Model for the In Vivo Assembly of Nascent L^d Class I Molecules and for the Expression of Unfolded L^d Molecules at the Cell Surface

By Joseph D. Smith, Nancy B. Myers, John Gorka,* and Ted H. Hansen

*From the Department of Genetics and *The Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Missouri 63110*

Summary

To characterize the process of class I assembly and maturation, we have studied the L^d molecule of the mouse. Previous studies have shown that a significant proportion of intracellular and surface L^d molecules can be detected in an alternative conformation designated L^dalt[1]. Nascent L^dalt molecules are non–peptide ligand associated and are weakly associated with β_2-microglobulin (β_2m). Unexpectedly, when monoclonal antibodies were added directly to the lysis buffer, significant amounts of L^dalt/β_2m heterodimer were detected, suggesting that β_2m association is not necessarily sufficient to induce L^d conformation. By contrast, addition of peptide to cell lysates rapidly induced the folding of β_2m-associated L^dalt to conformed L^d. Furthermore, the time course and dynamics of this conversion correlated precisely with peptide binding to L^d. The precursor–product relationship of L^dalt and conformed L^d was also visualized in vivo by pulse-chase analysis of BALB/c splenocytes. To investigate the factors that regulate intracellular transport of class I molecules, expression of L^d was studied in the peptide transport–deficient cell line, RMA.S-L^d, and in $\beta_2m^{-/-}$ splenocytes. In contrast to wild-type cell lines, both L^dalt and conformed L^d are poorly expressed at the cell surface of RMA.S-L^d and $\beta_2m^{-/-}$ splenocytes. Therefore, surface expression of L^dalt is dependent upon the concomitant expression of conformed L^d molecules. To determine whether surface L^dalt molecules can result from melting of conformed L^d molecules, surface L^d molecules were loaded with several different known L^d peptide ligands. Complexes of L^d with different ligands were found to have dramatically disparate surface half-lives. Importantly, the L^d peptide complexes that turned over the most rapidly resulted in the most gain in surface L^dalt, implying that peptide dissociation can induce the accumulation of nonconformed L^d heavy chains at the cell surface.

Class I MHC are membrane-bound, cell surface glycoproteins of 45,000 mol wt. Class I heavy chains are composed of an $\alpha1\alpha2$ outer domain and a membrane proximal $\alpha3$ domain. These class I heavy chains associate with a non- MHC-encoded, non–membrane, bound light chain, β_2-microglobulin (β_2m).[1] Class I β_2m heterodimers bind peptide ligands and present them at the cell surface to CTL (1–3). Early studies of class I biosynthesis emphasized the importance of β_2m for correct class I heavy chain tertiary structure and surface expression. In accordance with this notion, class I molecules expressed in the β_2m-negative Daudi cell line are nonconformed and are not transported to the cell surface (4–6). By contrast, nonconformed class I heavy chains expressed in wild-type cell lines associate in vivo with β_2m, acquire conformational epitopes of mature folded class I molecules, and are transported to the cell surface (7). Thus, β_2m appears to be required for human class I heavy chain folding and intracellular transport.

Further studies of class I assembly have noted allele-specific differences in either the strength of class I heavy chain association with β_2m or in the kinetics of class I transport (8–10). In general, however, the premise of this original proposal has remained largely unchanged. One intriguing exception to this paradigm of class I assembly and surface transport has been noted for the murine class I molecule D^b. Unlike other class I molecules that were examined, D^b class I heavy chains expressed by the β_2m-negative murine cell line RIE-D^b were transported to the cell surface (11). D^b class I heavy chains expressed by RIE-D^b, however, are not recognized by mAb to the $\alpha1\alpha2$ domains (11). These results suggest that D^b heavy chains do not require β_2m association for endoplasmic reticulum (ER) egress, but do require β_2m for stable $\alpha1^+$ $\alpha2^+$ conformation.

[1] *Abbreviations used in this paper:* Bfa, brefeldin A; ER, endoplasmic reticulum; L^dalt, alternative L^d; β_2m, β_2-microglobulin; hβ_2m, human β_2m; PAS, protein A–Sepharose.

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More recent reports have highlighted the critical role of peptide ligand in class I structure and surface expression. The original support for this hypothesis came from study of RMA.S (12). RMA.S is a cell line immunoselected to lack class I at the cell surface. This cell line has been reported to be defective in peptide transport (12–14). However, the defect in RMA.S can be complemented either by incubating intact RMA.S cells with exogenous peptide to increase class I surface expression (12, 13) or by adding peptide ligand to RMA.S cell detergent lysates to increase the proportion of β_2m-associated class I heavy chains as detectable by conformationally dependent mAb (14). From these data, current opinion has evolved that peptide, β_2m, and heavy chain each contribute to the conformational stability and surface expression of the class I trimolecular complex.

Recently, D^b class I folding has been studied in vitro in cell lines with mutations that affect either peptide transport (e.g., RMA.S) or β_2m expression (e.g., RIE-D^b). The model which has been proposed from these studies (14, 15) describes an equilibrium between nonconformed $\alpha3^+\alpha1^-\alpha2^-$ D^b heavy chains and conformed $\alpha3^+\alpha1^+\alpha2^+$ D^b heavy chains which is influenced by peptide and β_2m. It has been suggested that peptide or β_2m, alone, is sufficient for $\alpha3^+\alpha1^+\alpha2^+$ D^b heavy chain conformation. But, together, peptide and β_2m favor stable $\alpha3^+\alpha1^+\alpha2^+$ D^b conformation. Although this proposal elegantly describes an in vitro pathway of class I assembly, the physiologic details of this model have not been completely explored owing to lack of D^b monoclonal reagents with specific reactivity for $\alpha3^+\alpha1^-\alpha2^-$ D^b.

Our investigation of class I assembly has focused upon the murine class I molecule H-2L^d. L^d molecules exist in two distinct antigenic conformations that are present both intracellularly and at the cell surface (16–18). These L^d molecular forms, which we designated alternative (L^{d}alt) and conformed L^d, are specifically distinguished by mAb (17, 18). Conformed L^d molecules are both β_2m and peptide ligand associated (16–18). By contrast, L^{d}alt molecules are weakly β_2m associated and are not peptide ligand associated (16–18). However, in biosynthetic lysates, exogenous peptide induces a titratable gain in L^d molecules in parallel with the loss of L^{d}alt (18). From these results, we have proposed that L^{d}alt are partially folded class I heavy chains awaiting constituents such as peptide and β_2m to complete their assembly and folding into mature class I molecules.

In this report we describe our further investigation into the role of peptide and β_2m in L^d antigenic structure and intracellular transport. From these studies, we define a role for peptide ligand and β_2m in L^d conformation and, in addition, we propose a physiologic pathway of L^d class I assembly, which explains the expression of L^{d}alt molecules both intracellularly and at the cell surface.

Materials and Methods

Mice and Cell Lines. L-L^d cells were generated by introducing the L^d gene into murine Ltk$^-$ DAP-3 (H-2^k) fibroblast cells. RMA.S-L^d was generated by introducing an L^d cDNA into the RMA.S cell line (19). Cell lines were maintained at 37°C, 6.5% CO_2 in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 5% FCS/5% bovine serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, and 100 U/ml of penicillin/streptomycin. $\beta_2m^{-/-}$ mice, a generous gift from Dr. Oliver Smithies (University of North Carolina, Chapel Hill, NC) were backcrossed onto BALB/c background. Mice were housed in the animal facility of Dr. Donald Shreffler, Washington University School of Medicine.

mAb. For detection of L^d molecules, mAb 30-5-7 (α2 domain) (20), mAb 64-3-7 (α1 domain) (21), and mAb 28-14-8 (α3 domain) (20) were used. All mAb are of the IgG2 isotype. W6/32 is a mouse mAb that recognizes a determinant present on β_2m associated human class I heavy chains (22). For immunoprecipitates from $\beta_2m^{-/-}$ splenocytes, immunopurified antibodies were used to avoid introducing β_2m at the time of immunoprecipitation.

Flow Cytometry. Flow cytometry was performed as previously described (17). Briefly, cells were incubated with saturating concentrations of mAb, washed, and incubated with a saturating concentration of fluorescein-conjugated, Fc-specific, affinity purified F(ab')2 fragment of goat anti-mouse IgG (Organon Teknika-Cappel, West Chester, PA). Labeled cells were analyzed using a FACScan (Becton Dickinson & Co., Mountain View, CA). Mean fluorescence values were converted from logarithmic amplification by linear regression analysis using Consort 30 software (Becton Dickinson & Co.).

Peptide Synthesis. Peptides were synthesized using Merrifield's solid-phase method (23) on a peptide synthesizer (model 431A, Applied Biosystems Inc., Foster City, CA). Peptides were purified (>90%) by reverse-phase HPLC and subjected to purity assessment techniques as described (24).

Iodination. 0.5–1 mg of peptide or 20 μg of human β_2m (hβ_2m) (Calbiochem Corp., La Jolla, CA) were iodinated using Iodo-Beads (Pierce Chemical Co., Rockford, IL) and 1–5 mCi of $Na^{125}I$ (New England Nuclear, Boston, MA; 1 Ci = 37 GBq). Iodination reaction was allowed to proceed for 30 min (peptide) or 15 min (hβ_2m) at room temperature. Radiolabeled peptide or hβ_2m were recovered by transferring the reaction mixture to a 1-ml packed AG-1X8 ion exchange resin column (Bio-Rad Laboratories, Richmond, CA) to bind free ^{125}I. Peptides were labeled to specific activities between 0.4 and 1 $\times$ 10^{16} cpm/mol. hβ_2m was labeled to 9 $\times$ 10^6 cpm/μg.

Immunoprecipitation. Immunoprecipitation of class I molecules with specific mAb was performed essentially as previously described (17) with some modifications. 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). Lysates were untreated or supplemented with peptide ligand, and L^d molecules were immunoprecipitated either directly or from precleared lysates. For preclearance, lysates were incubated two times with an equal volume of 10% (vol/vol) IgGsorb (The Enzyme Center, Malden, MA) and centrifuged, and supernatants were incubated with a saturating volume of mAb for 35 min on ice. Class I complexes were immunoprecipitated with protein A–Sepharose (PAS, Pharmacia, Inc., Piscataway, NJ) beads by incubating samples 35 min on ice. Immunoprecipitated complexes were washed three times in 3 ml cold Tris-buffered saline containing 1% NP-40 and 0.2 M PMSF. In direct immunoprecipitates, mAb were added to detergent without IgGsorb preclearance. Samples were otherwise processed as described above. Complexes of mAb-class I were eluted from PAS by incubation of 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 cen-

trifugation to remove PAS. SDS-PAGE was performed on a 10–15% linear gradient gel with a Laemmli buffer system, except for the double-labeling experiment in which samples were separated in a 10–20% polyacrylamide gradient.

For pulse-chase experiments, cells at 2 × 10^7/ml were preincubated in methionine-free medium for 20 min at 37°C. [^{35}S]methionine was added at 500 μCi/ml and incubation was continued for 5 min at 37°C. To begin the chase, samples were diluted 10-fold into prewarmed (37°C) RPMI medium (containing fivefold excess methionine) and incubation was continued at 37°C. The chase was quenched by diluting samples into a eightfold excess volume of 4°C PBS. Samples were subsequently washed one additional time with PBS and maintained on ice until they were processed for immunoprecipitation. Immunoprecipitation was as above and included two IgGsorb preclearance steps.

Densitometric analysis of class I heavy chain immunoprecipitates was performed by scanning autoradiographs on an Apple Scanner. The program and computer were kindly made available to us by Dr. Garrett Brodeur, Washington University School of Medicine. For this analysis, a computer-based program was used to analyze images from autoradiograms (Densitometer-on-a Disk, or DoaD, Imagenetics and Amoco Technology Co., Naperville, IL).

Kinetics of Peptide Association. Iodinated peptides were added to L-L^d cell lysates. The binding reaction was stopped by centrifuging lysates through spun columns packed with Sephadex G25 (superfine) (Pharmacia, Inc.) for 4 min at 2,000 rpm. Spun column eluate was divided and immunoprecipitated with mAb 30-5-7 or mAb 64-3-7. L^d-specific peptide counts per minute were determined by counting immunoprecipitates on an ICN Isomedic gamma counter and subtracting counts per minute associated with 64-3-7 immunoprecipitates from counts per minute of 30-5-7 immunoprecipitates.

Association of Iodinated hβ_2m with Class I Molecules in Cell Lysates. To monitor the association of hβ_2m with L^d in cell lysates, cells were lysed in Tris-buffered saline containing 0.5% NP-40 and freshly added PMSF. Iodinated hβ_2m was added at the time of cell lysis and cell lysates were incubated overnight at 4°C. Before immunoprecipitation cell lysates were centrifuged for 15 min at 14,000 rpm and the supernatant precleared two times with an equal volume of 10% (vol/vol) IgGsorb. Class I complexes were immunoprecipitated from precleared lysates as described above. Association of iodinated hβ_2m to L^d was determined in an Isomedic gamma counter (ICN Biomedicals, Inc., Costa Mesa, CA).

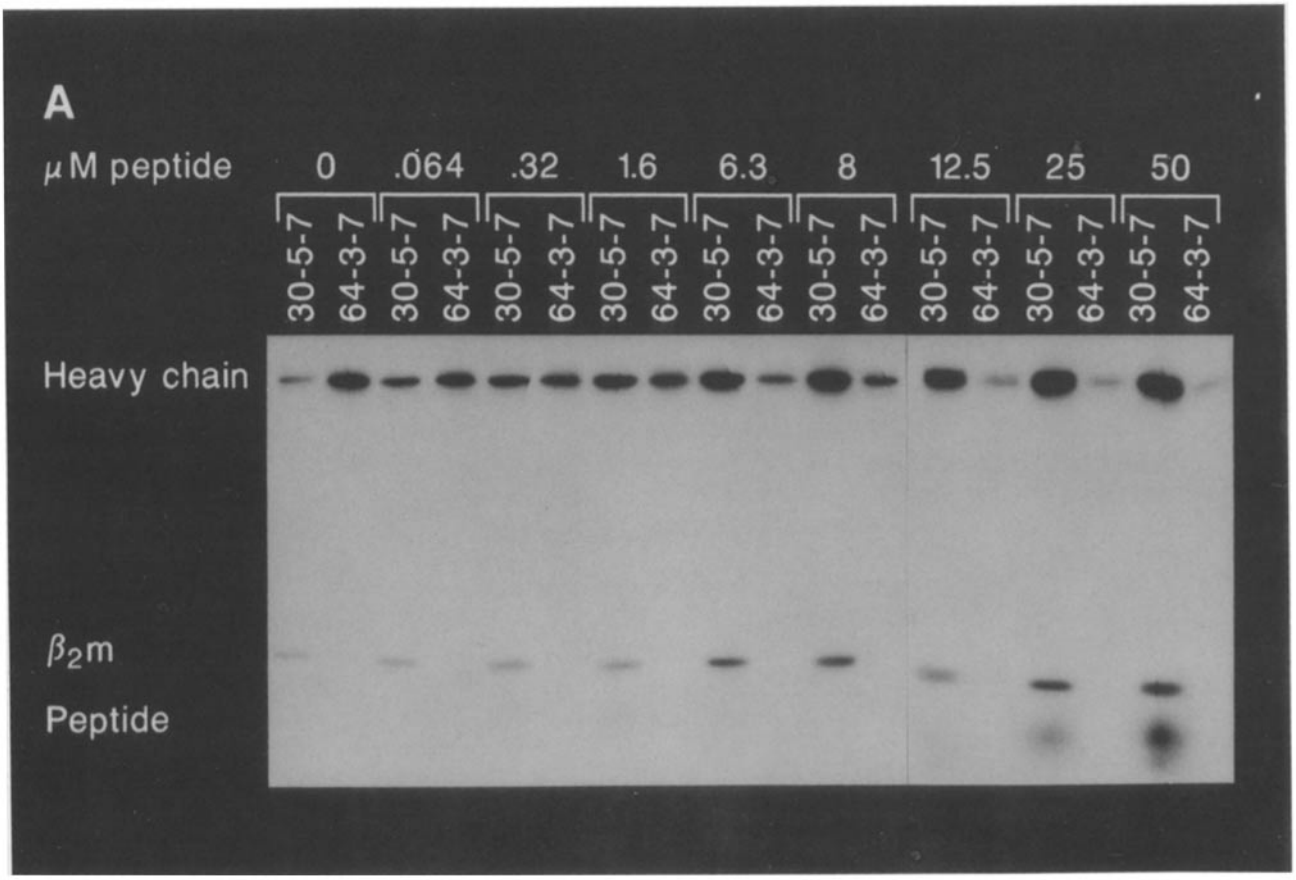
Results

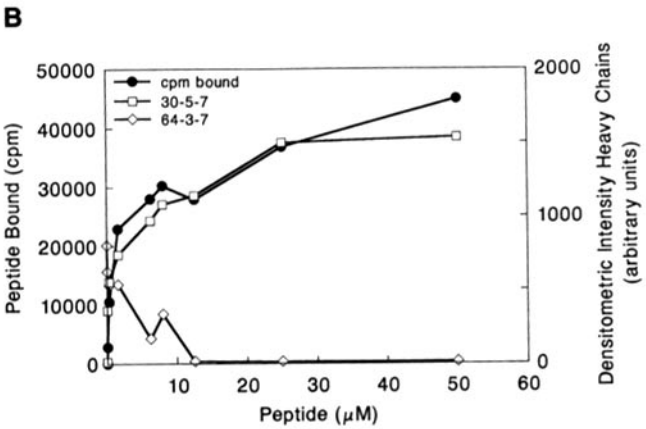
H-2L^d molecules exist in two distinct antigenic conformations that are distinguished by the mAb 64-3-7 and the mAb 30-5-7. 64-3-7$^+$ L^dalt molecules are weakly β_2m associated and are not associated with peptide ligand (16–18). By contrast, 30-5-7$^+$ L^d are both β_2m and peptide ligand associated (17, 18). In previous experiments, we observed that peptide-treated biosynthetic lysates exhibited a dramatic dose-dependent increase in 30-5-7$^+$ L^d forms in conjunction with the loss of 64-3-7$^+$ L^dalt forms (18). We have proposed that these events are directly linked and that exogenous peptide folds 64-3-7$^+$ L^dalt to 30-5-7$^+$ L^d in detergent lysates. To examine this hypothesis more critically, we have investigated the binding and kinetic parameters of peptide ligand association to L^d.

The Gain of 30-5-7$^+$ L^d Forms and Loss of 64-3-7$^+$ L^dalt Forms in L-L^d Cell Lysates Is Directly Associated with Peptide Binding to L^d. The peptide that we used in this study is derived from CMV (pp89 168-176) and has been defined previously by others as optimally sized (25), immunodominant (26), and the naturally processed L^d ligand (27). To study the association of CMV peptide with L^d, iodinated CMV peptide was added to biosynthetically labeled L-L^d detergent lysates over a range of peptide concentrations. Immunoprecipitates of 64-3-7$^+$ L^dalt and 30-5-7$^+$ L^d from these lysates were then compared by SDS-PAGE analysis. As shown in Fig. 1 *A*, iodinated CMV peptide migrates to the front of the gel as a loose doublet, which may reflect differences in the mobility of mono- or di-iodinated CMV peptide. In any case, CMV peptide is specifically present in 30-5-7$^+$ L^d immunoprecipitates, and not 64-3-7$^+$ L^dalt immunoprecipitates, at every peptide concentration tested. Moreover, iodinated CMV peptide induces a dose-dependent increase of 30-5-7$^+$ L^d in the lysate and, in the same fashion, a dose-dependent loss of 64-3-7$^+$ L^dalt (Fig. 1 *A*). In parallel to the gain of 30-5-7$^+$ L^d in the lysate, CMV peptide also increases the amount of β_2m in 30-5-7 immunoprecipitates (Fig. 1 *A*). Thus, exogenous peptide increases the proportion of β_2m-associated 30-5-7$^+$ L^d forms in the cell lysates at the expense of 64-3-7$^+$ L^dalt forms.

To quantify the binding of iodinated peptides to L^d molecules in L-L^d biosynthetic lysates, 30-5-7$^+$ L^d and 64-3-7$^+$ L^dalt immunoprecipitates from the experiment shown in Fig. 1 *A* were compared. For this comparison, radioactivity was measured by gamma counter. As [^{35}S]methionine does not register on a gamma counter, radioactive counts detected by this instrument reflect the specific binding of peptide ligands to L^d molecules. The results of this experiment are plotted in Fig. 1 *B* and compared to densitometric tracings of 30-5-7$^+$ L^d or 64-3-7$^+$ L^dalt heavy chain immunoprecipitates at the same peptide concentrations. This comparison shows that the binding of iodinated peptide to L^d molecules, the gain of 30-5-7$^+$ L^d, and the loss of 64-3-7$^+$ L^dalt molecules in cell lysates occur at an almost identical half-maximal peptide concentration (~2 μM). This finding strongly suggests that peptide binding to L^d in cell lysates is directly responsible for the gain of 30-5-7$^+$ L^d and loss of 64-3-7$^+$ L^dalt forms in cell lysates.

Iodinated CMV Peptide Rapidly Binds to L^d in Cell Lysates. We next investigated the association kinetics of CMV peptide binding to L^d. For this experiment, unlabeled L-L^d detergent lysates were incubated with a fixed concentration of iodinated CMV peptide for varying periods of time and the binding reaction was stopped by separating cell lysates over a Sephadex column to enrich for L^d bound peptide. The specific association of peptide ligand with L^d was calculated by comparing 30-5-7 and 64-3-7 immunoprecipitates. As the immunoprecipitation step occurs slowly relative to peptide association to L^d, this protocol has been designed to minimize peptide association and dissociation to L^d during immunoprecipitation. For example, Sephadex column purification minimizes the free peptide concentration and thus reduces peptide association to L^d during immunoprecipitation. In addition, immunoprecipitation is performed at 4°C so that little class I peptide dissociation occurs. In fact, L^d-CMV





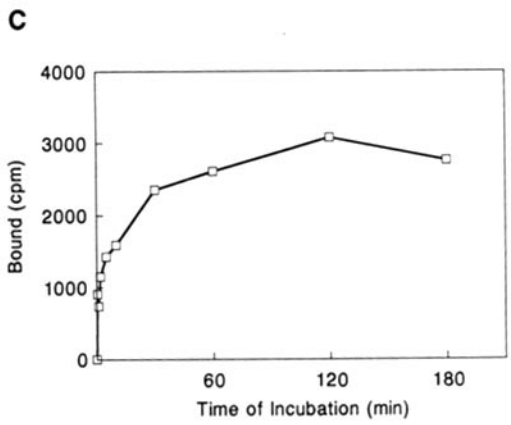


Figure 1. The binding of iodinated CMV peptides to L^d molecules in L-L^d cell lysates occurs rapidly and promotes the gain of 30-5-7$^+$ L^d and loss of 64-3-7$^+$ L^dalt class I heavy chains in cell lysates. (*A*) Dose dependency of the effect of iodinated CMV peptide on 64-3-7$^+$ and 30-5-7$^+$ forms of L^d in L-L^d biosynthetic lysates. L-L^d cells were labeled with [^{35}S]methionine for 45 min, divided into nine aliquots, and solubilized at 5 × 10^6 cells/500 μl in 0.5% NP-40 lysis buffer. Each aliquot was incubated in the presence or absence of iodinated CMV peptide (at the peptide concentrations given along top of figure) overnight at 4°C. Immunoprecipitates of mAb 30-5-7 or mAb 64-3-7 were compared on 10–20% polyacrylamide gels. Class I heavy chain, β_2m, and peptide are indicated. In the same experiment, free ^{125}I-CMV peptide ran at the front of the gel as a loose doublet (data not shown). (*B*) Quantitative comparison of the specific binding of iodinated CMV peptides to L^d molecules and the reciprocal effect of peptide on 30-5-7$^+$ L^d or 64-3-7$^+$ L^dalt forms in the lysate. The specific association of peptide to L^d (●) was determined by subtracting 64-3-7–precipitable counts per minute from 30-5-7–precipitable counts per minute. Iodinated peptide cpm in 64-3-7$^+$ immunoprecipitates never exceeded 100. Total iodinated CMV peptide bound counts (*left axis*) are compared to densitometric intensity of 30-5-7$^+$ L^d (□) or 64-3-7$^+$ L^dalt (◇) class I heavy chains shown in gel above (*right axis*). (*C*) Kinetics of CMV peptide binding to L^d in L-L^d cell lysates (□). 2 μM ^{125}I-CMV peptide was added to 10 aliquots of 5 × 10^6 L-L^d/500 μl of 0.5% NP-40 lysis buffer. Peptide association to L^d was quenched by centrifuging samples through a Sephadex column. The amount of post-Sephadex column peptide ranged from 0.02 to 0.04 μM, 35–40% of which was associated with L^d in immunoprecipitates. From initial titration experiments, this purification would assure that counts in L^d immunoprecipitates reflect precolumn binding. The specific association of peptide with L^d was calculated as described above. The results of this experiment are representative of three similar experiments.

complexes are >95% stable over a 2-h incubation at 4°C (data not shown). Thus, class I peptide association in cell lysates can be accurately measured by employing Sephadex column separation and class I immunoprecipitation. As shown in Fig. 1 *C*, little CMV peptide is associated with L^d at the zero time point confirming the efficiency of column purification. Thereafter, peptide rapidly associates with L^d with a half-time of ~2 min and reaches a plateau by ~2 h.

β_2m-associated 64-3-7$^+$ L^dalt Are Rapidly Converted to 30-5-7$^+$ L^d by Peptide Ligand. The CMV peptide-induced gain of β_2m-associated 30-5-7$^+$ L^d forms in cell lysates (Fig. 1 *A*), can be explained by two distinct but not mutually exclusive possibilities. One possibility is that peptide stabilizes 30-5-7$^+$ L^d molecules in the cell lysate that would otherwise melt to 64-3-7$^+$ L^dalt. Alternatively, peptide may promote the conversion of 64-3-7$^+$ L^dalt to 30-5-7$^+$ L^d. To study the peptide-induced folding of 64-3-7$^+$ L^dalt, independent of stabilizing effects of peptide on 30-5-7$^+$ L^d, we first assessed the stability of L^d molecules in detergent lysates. For this analysis, mAb were added directly to detergent lysates that were not precleared in order to more accurately assess the conformation of class I molecules in the cell (15). In Fig. 2 (*top*), direct immunoprecipitates of 30-5-7$^+$ L^d and 64-3-7$^+$ L^dalt are compared from L-L^d biosynthetic lysates incubated 0, 30, or 90 min on ice. The mAb 28-14-8, which binds to the α3 domain of L^d, was also included in this analysis as a control antibody that binds both L^d and L^dalt populations (17). As shown in Fig. 2, the number of 30-5-7$^+$ L^d molecules decreases only a little with time. During the same 90-min detergent incubation, 64-3-7$^+$ L^dalt remain almost unchanged, as do 28-14-8 immunoprecipitates (Fig. 2, *top*). This observation suggests that L^d molecules are quite stable over a 90-min detergent incubation at 4°C.

In the same experiment, CMV peptide was added at 30 min postlysis and the effect of peptide on L^d/L^dalt ratios was determined at 15 or 30 min, or after overnight incubation at 4°C (Fig. 2, *bottom*). Within 15 min of exogenous peptide addition, 30-5-7$^+$ L^d immunoprecipitates are increased and 64-3-7$^+$ L^dalt are decreased. As 30-5-7$^+$ L^d and 64-3-7$^+$ L^dalt are otherwise stable between 30 and 90 min postlysis in the absence of exogenous peptide (Fig. 2, *top*), this finding implies that peptide has folded 64-3-7$^+$ L^dalt forms to 30-5-7$^+$ L^d. Furthermore, the effect of CMV peptide on L^d/L^dalt ratios was as complete at the 15-min time point as it was after overnight incubation. Therefore, CMV peptide-induced conversion of 64-3-7$^+$ L^dalt to 30-5-7$^+$ L^d occurs with rapid kinetics similar to iodinated peptide association to L^d (Fig. 1 *C*).

Also shown in the experiment in Fig. 2, β_2m is present in 64-3-7$^+$ L^dalt immunoprecipitates (Fig. 2, *top*). By comparison to immunoprecipitates of precleared lysates, this association is easily detected in direct immunoprecipitates (compare Figs. 1 and 2). Interestingly, 64-3-7$^+$ L^dalt association with β_2m is relatively stable over 90 min of incubation at 4°C (Fig. 2, *top*). However, exogenous peptide both reduces the number of β_2m associated 64-3-7$^+$ L^dalt in cell lysates and increases the number of β_2m associated 30-5-7$^+$ L^d (Fig. 2, *bottom*). This finding implies that β_2m-associated 64-3-7$^+$ L^dalt heterodimers in L-L^d detergent lysates are readily converted by peptide ligand to 30-5-7$^+$ L^d. It is also possible that some of the gain of 30-5-7$^+$ L^d may be the result of peptide driving the assembly of free L^dalt heavy chains and β_2m. It is interesting that the amount of β_2m in 28-14-8 immunoprecipitates is increased by exogenous CMV peptide (Fig. 2). This result suggests that β_2m has a greater affinity for peptide-associated 30-5-7$^+$ L^d heavy chains than for free 64-3-7$^+$ L^dalt heavy chains and is consistent with the more prominent β_2m association in 30-5-7$^+$ L^d immunoprecipitates than in 64-3-7$^+$ L^dalt immunoprecipitates.

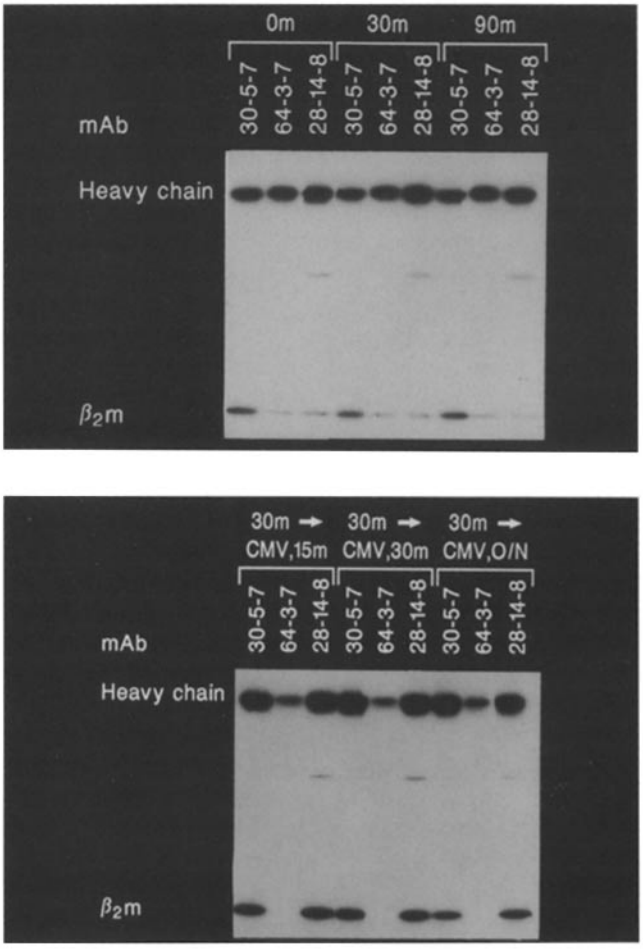
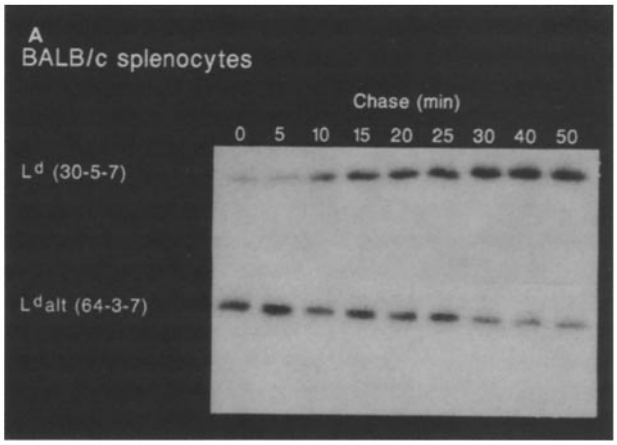


Figure 2. Exogenous CMV peptide rapidly promotes the conversion of β_2m associated 64-3-7$^+$ L^dalt to 30-5-7$^+$ L^d in L-L^d cell lysates. 4.5 × 10^7 L-L^d were biosynthetically labeled with [^{35}S]methionine for 45 min at 37°C. Cells were divided into 18 aliquots and each aliquot was lysed in a final volume of 250 μl of 0.5% NP-40 lysis buffer. For 4°C detergent incubations in the absence of peptide (*top*), mAb 30-5-7, 64-3-7, or 28-14-8 were added at 0, 30, or 90 min postlysis without prior preclearance of cell lysates with IgGsorb. In CMV peptide-treated lysates (*bottom*), cells were lysed in 0.5% NP40, and 30 min post-lysis CMV peptide in 0.5% NP-40 lysis buffer was added to give a final concentration of 500 μM CMV. 30-5-7, 64-3-7, and 28-14-8 direct immunoprecipitates were compared from lysates after peptide addition at 15 or 30 min, or after overnight incubation at 4°C. Similar results were obtained in other experiments with a different L^d peptide ligand derived from lymphochoriomenigitis virus (data not shown).

Iodinated hβ_2m Associates with Both 64-3-7$^+$ L^dalt and

30-5-7$^+$ L^d Cell Lysates. To extend the above observation that β_2m associates with both 64-3-7$^+$ L^dalt and 30-5-7$^+$ L^d, iodinated hβ_2m was added to L-L^d cell lysates or to cell lysates from the untransfected control cell line DAP-3. As shown in Fig. 3, iodinated hβ_2m specifically associated in a dose-dependent fashion to both 64-3-7$^+$ L^dalt and 30-5-7$^+$ L^d. Furthermore, in data not shown, excess unlabeled hβ_2m was found to specifically inhibit the association of labeled hβ_2m with both 64-3-7$^+$ L^dalt and 30-5-7$^+$ L^d. It is noteworthy that the association of iodinated hβ_2m is more prominent in immunoprecipitates of 30-5-7$^+$ L^d than in immunoprecipitates of 64-3-7$^+$ L^dalt. This may, in part, reflect a weaker affinity of β_2m for non-peptide ligand-associated L^dalt than for peptide-associated L^d heavy chains as suggested above (Fig. 2). However, 64-3-7$^+$ L^dalt clearly show significant β_2m association as detected in immunoprecipitates with either endogenous murine β_2m (Fig. 2) or human β_2m (Fig. 3). The detection of β_2m/64-3-7$^+$ L^dalt heterodimers is significant, because it implies that β_2m association need not be sufficient for class I heavy chain folding.

64-3-7$^+$ L^dalt Are the Precursor of 30-5-7$^+$ L^d in BALB/c Splenocytes. The results discussed above strongly suggest that in vitro peptide ligand can convert 64-3-7$^+$ L^dalt to 30-5-7$^+$ L^d. To extend these findings in vivo, we have examined the antigenic relationship of these molecules in BALB/c splenocytes. In the experiment shown in Fig. 4, BALB/c splenocytes were pulsed with labeled methionine for 5 min and then chased in the presence of excess unlabeled methionine. Splenocytes were followed over a 50-min chase period during which 30-5-7 and 64-3-7 immunoprecipitates were compared. As shown in Fig. 4 *A*, both 30-5-7$^+$ L^d and 64-3-7$^+$ L^dalt forms are present even at the earliest time points (Fig. 4 *A*). However, with increasing time of chase the amount of 30-5-7$^+$ L^d increases and the amount of 64-3-7$^+$ L^dalt decreases. The criss-cross relationship of 64-3-7$^+$ L^dalt and 30-5-7$^+$ L^d over the course of the pulse-chase analysis suggests that 64-3-7$^+$ L^dalt fold in vivo into 30-5-7$^+$ L^d (Fig. 4 *B*).



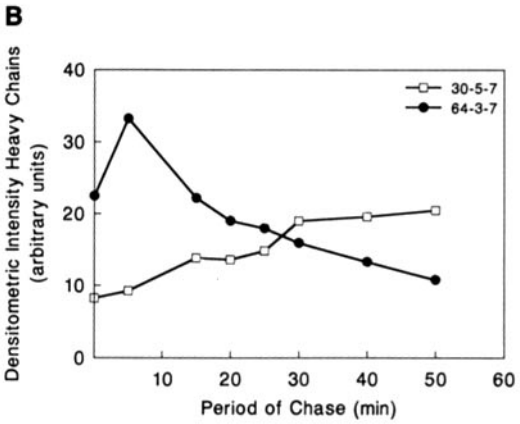


Figure 4. 64-3-7$^+$ L^dalt are the precursor of 30-5-7$^+$ L^d in BALB/c splenocytes. (*A*) Pulse-chase experiment comparing the maturation of 64-3-7$^+$ L^dalt and 30-5-7$^+$ L^d. 9 × 10^7 BALB/c splenocytes were labeled with [^{35}S]methionine for 5 min at 37°C. For chase, aliquots of 1 × 10^7 cells were transferred to a 10-fold volume of RPMI containing fivefold excess methionine, and incubation was continued at 37°C. For each time point, 30-5-7 and 64-3-7 immunoprecipitates were analyzed by 10–15% gradient SDS-PAGE. (*B*) Quantitative comparison of 30-5-7$^+$ L^d (□) or 64-3-7$^+$ L^dalt (●) heavy chain immunoprecipitates.

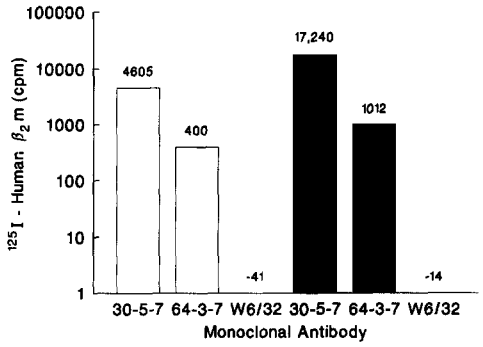


Figure 3. Exogenous hβ_2m associates with both 64-3-7$^+$ L^dalt and 30-5-7$^+$ L^d in L-L^d cell lysates. 12 × 10^6 DAP-3 or L-L^d cells were lysed in 450 μL 0.5% NP-40 containing either 0.1 μM (□) or 1.0 μM (■) iodinated hβ_2m. The specific association of iodinated hβ_2m to L^d was determined by subtracting DAP-3–precipitable counts per minute from L-L^d–precipitable counts per minute. The actual precipitable counts per minute from L-L^d or DAP-3 cell lysates were respectively: 0.1 μM hβ_2m, 30-5-7 (4679, 74), 64-3-7 (426, 26), W6/32 (84, 125); 1.0 μM hβ_2m, 30-5-7 (17726, 486), 64-3-7 (1510, 498), W6/32 (677, 691).

30-5-7$^+$ L^d Are Detectable in Direct Immunoprecipitates of RMA.S-L^d and $\beta_2m^{-/-}$ Splenocytes. To investigate the relative contributions of peptide and β_2m to L^d conformation and intracellular transport, L^d expression was assayed in the peptide transport-deficient cell line RMA.S-L^d and in β_2m-deficient splenocytes. L-L^d cells were also included in this analysis as a wild-type control. L^d molecules in these three different cell types were biosynthetically labeled and immunoprecipitates were compared from cell lysates at 0 min, 4 h, or after overnight incubation. Surprisingly, 30-5-7$^+$ L^d were detected in direct immunoprecipitates from all three cell lines (Fig. 5, 0 min [*0m*]), indicating that neither optimal peptide loading nor β_2m association are absolute requirements for in vivo folding of L^d. However, the majority of 30-5-7$^+$ L^d in RMA.S-L^d and $\beta_2m^{-/-}$ splenocytes are unstable and quickly lost with 4°C incubation (Fig. 5, *middle* and *bottom* panels; compare *0m*, *4h*, and *O/N*). Thus, op-

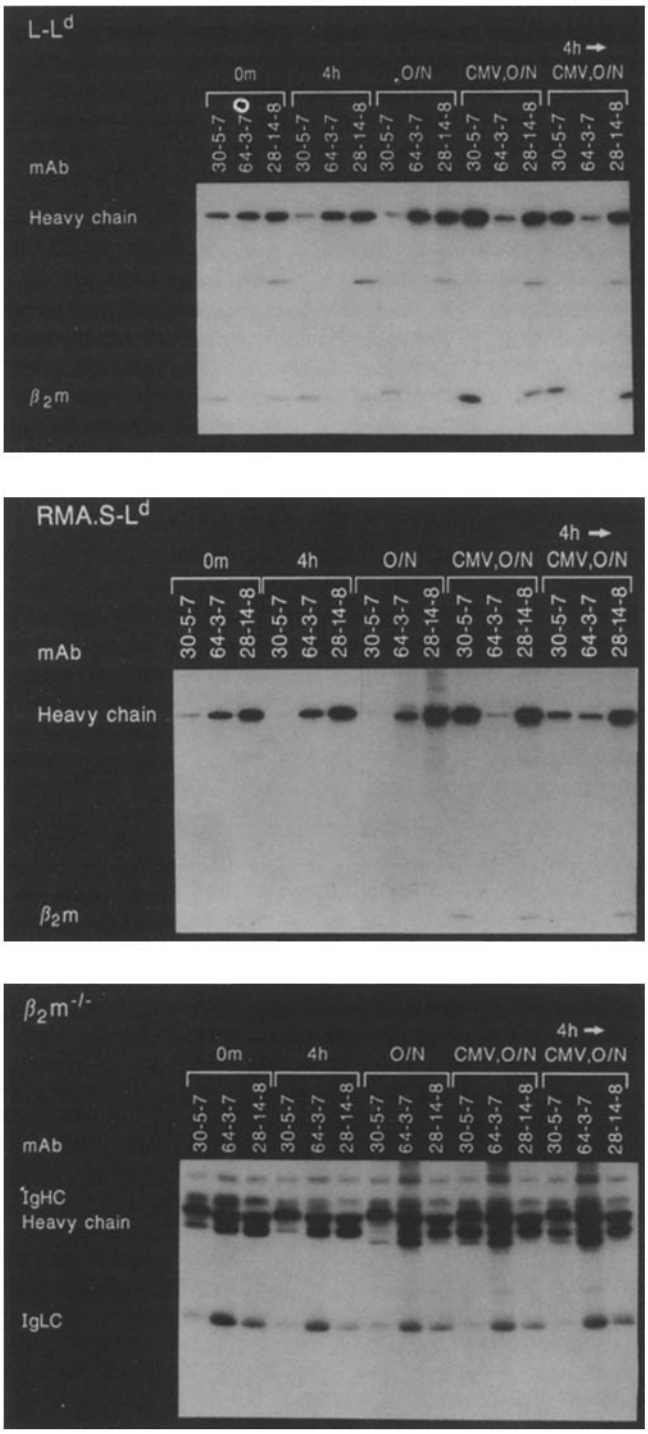


Figure 5. 30-5-7$^+$ L^d molecules are detected in cell lysates in the absence of β_2m or under suboptimal peptide conditions. Immunoprecipitates of 30-5-7$^+$ L^d, 64-3-7$^+$ L^{d}alt, or 28-14-8$^+$ L^d(α3) were compared from L-L^d cells (*top*), RMA.S-L^d cells (*middle*), or β_2m$^{-/-}$ splenocytes (*bottom*). For each cell type, 3.75 × 10^7 cells were labeled with [^{35}S]methionine for 45 min. Cells were divided into 15 aliquots of 2.5 × 10^6 cells and each aliquot was detergent-lysed in a final volume of 133 μl of 0.5% NP-40 lysis buffer. For comparison of stability of class I molecules during 4°C detergent incubation, mAb 30-5-7, mAb 64-3-7, or mAb 28-14-8 was added at 0 min, 4 h, or after overnight incubation at 4°C. For CMV peptide-treated lysates, 400 μM CMV peptide was added to 2.5 × 10^6 cells in 133 μl 0.5% NP-40 lysis buffer, or at 4 h postlysis 33 μl CMV peptide was added in 0.5% NP-40 lysis buffer to cell lysates to give a final volume of 133 μl and final concentration of 400 μM peptide. Lysates were incubated overnight at 4°C before direct immunoprecipitation with mAb 30-5-7, 64-3-7, or 28-14-8. Because β_2m$^{-/-}$ splenocytes were not precleared before lysis, biosynthetically labeled immunoglobulin from B lymphocytes in this preparation were introduced into the immunoprecipitates. Endogenous immunoglobulin heavy chain (Ig HC) and light chain (Ig LC) are indicated on the left side of the figure.

timal peptide loading and β_2m association appear to be required for stable 30-5-7$^+$ L^d conformation. Interestingly, a proportion of 30-5-7$^+$ L^d expressed in L-L^d are also unstable (Fig. 5, *top* panel; compare *0m*, *4h*, and *O/N*) indicating that unstable 30-5-7$^+$ L^d complexes are not restricted to mutant cell lines. Indeed, some labile 30-5-7$^+$ L^d are present in all cell lines which we have examined including BALB/c splenocytes (data not shown). This finding may imply that peptide and/or β_2m are limiting for L^d assembly in all cell types. Alternatively, unstable 30-5-7$^+$ L^d may contain suboptimal peptide ligands. In the same experiment, immunoprecipitates with mAb 64-3-7 and 28-14-8 are unchanged or slightly increased with detergent incubation at 4°C (Fig. 5, compare *0m*, *4h*, and *O/N*).

The loss of 30-5-7$^+$ L^d which is detected in lysates from RMA.S-L^d cells, β_2m$^{-/-}$ splenocytes, and L-L^d cells after overnight incubation can be prevented by immediate addition of exogenous CMV peptide (Fig. 5, compare *0m*, *O/N* and *CMV,O/N*). This result implies that exogenous peptide can stabilize 30-5-7$^+$ L^d that would otherwise melt with overnight incubation. Exogenous peptide, in addition to stabilizing 30-5-7$^+$ L^d, also reduced 64-3-7$^+$ L^{d}alt immunoprecipitates (Fig 5, compare *0m*, *O/N*, and *CMV,O/N*). Therefore, exogenous peptide both stabilizes 30-5-7$^+$ L^d in cell lysates (Fig. 5) and promotes the conversion of 64-3-7$^+$ L^{d}alt to 30-5-7$^+$ L^d (Fig. 2). Interestingly, exogenous peptide alone, in β_2m$^{-/-}$ splenocytes appears to be sufficient to fold free L^{d}alt heavy chains (Fig. 5, *bottom*; compare *0m*, *4h*, *O/N*, and *4h→CMV, O/N*).

Although 30-5-7$^+$ L^d were lost with 4 h of incubation, this loss was reversible by addition of exogenous CMV peptide (Fig. 5, compare *0m*, *4h*, *O/N*, and *4h→CMV,O/N*). However, by delaying CMV peptide addition until 4 h postlysis, the gain of 30-5-7$^+$ L^d was not as dramatic as with immediate CMV peptide addition (Fig. 5, compare *CMV,O/N*, and *4h→CMV, O/N*). This result suggests that L^d molecules in cells lysates become refractory to acquisition of peptide-induced 30-5-7$^+$ L^d conformation with detergent incubation.

Both 30-5-7$^+$ L^d and 64-3-7$^+$ L^{d}alt Are Poorly Expressed at the Surface of RMA.S-L^d Cells and β_2m$^{-/-}$ Splenocytes. To study the surface expression of L^d molecules in the absence of β_2m or under suboptimal peptide conditions, β_2m$^{-/-}$ splenocytes and RMAS-L^d were examined by flow cytometry for 30-5-7$^+$ L^d and 64-3-7$^+$ L^{d}alt expression. As a positive control, L^d expression was also monitored on BALB/c splenocytes and L-L^d cells. Although both 30-5-7$^+$ L^d and 64-3-7$^+$ L^{d}alt are well expressed at the surface of L-L^d (Fig.

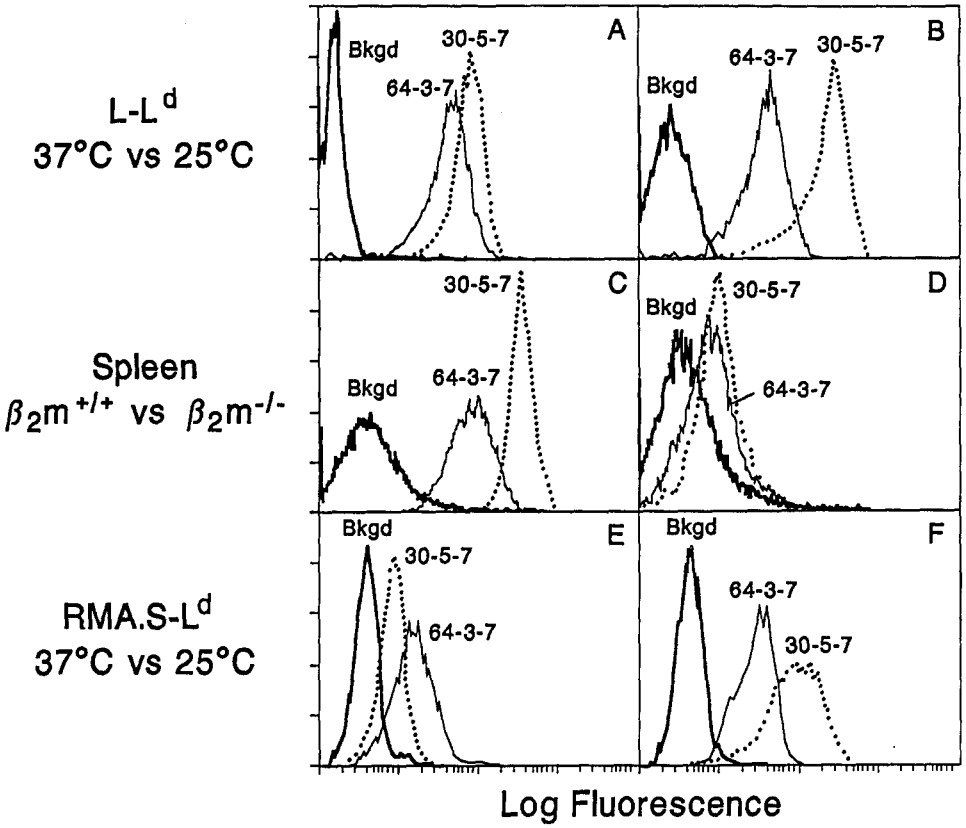


Figure 6. Surface expression of alternative forms of L^d on various cell types. Non-peptide-associated L^dalt molecules were detected with mAb 64-3-7 (–) and conformed L^d were detected with mAb 30-5-7 (···). Fluorometric histograms are shown for L-L^d cells grown at 37°C (*A*) or 25°C (*B*), $\beta_2m^{+/+}$ spleen cells (*C*) or $\beta_2m^{-/-}$ spleen cells (*D*), and RMA.S-L^d cells grown at 37°C (*E*) or 25°C (*F*). Background (*Bkgd*) was determined using the secondary antibody alone. Mean channels for Bkgd, 64-3-7, and 30-5-7 were respectively: *A* (2, 48, 77), *B* (8, 44, 236), *C* (16, 138 350), *D* (15, 18, 21), *E* (5, 19, 10), and *F* (6, 29, 99).

6 *A*), neither L^d form is expressed well at the surface of $\beta_2m^{-/-}$ splenocytes (Fig. 6 *D*) or RMA.S-L^d (Fig. 6 *E*). Compared to BALB/c splenocytes, 30-5-7$^+$ L^d expression is reduced by greater than 50-fold in $\beta_2m^{-/-}$ splenocytes (Fig. 6). The expression of 30-5-7$^+$ L^d is similarly decreased in RMA.S-L^d (Fig. 6 *E*) relative to RMA-L^d (data not shown). Although 30-5-7$^+$ L^d are weakly expressed at the surface of RMA.S-L^d, this expression is increased by 25°C incubation (Fig. 6 *F*). A comparison between the surface expression of 30-5-7$^+$ L^d molecules in different cell backgrounds (Fig. 6) and the stability of 30-5-7$^+$ L^d in biosynthetic lysates (Fig. 5) suggests that in the absence of β_2m or under suboptimal peptide conditions 30-5-7$^+$ L^d are unstable and poorly expressed at the cell surface. Moreover, the low surface expression of 64-3-7$^+$ L^dalt in RMA.S-L^d cells and $\beta_2m^{-/-}$ splenocytes implies that surface expression of these molecules, like 30-5-7$^+$ L^d, is limited by the intracellular availability of peptide and β_2m. Indeed, surface 64-3-7$^+$ L^dalt expression appeared to correlate with the relative amount of 30-5-7$^+$ L^d expressed on the cell surface (Fig. 6 and reference 17). This observation raised the possibility the 64-3-7$^+$ L^dalt are melted 30-5-7$^+$ L^d forms that arise during post-ER transport or at the cell surface.

Rapid Turnover of 30-5-7$^+$ L^d-Peptide Complexes at the Cell Surface Results in the Appearance of 64-3-7$^+$ L^dalt. To determine whether peptide dissociation is responsible for the accumulation of surface L^dalt, the turnover of various L^d/peptide complexes was compared. L-L^d cells were incubated overnight with four different peptide ligands: CMV (25), lymphocytic choriomeningitis virus peptide (LCMV) (28), tum$^-$ (29), or the endogenous peptide P29 (30). As expected, each of these ligands increased surface expression of L^d 4 to sixfold (Fig. 7 *A*). This level of induction insured a high percentage of the L^d molecules were loaded with the respective exogenous ligand with which the cells were cultured. Previous studies have shown that this peptide-induced increase in L^d expression is attributable to exogenous peptide binding to accessible ligand binding sites on surface L^d and thereby dramatically prolonging their half-life (18, 31). To compare the turnover of the L^d molecules bound by each of these four different ligands, cells were washed after overnight incubation with peptide and treated with Brefeldin A (Bfa) to prevent new arrival of L^d at the cell surface. As shown in Fig. 7 *A*, the turnover of the different L^d/peptide complexes varied considerably. For example, P29/L^d complexes were stable during the 2-h incubation, whereas the CMV/L^d complexes displayed a half-life of <1 h. Importantly, the turnover rate of these complexes correlated precisely with the amount of 64-3-7$^+$ L^dalt detected (Fig. 7 *B*). Thus peptides yielding the least stable L^d complexes resulted in the gain of the most 64-3-7$^+$ L^dalt. It should be noted that the magnitude of loss of 30-5-7$^+$ L^d was greater than the increase in 64-3-7$^+$ L^dalt during the 2-h incubation (compare scale of Fig. 7 *A* with that of 7 *B*). Furthermore, the gain of 64-3-7$^+$ L^dalt appeared to be somewhat transient. It was surprising to us that L^dalt were unstable under these conditions, as they appeared very stable in other assays in the continual presence of Bfa and exogenous peptide (18,

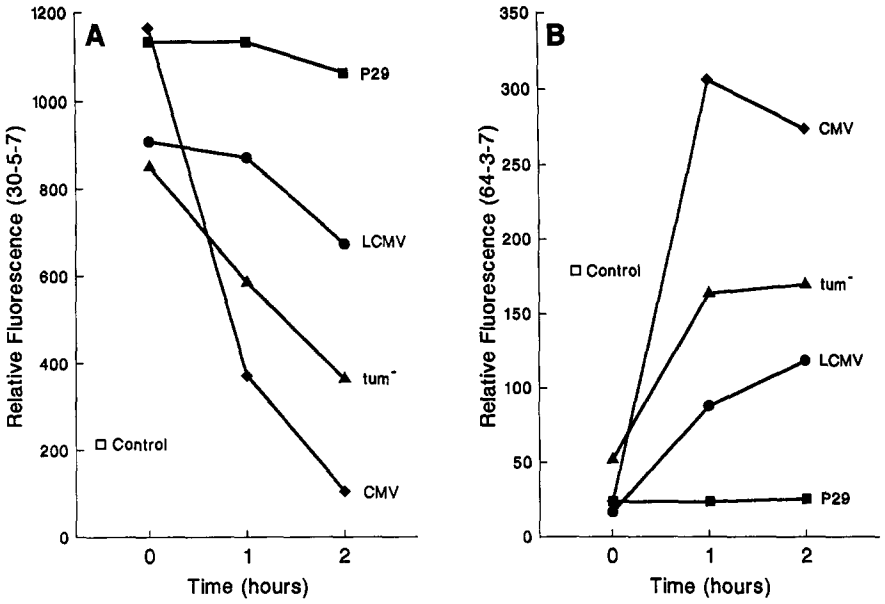


Figure 7. Comparison of the turnover rate of surface L^d occupied by various peptide ligands. L-L^d cells were cultured overnight with saturating concentrations (250 μM) of four different known L^d ligands: P29 (■), YPNVNIHNF (30), LCMV (●), RPQASGVYM (28); tum⁻ (▲), TQNHRALDL (29); and CMV (◆), YPHFMPTNL (25). Cells were washed to remove unbound peptide and treated with Bfa (5 μg/ml) (Epicentre Technologies, Madison, WI) to prevent new arrival of class I at the cell surface. After 0, 1, or 2 h cells were tested by flow cytometry to measure the expression of 30-5-7$^+$ L^d (*A*) or 64-3-7$^+$ L^dalt (*B*). The level of expression of 30-5-7$^+$ L^d and 64-3-7$^+$ L^dalt on control and untreated L-L^d cells is indicated by the box (□) shown in *A* and *B*.

and data not shown). However, it is not completely clear what factors regulate the turnover rate of L^dalt at the cell surface and whether other conformational variants may be involved in class I surface turnover (32). In any case, the results shown in Fig. 7 suggest that bound peptide can determine the cell surface stability of conformed L^d and also that peptide dissociation from conformed L^d can lead to the accumulation of L^dalt at the cell surface. Thus, mAb 64-3-7 detects both partially folded nascent L^d molecules awaiting peptide to undergo de novo folding as well as unfolded L^d molecules accumulating at the cell surface after peptide dissociation.

Discussion

In this report we have investigated the relative contributions of peptide and β_2m to L^d conformation and cell surface transport. This study exploits the availability of a unique mAb, 64-3-7, that detects non-peptide-associated, partially folded forms of L^d, designated L^dalt. L^dalt heavy chains are present both intracellularly and at the cell surface of all L^d-expressing cell lines that we have examined (17). In a previous investigation (18), we found that addition of peptide ligands to cell lysates results in increases in folded L^d as detected by conformation-dependent mAb such as 30-5-7. Accompanying this increase, a decrease in L^dalt was also observed. Based on this result we proposed that L^dalt molecules are partially folded class I heavy chain intermediates awaiting peptide and β_2m to complete their folding to mature conformed class I molecules. Strong evidence in support of this hypothesis is presented here. For instance, we have compared the binding of iodinated peptides to L^d molecules, the gain of L^d forms, and the loss of L^dalt forms in cell lysates over a range of peptide concentrations (Fig. 1 *A*). These three effects of exogenous peptide on L^d molecules appear to be directly related as they occur at an almost identical half-maximal peptide concentration (Fig. 1 B). In addition, the time course of iodinated CMV peptide binding to L^d in L-L^d lysates was rapid (Fig. 1 *C*), as is the peptide-induced loss of L^dalt and the gain of conformed L^d in cell lysates (Fig. 2). Together, these results strongly suggest that peptide binding to L^dalt is directly linked to their conversion to 30-5-7$^+$ L^d. These observations validate this as a system to monitor peptide induced in vitro folding of class I molecules.

Although peptide binding to class I is reported to be rapid under many experimental conditions (33–36), the peptide occupancy of class I molecules in many of these experimental systems is unknown (33, 35, 36). Peptide association to partially folded, "empty," and peptide-occupied class I complexes may differ. Indeed, for peptide-occupied class I, the binding of exogenous peptide can depend upon the dissociation rate of the prebound peptide (31, 37). In this respect, we suggest that our experimental system is uniquely suited to studying the physiological interaction of peptide ligand with nascent class I heavy chains.

In vivo, L^d increase and L^dalt decrease with increasing time of chase in BALB/c splenocytes (Fig. 4). In addition, similar results were derived from study of P815 cells and L-L^d cells plus or minus cyclohexamide to prevent nascent chain elongation (data not shown). We suggest that L^dalt associate in vivo with peptide and β_2m and fold to conformed L^d. This explanation of L^d class I folding parallels earlier findings of human class I assembly. These parallels include a population of non-conformed class I heavy chains which associate in vivo with β_2m to acquire conformational epitopes of mature folded class I molecules (7). Our interpretation of L^d pulse-chase experiments is supported by in vitro analysis of L^d folding (Figs. 1 and 2) and has been extended to include a role for peptide ligand.

Although we favor the notion that L^dalt assemble in vivo with β_2m and peptide to fold to conformed L^d, this does not exclude alternative fates for these molecules. In fact, in pulse-chase experiments with longer pulses, L^dalt molecules accumulate during the chase period so that it is difficult to define a precursor–product relationship between these two

L^d molecular forms (17). Perhaps the intracellular availability of peptide and β_2m determines the efficiency of L^dalt folding to conformed L^d. Investigation of RMA.S-L^d and $\beta_2m^{-/-}$ splenocytes supports this possibility. In these cell lines, the intracellular ratio of L^d molecules is shifted in favor of L^dalt and most L^d are unstable (Fig. 5). Therefore peptide and β_2m may influence L^d antigenic structure. However, the finding that conformed L^d are present in these cell lines suggests that neither optimal peptide availability nor β_2m may be strictly necessary for L^d conformation.

Three lines of evidence support the hypothesis that peptide is sufficient for L^d conformation. First, conformed L^d are present both intracellularly and at the cell surface of $\beta_2m^{-/-}$ splenocytes (Figs. 5 and 6). Second, exogenous CMV peptide, alone, is sufficient to fold free L^dalt heavy chains to L^d in $\beta_2m^{-/-}$ splenocytes (Fig. 5). Third, $\beta_2m^{-/-}$ splenocytes are lysed by alloreactive L^d CTL clones specific for the 2C peptide (38, J. Connolly, personal communication), implying that surface L^d are peptide occupied. In contrast, there is some controversy about the ability of D^b to fold with peptide alone. In early reports, conformed D^b molecules were not detected in RIE-D^b cell lysates implying that β_2m was necessary for D^b class I heavy chain folding (11). However, in a recent report, conformed D^b molecules were detected by fluorometric analysis of $\beta_2m^{-/-}$ splenocytes (39). In addition, evidence has been presented that exogenous peptide is sufficient to fold free D^b heavy chains in RIE-D^b cell lysates (15). Therefore, peptide appears to be sufficient to fold both free D^b and L^d heavy chains. Further experiments will be required to determine the extent to which different class I allele products may differ either in their ability to fold or to be transported to the cell surface in the absence of β_2m. In this regard, we detect conformed D^d molecules in direct immunoprecipitates from $\beta_2m^{-/-}$ splenocytes but D^d molecules are poorly if at all expressed at the cell surface of these same cells (data not shown).

The sufficiency of β_2m for L^d folding and surface transport is less clear. Because $\alpha3^+\alpha1^+\alpha2^+$ D^b have been detected in RMA.S cell lysates, it was previously proposed that β_2m is sufficient for D^b folding (14, 15). We also detect conformed L^d in RMA.S-L^d cell lysates (Fig. 5), implying that β_2m may be sufficient for L^d heavy chain folding. However, it remains possible that conformed class I molecules in RMA.S cell lysates are peptide occupied to some degree (40, 41). The association of β_2m with L^dalt reported here is very intriguing (Figs. 2 and 3). It is important to note that the significant association of endogenous murine β_2m to L^dalt detected here, but not in earlier studies (17, 18), is attributable to the fact that in these analyses we added mAb directly to the lysis buffer. This method was previously described by others to more accurately reflect the in vivo assembly of β_2m with class I (15). The detection of L^dalt/β_2m complexes has three important implications. First, this result suggests that β_2m association alone is insufficient to induce L^d heavy chain folding. Second, this finding implies that β_2m association with L^d can precede peptide binding. Indeed, in vitro, L^dalt-β_2m heterodimers are readily folded by peptide ligand to L^d (Fig. 2). Finally, this observation suggests that peptides may be limiting in vivo for L^d trimolecular assembly. In fact, β_2m assembled L^dalt are readily detected in all cell types including BALB/c splenocytes (data not shown) and an even more striking association is detected for β_2m to L^qalt (Smith, J. D., manuscript in preparation). If peptide is limiting for L^d assembly, then this finding may have general implications for the availability of peptide for class I assembly. The association of β_2m with unfolded L^dalt heavy chains would appear to distinguish our findings from the elegant pathway of D^b assembly proposed earlier (14, 15). However, the general features of our findings are in complete accordance with their model including: (*a*) an equilibrium between unfolded and folded class I forms and (*b*) the influence of β_2m and peptide in facilitating class I heavy chain folding. The significant distinction between our results and the earlier findings of D^b folding (14, 15) is that peptide appears sufficient for L^d folding whereas β_2m does not.

In addition to influencing the antigenic nature of L^d molecules, peptide and β_2m also affect L^d surface expression. Compared to RMA-L^d (data not shown) and BALB/c splenocytes (Fig. 6 *C*), both L^d and L^dalt molecules are poorly expressed at the cell surface of RMA.S-Ld and $\beta_2m^{-/-}$ splenocytes (Fig. 6). The correlation between L^d and L^dalt surface expression on all cell type suggests that surface L^dalt may be derived from conformed L^d that melt during post-endoplasmic reticulum transport or at the cell surface. Thus, the small number of conformed L^d present at the surface of RMA.S-L^d and $\beta_2m^{-/-}$ splenocytes could account for low surface expression of L^dalt molecules on these cell lines.

We have investigated the capacity of surface, conformed L^d molecules to denature to L^dalt. For these experiments, live cells were incubated with L^d ligands to increase surface L^d expression and the fate of these molecules was monitored over time. A direct correlation was found between the turnover rate of L^d-peptide complexes and the gain of L^dalt at the surface (Fig. 7). This finding supports the hypothesis that L^dalt molecules accumulate at the cell surface as a result of peptide dissociation from conformed L^d molecules. Indeed, this is the first demonstration, of which we are aware, that cell surface turnover of peptide occupied class I molecules can result in the appearance of unfolded class I heavy chains at the cell surface. In more general terms, the surface expression of unfolded class I heavy chains has been described for D^b, K^b, and D^d (32, 42, 43). However, in these studies, unfolded class I heavy chains were induced at the cell surface by incubating cells at reduced temperature and then shifting the cells to physiologic temperature in order to study melted class I forms. The above mentioned studies do support the notion that surface expression of unfolded class I heavy chains is not restricted to certain unique murine class I alleles. The surface expression of unfolded class I heavy chains also includes human cell lines, such as activated B and T cells (44, 45). In this regard, we have recently detected denatured forms of several different HLA class I molecules on resting peripheral blood lymphocytes and provide evidence that they result from peptide dissociation (Carreno B., and T. H. Hanson, manuscript submitted). Thus, surface expression of denatured

free class I heavy chain appears to be extensive and may not be absolutely restricted to certain unique cell lineages.

In conclusion, many of the principles of L^d trimolecular assembly and expression which we describe here are consistent with observations in the literature for other murine or human class I alleles. These include the existence of partially folded class I heavy chains that appear to associate in vivo with peptide and β_2m to acquire conformational epitopes of mature class I molecules. In this report, we have discussed some of the parallels between L^d and D^b in their association with β_2m and their peptide-induced folding. In addition, unfolded D^b molecules expressed in the β_2m-negative cell line RIE-D^b share several properties with L^dalt. These include: (*a*) their detection by mAb to the $\alpha 3$ domain and not mAb to the $\alpha 1\alpha 2$ domain, (*b*) their expression both intracellularly and at the cell surface, and (*c*) their ability to associate with peptide and β_2m in cell lysates to acquire $\alpha 1\alpha 2$ class heavy chain conformation. Originally, surface expression of nonconformed D^b molecules was taken as evidence that these molecules assumed an alternative conformation distinct from folded class I molecules that permitted their intracellular transport (46). However, it is now clear that nonconformed D^b are inefficiently expressed at the surface of RMA.S cells (12) and β_2m-negative splenocytes (39) and, in addition, these two cell types and RIE-D^b cells all express small numbers of conformed D^b (12, 13, 39). We suggest that nonconformed D^b, like L^dalt, may accumulate at the cell surface from melting of conformed class I molecules. If this is the case, then class I molecules may accumulate at the cell surface of peptide-deficient or β_2m-deficient cell lines to a greater or lesser degree depending on their ability to efficiently fold in the absence of peptide or β_2m and exit the ER. Thus the unique abilities of D^b (11) and L^d to be expressed at the cell surface in the absence of β_2m may be explained by their capacity to fold intracellularly with peptide alone. If valid, this assumption would predict that it is the folding state of the class I heavy chain and not β_2m-association that permits ER egress.

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Address correspondence to Dr. Ted H. Hansen, Department of Genetics, Washington University School of Medicine, Box 8232, 4566 Scott Avenue, St. Louis, MO 63110.

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