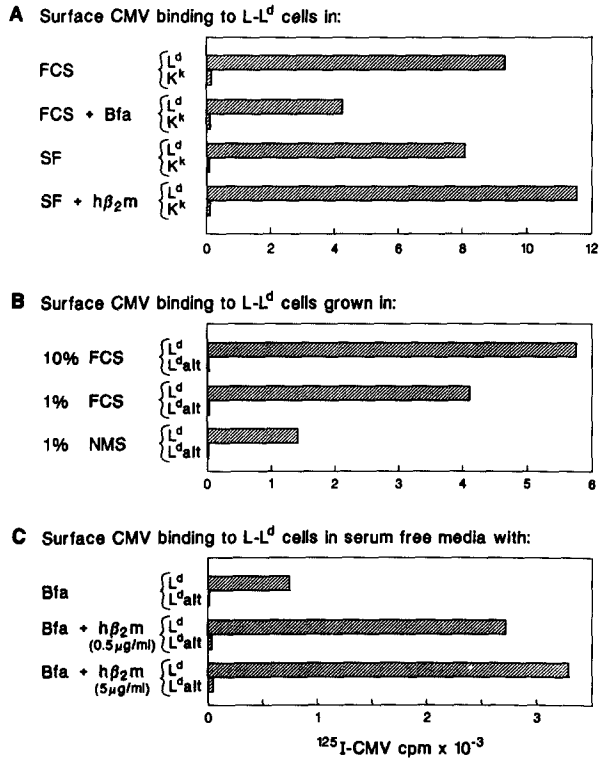


Figure 6. Surface CMV binding to L-L^d cells grown under various conditions. All binding assays were performed at 37°C as detailed in Materials and Methods. (*A*) Binding of CMV peptide to L-L^d cells grown in FCS (10%), FCS (10%) plus Bfa, SF, or SF plus hβ_2m (18 μg/ml). After culturing cells with peptide for 4 h, binding to surface L^d or K^k molecules was assessed by precoating cells before lysis with mAb 30-5-7 or 11-4-1, respectively. This method is described in detail in the legend of Fig. 1 *A*. Cells used for the assay shown in *A* were previously maintained in DME + 10% FCS. (*B*) Binding of CMV peptide to L-L^d cells grown in 10% FCS, 1% FCS, or 1% NMS. In this assay, cells were grown under the indicated conditions for 3 d before the binding assay as well as during the 4-h binding assay. Peptide binding to surface L^d or L^{d}alt molecules assessed by precoating cells before lysis with mAb 30-5-7 or 64-3-7, respectively. (*C*) Binding of CMV peptide to L-L^d cells grown in SF media with Bfa, Bfa + 0.5 μg/ml hβ_2m (Calbiochem Corp. La Jolla, CA), or Bfa + 5 μg/ml hβ_2m (Calbiochem Corp.). In this assay cells were cultured in SF media for 3 d before the binding assay and were cultured under indicated conditions during the binding assay. Binding to surface L^d or L^{d}alt molecules was assessed by precoating cells before lysis with mAb 30-5-7 or 64-3-7, respectively.

were incubated in SF media with labeled-CMV peptide, significant binding to surface L^d was observed (85% of the FCS control). This result indicates that exchange for exogenous β_2m appears not to be the major factor in determining surface binding to L^d. It should be noted that the cells used for the assay shown in Fig. 6 *A* were grown in media containing 10% FCS before their 4-h incubation with labeled peptide under indicated conditions. Thus, entering the peptide binding assay the surface L^d molecules would be expected to be heavily β_2m associated. Interestingly, the purified hβ_2m augmented the binding of peptide in SF media, probably by maintaining a higher level of β_2m-associated L^d during the 4-h assay.

To further explore the role of β_2m association in peptide binding to L^d, L-L^d cells were grown for 3 d in either 10% FCS, 1% FCS, or 1% normal mouse serum (NMS). In our previous immunoprecipitation studies (18), surface 30-5-7$^+$ L^d molecules were detected as predominantly free heavy chains when cells were grown in NMS as compared to heterodimers with bovine β_2m when grown in FCS. In spite of these imposed differences in β_2m-association, the amount of 30-5-7$^+$ L^d and the ratio of 30-5-7$^+$ L^d to 64-3-7$^+$ L^d was comparable on cells grown in 10% FCS, 1% FCS or 1% NMS (fluorometric data not shown). However, the ability of cells under each of these conditions to bind peptide was clearly different (Fig. 6 *B*). Correlating with their expected level of β_2m association (18), peptide binding to surface L^d was highest on cells grown in 10% FCS, next highest on cells grown in 1% FCS, and lowest on cells grown in 1% NMS. As an alternative method to assess the role of β_2m association, L-L^d cells were grown in SF medium for 3 d before the binding assay in order to maximize β_2m dissociation and to eliminate the potential to bind bovine β_2m. After growth in SF, cells were treated with Bfa to prevent new L^d expression during the binding assay. During the 4-h incubation with peptide, aliquots of these cells were grown with no β_2m, 0.5 μg/ml hβ_2m, or 5 μg/ml hβ_2m as indicated in Fig. 6 *C*. Precipitates from the control cells with no exogenous β_2m showed a small but significant amount of peptide binding to 30-5-7$^+$ L^d. This binding could be attributed to either surface expression of intracellular L^d-β_2m complexes beyond the Bfa block or the failure of all L^d molecules to dissociate in SF media. In any case, the addition of purified hβ_2m to SF media was found to greatly enhance the specific peptide binding to L^d (Fig. 6 *C*). In total, these experiments measuring direct peptide binding indicate that new surface expression of L^d and exchange for exogenous β_2m are not required for peptide binding (Fig. 6 *A*). However, when new expression is blocked and cells are grown for an extended period without serum, peptide binding in the absence of exogenous β_2m was ~22% that seen in the presence of hβ_2m (Fig. 6 *C*). It is noteworthy that this 22% binding did not occur in the lysate since all the assays shown in Fig. 6 were performed using Ab-coated cells to detect exclusively surface class I molecules. What is strikingly apparent from each of the experiments shown in Fig. 6 is that peptide binding correlates precisely with the amount of β_2m associated with L^d, implying that mainly class I:β_2m heterodimers bind peptide.

Discussion

Several recent publications have studied the association of class I molecules with peptide by culturing live cells in media containing known peptide ligands (6–10). The initial experiments monitored peptide association indirectly by quantitating the peptide-induced increase in surface class I expression. Culturing cells with peptide resulted in the specific increase of class I molecules on immunoselected cells such as RMA.S (11) or LBL721.174 (37) or of L^d (25) molecules on unselected cells. The mechanism of this induction was origi-

nally thought to result from retrograde peptide transport to the ER. Therein, the fed-peptide would facilitate the folding, assembly, and/or transport of the class I H chain. Consistent with this notion, immature (Endo H^s) class I H chains were affected by peptide feeding. However, a subsequent experiment by Townsend and colleagues (13) showed that peptide-induced folding of class I could have occurred in the cell lysate and not in the intact live cells. Thus, there was no need to hypothesize retrograde peptide transport. Furthermore, the observation that peptide stabilized class I expression on RMA.S cells provided a viable mechanism to explain the surface increase in class I expression by peptide. In accordance with this conclusion, we report here that peptide ligands significantly prolong the half-life of the uncharacteristically labile surface L^d molecule. Therefore, peptide-induced class I expression on live cells certainly affects surface molecules and needs not implicate retrograde peptide transport.

Inspired by these earlier peptide-feeding protocols, several groups have incubated various cell types with labeled peptide ligands for different class I molecules. Specific binding was then measured by immunoprecipitating the class I molecules from the lysates of the peptide-fed cells (6–10). Although each of these reports provides direct evidence for specific peptide binding to class I, in none of these studies was the mechanism elucidated. Mechanistic dilemmas were encountered due to two findings: first, cell inhibitory drugs such as Bfa, cycloheximide, or sodium azide have frequently yielded equivocal results; and second, the aforementioned findings of Townsend raised questions of whether binding occurs to intact cells or in the cell lysate. Two experiments reported here are relevant to this issue. Reciprocal lysate mixtures from peptide-fed versus unfed, L^d-positive versus L^d-negative cells were used to quantitate the amount of binding occurring in a lysate. This experiment indicated that at most 5% of the peptide binding occurred exclusively in the lysate of peptide-fed cells. This finding is in agreement with similar results obtained in studies of B27 (7). As an alternative approach, cells previously incubated with iodinated peptide were coated with mAb before lysis and thus only surface L^d molecules were detected. Using this approach, surface L^d molecules could be shown to account for at least 80% of the total (surface + lysate) peptide binding. We therefore feel confident that at least in our system most of the peptide ligand binding occurs to surface L^d molecules on live intact cells. Using this assay system we go on to show that the binding of ligand to surface L^d does not require new expression of class I nor exchange for heterologous β_2m. However, without both new expression of L^d and exogenous β_2m, the peptide binding to L^d falls off precipitously. Furthermore, this binding capability can be restored by adding purified hβ_2m. The reduced but significant peptide binding to L^d in the presence of Bfa agrees with a recent study of B27 (7), and the observation of significant peptide binding to L^d in the absence of exogenous β_2m is consistent with studies of D^b (8) and B27 (7). It should be noted, however, that these latter studies did not quantitate ligand binding with or without exogenous β_2m. Perhaps the most striking feature of our data is that there is a precise quantitative correlation between the amount of β_2m association and the ability to bind peptide. We thus conclude that β_2m exchange is not a prerequisite for peptide binding to surface class I, but that β_2m association promotes binding.

It has been speculated that ligand binding is to empty class I molecules in these assays using live cells. Indeed, there is good suggestive evidence supporting this conclusion from studies using RMA.S. In functional studies, RMA.S cells grown at 25°C were found to express high levels of surface class I molecules incapable of presenting endogenous antigens to CTL (12). Furthermore, in structural studies these class I molecules expressed by RMA.S at 25°C were found to bind labeled-exogenous peptide ligands and were found to be thermo-labile at 37°C in the absence of exogenous peptide (12). Thus, these studies provide structural and functional evidence consistent with the notion that class I molecules expressed by RMA.S cells grown at 25°C are empty. Given the validity of this conclusion, it is tempting to speculate that L^d molecules may also have a propensity to be expressed with empty ligand binding sites. Like RMA.S-expressed class I molecules, surface binding of peptide to L^d can be readily demonstrated and exogenous peptide dramatically enhances the surface stability of L^d. In addition, culturing cells at 23–26°C substantially increases 30-5-7^+ L^d expression 1.5- to 4-fold, in contrast to other class I molecules that showed modest if any increases (W-R. Lie, unpublished observations). However, under SF conditions 30-5-7^+ L^d are stably expressed and at levels equal to normal serum conditions. This result is in direct contrast to the extremely labile K^b/D^b molecules expressed by RMA.S cells at 25°C under SF conditions (38). Therefore our data favor the contention that at 37°C, 30-5-7^+ L^d are occupied by endogenous peptides.

The unique inducibility of L^d at 25°C or with peptide ligand could imply that there is a smaller pool of endogenous peptides capable of binding L^d compared with other class I molecules. It should be noted that in functional studies the L^d molecule has been found to be the restriction element for several CTL responses (35, 36, 39–42). Thus, these putative differences in the ability of L^d to bind peptide do not impair its physiological function. Perhaps the weak affinity of L^d for mouse β_2m causes it to be more selective in ligand binding than other class I molecules. In support of the uniqueness of the interaction of nascent L^d molecules with β_2m, we report here that L^d-peptide complexes readily exchange mouse β_2m for hβ_2m in cell lysates. By contrast, Townsend et al. (13) found that D^b-peptide complexes retain mouse β_2m in RMA.S cell lysates. In agreement with their studies, we found that the de novo folding of L^d molecules in cell lysates was highly influenced by peptide ligand. Given this result it is possible that all 30-5-7^+ L^d molecules synthesized at 37°C initially contain (or require) a peptide to attain proper folding. However, once folded the weak affinity for β_2m could render L^d more vulnerable for peptide exchange.

Perhaps the most intriguing result presented in this study is the ambivalent nature of the 64-3-7^+ form of L^d. Our studies of cell lysates show that peptide converts immature 64-3-7^+ L^d to 30-5-7^+ L^d, clearly establishing their precursor/product relationship. Furthermore, this conversion is

extensive if not complete for nascent 64-3-7$^+$ L^d molecules. By contrast, our attempts to convert mature or surface forms of 64-3-7$^+$ L^d to 30-5-7$^+$ L^d have been unsuccessful. These failures to see conversion were observed in: (*a*) fluorometric comparisons of surface L^d forms on cells treated with Bfa +/− peptide (Fig. 5), (*b*) fluorometric analysis of sodium azide–treated cells +/− peptide ligand (data not shown), (*c*) cells grown in SF media +/− purified β_2m (Fig. 6, data not shown), and (*d*) immunoprecipitates of surface ^{125}I-labeled L^d forms treated with peptide in the cell lysate (data not shown). No evidence for conversion was observed in each of the above experiments, suggesting that unlike immature 64-3-7$^+$ L^d, mature forms are refractory to peptide-induced folding. Furthermore, direct evidence showing that only immature forms of L^d are peptide convertible was shown in pulse-chase experiments (Figs. 3 and 4).

These characterizations of the 64-3-7$^+$ L^d molecules present some interesting comparisons in regard to previous reports of denatured heavy chains detected in/on cells. In studies using lysates of RMA.S, nascent heavy chains were detected by either a mAb to the α3 domain (e.g., 28-14-8, [α3] D^b), or a heteroantiserum to a cytoplasmic domain of K^b (43). These latter reagents, in contrast to 64-3-7, detect both nonconformed and conformed heavy chains. Carrying this analogy with RMA.S further, one might predict that surface 64-3-7 is equivalent to melted class I molecules on RMA.S. However, this appears not to be the case. Melted surface class I molecules on RMA.S can only be stably detected with the aforementioned heteroantiserum and not α3 domain antibodies, whereas 64-3-7$^+$ L^d molecules stably express α3 serological epitopes (e.g., 28-14-8). More importantly, there is considerable evidence that conformed class I molecules on RMA.S melt and become detectable with this heteroantiserum (21), whereas we have no evidence suggesting that conformed L^d molecules melt and become detectable with mAb 64-3-7. If 64-3-7$^+$ L^d were melted 30-5-7$^+$ L^d, then adding peptide to Bfa-treated cells, adding hβ_2m to cells in SF media, or growing cells at 25°C would be expected to retard melting, leading to decreased amounts of surface 64-3-7$^+$ L^d. However, no reductions were seen in these experiments. Perhaps the implication of the studies of RMA.S is that the truly melted form of surface L^d cannot be detected by either 64-3-7 or 28-14-8, but only by reagents like the heteroantiserum to the cytoplasmic domain. The better analogy for surface 64-3-7$^+$ L^d is the D^b molecule expressed by the β_2m^- cell line RIE. RIE.D^b and 64-3-7$^+$ L^d share several properties including: (*a*) their detection with α3 domain mAb, (*b*) their lack of β_2m association, and (*c*) an undefined function. In addition, we found the RIE.D^b molecules like 64-3-7$^+$ L^d are stably expressed on the surface ($t_{1/2}$ ~6 h) and are not readily convertible to proper conformation with exogenous β_2m and/or peptide ligand (data not shown). These findings imply that the fates of both 64-3-7$^+$ L^d and D^b expressed by RIE were determined intracellularly.

Based on the findings reported here and these comparisons to studies of RMA.S or RIE.D^b cells, we propose that structural distinctions between the alternative forms of L^d are determined by whether or not peptide/β_2m-induced folding occurs. Furthermore, there is a critical time frame in which this folding must occur, as indicated by our pulse-chase experiments. The results imply either that the ER microenvironment is required for proper folding or that structural features of nascent L^d molecules, or their associated proteins, render them more susceptible to peptide/β_2m-induced folding. What is remarkable is that these properties are maintained in cell lysates. Once 64-3-7$^+$ L^d molecules are expressed on the cell surface they appear structurally inert. In contrast, surface 30-5-7$^+$ L^d molecules that are β_2m associated readily bind exogenous peptide ligand.

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References

1. Townsend, A.R.M., J. Rothbard, F.M. Gotch, G. Bahadur, D. Wraith, and A.J. McMichael. 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined by short synthetic peptides. *Cell.* 44:959.
2. Rötzschke, O., K. Falk, K. Dores, H. Schild, M. Nosda, J. Metzger, G. Jung, and H.G. Rammensee. 1990. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature (Lond.).* 348:252.
3. Van Bleek, G.M., and S.G. Nathenson. 1990. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2K^b molecule. *Nature (Lond.).* 348:213.
4. Nuchtern, J.G., J.S. Bonifacino, W.E. Biddison, and R.D.

Klausner. 1989. Brefeldin A implicates egress from endoplasmic reticulum in class I restricted antigen presentation. *Nature (Lond.).* 339:223.
5. Yewdell, J.W., and J.R. Bennink. 1989. Brefeldin A specifically inhibits presentation of protein antigens to cytotoxic T lymphocytes. *Science (Wash. DC).* 244:1072.
6. Schumacher, T.N.M., M.-T. Heemels, J.J. Neefjes, W.M. Kast, C.J.M. Melief, and H.L. Ploegh. 1990. Direct binding of peptide to empty MHC class I molecules on intact cells and in vitro. *Cell.* 62:563.
7. Benjamin, R.J., J.A. Madrigal, and P. Parham. 1991. Peptide binding to empty HLA-B27 molecules of viable human cells. *Nature (Lond.).* 351:74.
8. Christinck, E.R., M.A. Luscher, B.H. Barber, and D.B. Williams. 1991. Peptide binding to class I MHC on living cells and quantitation of complexes required for CTL lysis. *Nature (Lond.).* 352:67.
9. Lie, W.R., N.B. Myers, J.M. Connolly, J. Gorka, D.R. Lee, and T.H. Hansen. 1991. The specific binding of peptide ligand to L^d class I major histocompatibility complex molecules determines their antigenic structure. *J. Exp. Med.* 173:449.
10. Leuscher, I.F., P. Romero, J.-C. Cerottini, and J.L. Maryanski. 1991. Specific binding of antigenic peptides to cell associated MHC class I molecules. *Nature (Lond.).* 351:72.
11. Townsend, A., C. Ohlen, J. Bastin, H.-G. Ljunggren, L. Foster, and K. Karre. 1989. Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature (Lond.).* 340:443.
12. Ljunggren, H.-G., N.J. Stam, C. Ohlen, J.J. Neefjes, P. Hoglund, M.-T. Heemels, J. Bastin, T.N.M. Schumacher, A. Townsend, K. Karre, and H.L. Ploegh. 1990. Empty MHC class I molecules come out in the cold. *Nature (Lond.).* 346:476.
13. Townsend, A., T. Elliott, V. Cerundolo, L. Foster, B. Barber, and A. Tse. 1990. Assembly of MHC class I molecules analyzed *in vitro*. *Cell.* 62:285.
14. Kvist, S., and U. Hamann. 1990. A nucleoprotein peptide of influenza A virus stimulates assembly of HLA-B27 class I heavy chains and β_2-Microglobulin translated *in vitro*. *Nature (Lond.).* 348:446.
15. Krangel, M.S., H.T. Orr, and J.L. Strominger. 1979. Assembly and maturation of HLA-A and HLA-B antigens *in vitro*. *Cell.* 18:979.
16. Hyman, R., and V. Stallings. 1976. Characterization of a TL^- variant of a homozygous TL^+ mouse lymphoma. *Immunogenetics.* 3:75.
17. Allen, H., J. Fraser, S. Calvin, and R. Flavell. 1986. β_2-microglobulin is not required for cell surface expression of the murine class I histocompatibility antigen $H\text{-}2D^b$ or of a truncated $H\text{-}2D^b$. *Proc. Natl. Acad. Sci. USA.* 83:7447.
18. Myers, N.B., W.-R. Lie, M. Nett, R.J. Rubocki, and T.H. Hansen. 1989. The conformation of L^d induced by β_2-microglobulin is fixed during *de novo* synthesis and irreversible by exchange or dissociation. *J. Immunol.* 142:2751.
19. Rubocki, R.J., J.M. Connolly, T.H. Hansen, R.W. Melvold, M.S. Kim, W.H. Hildebrand, and J. Martinko. 1991. Mutation at amino acid position 133 of $H\text{-}2D^d$ prevents β_2m association and immune recognition but not surface expression. *J. Immunol.* 146:2352.
20. Elliott, T., V. Cerundolo, J. Elvin, and A. Townsend. 1991. Peptide-induced conformational change of the class I heavy chain. *Nature (Lond.).* 351:402.
21. Ortiz-Navarrete, V., and G.J. Hammerling. 1991. Surface appearance and instability of empty H-2 class I molecules under physiological conditions. *Proc. Natl. Acad. Sci. USA.* 88:3594.
22. Rock, K.L., L.E. Rothstein, S.R. Gamble, and B. Benacerraf. 1990. Reassociation with β_2-microglobulin is necessary for K^b class I major histocompatibility complex binding of exogenous peptides. *Proc. Natl. Acad. Sci. USA.* 87:7517.
23. Vitiello, A., T.A. Potter, and L.A. Sherman. 1990. The role of β_2-microglogulin in peptide binding by class I molecules. *Science (Wash. DC).* 250:1423.
24. Kozlowski, S., T. Takeshita, W.-H. Boehncke, H. Takahaski, L.F. Boyd, R.N. Germain, J.A. Berzofsky, and D.H. Margulies. 1991. Excess β_2 microglobulin promoting functional peptide association with purified soluble class I MHC molecules. *Nature (Lond.).* 349:74.
25. Lie, W.-R., N.B. Myers, J. Gorka, R.J. Rubocki, J.M. Connolly, and T.H. Hansen. 1990. Peptide ligand-induced conformation and surface expression of the L^d class I MHC molecule. *Nature (Lond.).* 344:439.
26. Ozato, K., T.H. Hansen, and D.H. Sachs. 1980. Monoclonal antibodies to mouse MHC antigens. II. Antibodies to the $H\text{-}2L^d$ antigen, the product of a third polymorphic locus of the mouse major histocompatibility complex. *J. Immunol.* 125:2473.
27. Shiroishi, T., G.A. Evans, E. Appella, and K. Ozato. 1985. In vitro mutagenesis of a mouse MHC class I gene for the examination of structure-function relationships. *J. Immunol.* 134:623.
28. Ozato, K., N. Maher, and D.H. Sachs. 1980. Hybridomas cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* 124:533.
29. Oi, V.T., P.P. Jones, J.W. Goding, L.A. Herzenberg, and L.A. Herzenberg. 1978. Properties of monoclonal antibodies to mouse Ig allotypes, H-2, and Ia antigens. *Curr. Top. Microbiol. Immunol.* 81:115.
30. Ozato, K., N.M. Mayer, and D.H. Sachs. 1982. Monoclonal antibodies to mouse major histocompatibility complex antigens. IV. A series of hybridoma clones producing anti-$H\text{-}2^d$ antibodies and an examination of expression of $H\text{-}2^d$ antigens on the surface of these cells. *Transplantation (Baltimore).* 34:113.
31. Cullen, S.E., and B.D. Schwartz. 1976. An improved method for isolation of H-2 and Ia alloantigens with immunoprecipitation induced by protein A-bearing staphylococci. *J. Immunol.* 117:136.
32. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
33. Merrifield, R.B. 1963. Solid phase peptide synthesis I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 85:2149.
34. Gorka, J., D.W. McCourt, and B.D. Schwartz. 1989. Automated synthesis of a C-terminal photoprobe using combined Fmoc and t-Boc synthesis strategies on a single automated peptide synthesizer. *Peptide Res.* 2:376.
35. Reddehase, M.J., J.B. Rothbard, and U.H. Koszinowski. 1989. A pentapeptide as minimal antigenic determinant for MHC class I-restricted T lymphocytes. *Nature (Lond.).* 337:651.
36. Schultz, M., P. Aichele, M. Vollenweider, F.W. Bobe, F. Cardinaux, H. Hengartner, and R.M. Zinkernagel. 1989. Major histocompatibility complex-dependent T cell epitopes of lymphocytic choriomeningitis virus nucleoprotein and their protective capacity against viral disease. *Eur. J. Immunol.* 19:1659.
37. Cerundolo, V., J. Alexander, K. Anderson, C. Lamb, P. Cresswell, A. McMichael, F. Gotch, and A. Townsend. 1990. Presentation of viral antigen controlled by a gene in the major histocompatibility complex. *Nature (Lond.).* 345:449.

38. Rock, K.L., C. Gramm, and B. Benacerraf. 1991. Low temperature and peptides favor the formation of class I heterodimers on RMA-S cells at the cell surface. *Proc. Natl. Acad. Sci. USA.* 88:4200.
39. Ciavarra, R., and J. Forman. 1982. H-2L-restricted recognition of viral antigens: In the H-2^d haplotype, anti-vesicular stomatitis virus cytotoxic T cells are restricted solely by H-2L. *J. Exp. Med.* 156:778.
40. Orn, A., R.S. Goodnow, L. Hood, P.R. Brayton, J.G. Woodward, R.C. Harmon, and J.A. Frelinger. 1982. Product of a transferred H-$2L^d$ gene acts as restriction element for LCMV-specific killer T cells. *Nature (Lond.).* 297:415.
41. Reddehase, M.J., H.-J. Buhring, and U.H. Koszinowski. 1986. Cloned long-term cytolytic T-lymphocyte line with specificity for an immediate-early membrane antigen of murine cytomegalovirus. *J. Virol.* 57:408.
42. Lurquin, C., A. Van Pel, B. Mariame, E. DePlaen, J.-P. Szikora, C. Janssens, M.J. Reddehase, J. Lejeune, and T. Boon. 1989. Structure of the gene of tum^- transplantation antigen P91A: the mutated exon encodes a peptide recognized with L^d by cytolytic T cells. *Cell.* 58:293.
43. Smith, M.A., J.M. Robert-Parker, R.S. Hodges, and B.H. Barber. 1986. The preparation and characterization of anti-peptide heteroantisera recognizing subregions of the intracytoplasmic domain of class I H-2 antigens. *Mol. Immunol.* 23:1077.