# The Specific Binding of Peptide Ligand to $L^d$ Class I Major Histocompatibility Complex Molecules Determines Their Antigenic Structure

By Wen-Rong Lie,* Nancy B. Myers,* Janet M. Connolly,* John Gorka,‡ David R. Lee,§ and Ted H. Hansen*

*From the *Department of Genetics and ‡Howard Hughes Medical Institute Core Protein/Peptide Facility, Washington University School of Medicine, St. Louis, Missouri 63110; and the §Department of Molecular Microbiology and Immunology, University of Missouri Columbia School of Medicine, Columbia, Missouri 65212*

## Summary

To better understand the biological implications of the association of ligand with major histocompatibility complex class I molecules, we have studied the $L^d$ molecule of the mouse. The culturing of various nonselected cell lines with three different known $L^d$ peptide ligands resulted in a two- to fourfold specific increase in surface $L^d$ expression as detected by 10 of 11 different monoclonal antibodies (mAbs) recognizing $L^d$ epitopes. These findings suggest that $L^d$ molecules are not saturated with endogenous peptide ligands and thus have accessible binding sites. Exploiting this feature of $L^d$ we demonstrate that the physical association of $L^d$ with ligand is exquisitely specific, indicating that they function in determinant selection. In addition, a non–peptide-bound antigenic variant of $L^d$ was specifically detected with an exceptional mAb designated 64-3-7. In comparison with other $L^d$ molecules, 64-3-7$^+$ $L^d$ molecules are not peptide ligand inducible, are more susceptible to proteolysis, lack $\beta_2$ microglobulin association, and display a slower rate of oligosaccharide maturation. In spite of their deficiencies, the non–ligand-associated 64-3-7 $L^d$ molecules were detected on the surface of all cell types tested; however, they appear not to be recognized by alloreactive cytotoxic T lyphocytes.

Class I MHC molecules are highly polymorphic 45-kD membrane glycoproteins that associate noncovalently with β-2 microglobulin ($\beta_2$m),[1] a non–MHC-encoded, non–membrane-bound 11-kD polypeptide. Although each MHC haplotype of the mouse contains approximately 40 class I genes, only a few have known functions. For example, the *H-2*$^d$ haplotype, represented in the BALB/c inbred strain, expresses three class I molecules designated $K^d$, $D^d$, and $L^d$ that function as classical transplantation antigens. The K, D, and L molecules on virus-infected or allogeneic cells function as recognition structures for CTL. Crystallographic studies revealed that the highly polymorphic α1 and α2 domains of the class I molecule combine in an intricate folding pattern to form a single potential binding site (1–3). In fact the putative ligand binding site of the crystallized class I molecules was found to contain heterogeneous material estimated to be ~1–2 kD. In other studies using functional assays, virus-specific CTL were found to recognize a processed virus-derived peptide ligand in the context of a self class I molecule (4). There are now several examples where the specific virus-derived peptide has been identified for a given CTL clone (e.g., references 5, 6). Furthermore, these peptide ligands have been found to be between 5 and 20 amino acids in length. Thus, there is complete concordance between the crystallographic and functional studies. In spite of this knowledge, direct evidence of the binding of peptide to class I molecules has been very difficult to demonstrate using in vitro binding assays analogous to ones previously used to show class II/ligand association. Perhaps this difficulty results from the possibility that the binding site of isolated class I molecules is saturated with self-peptide and these ligands have a very slow rate of dissociation. Furthermore, functional recognition of a given peptide ligand appears to require binding to only a small percentage of the total class I molecules (7).

The association of class I molecules with peptide ligand could affect its surface expression and/or CTL recognition in a qualitative and/or quantitative manner. Of a qualitative nature, both the ligand and the class I molecule confer specificity to the interaction with the TCR, i.e., the CTL recognition of peptide is class I restricted. In spite of this exquisite specificity of the TCR recognition of the class I peptide complex in syngeneic responses, the role of peptide ligand in allogeneic CTL responses or antibody recognition has yet to be elucidated. Of a quantitative nature, the interaction of peptide ligand with class I molecules has recently

[1] *Abbreviations used in this paper:* Ag, antigen; $\beta_2$m, β-2 microglobulin; BFA, brefeldin A.


J. Exp. Med. © The Rockefeller University Press • 0022-1007/91/02/0449/11 $2.00
Volume 173 February 1991 449–459

been shown to facilitate surface expression of class I molecules in certain cell types (8–10). Because this peptide-induced expression was inhibitable by the drug brefeldin A (BFA), these findings were initially interpreted as an intracellular phenomenon (8, 10). Furthermore, immunoprecipitation results clearly showed peptide-specific induction of conformation of the $D^b$ $\alpha1/\alpha2$ domains and $\beta_2$m assembly (8). In separate lines of investigation, the drug BFA was used to provide evidence that peptide association with class I must occur in the endoplasmic reticulum (ER) in order to potentiate recognition by virus-specific CTL (11, 12). Thus, it is tempting to speculate that self or foreign peptides associate with newly synthesized class I molecules and this association is obligatory for egress of class I molecules from the ER and thus their plasma membrane expression. If this model were true, class I molecules with empty peptide binding sites might not exist on the cell surface.

In this communication we report several new observations relevant to these outstanding questions of the interaction of class I molecules with their peptide ligand. By culturing cells with labeled peptide ligand, significant levels of exquisitely specific binding to class I molecules was observed. Furthermore, antigenically distinct class I molecules were identified at the cell surface that lack peptide ligand binding, $\beta_2$m association, and alloreactive CTL recognition. Our findings implicate peptide ligand in the antigenic structure, intracellular transport, and function of class I molecules.

## Materials and Methods

*Mice and Cell Lines.* Splenocytes were obtained from BALB/c ($K^dD^dL^d$) or BALB/c-H-$2^{dm2}$ ($K^dD^d$) mice that were housed in the animal facility of Dr. Donald C. Shreffler, Washington University School of Medicine, St. Louis, MO. L-$L^d$ cells and R1.1-$L^d$ cells were generated by introducing the $L^d$ gene into murine $Ltk^-$DAP-3 (H-$2^k$) fibroblast cells and R1.1 (H-$2^k$) thymoma cells, respectively. The P815 (H-$2^d$) is a mouse mastocytoma cell line isolated from a DBA/2 mouse. 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, Logan, UT), 2 mM L-glutamine and 100 U/ml penicillin/streptomycin (DME-FCS). In certain experiments, L-$L^d$ cells were grown in HL-1 serum-free medium (Ventrex, Portland, ME) for 7–10 d before the assays.

*Peptide Synthesis.* Peptides were synthesized using Merrifield's solid-phase method (13) on a peptide synthesizer model 431A (Applied Biosystems, Inc. [ABI] Foster City, CA). All peptide synthesis reagents were of high purity (>99%) and supplied by ABI. The resin used for peptide synthesis was phenylacetamidomethyl (PAM) 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 ABI 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 Inc. (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 HF 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 (14).

*Flow Cytometry.* Flow cytometry was performed as previously described (15). 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 (FMF medium), and incubated with a saturating concentration of mAb or with FMF medium alone for 30 min at 4°C. The cells were washed three times with FMF medium and then incubated with a saturating concentration of fluorescein-conjugated $F(ab')_2$ fragment, goat anti-mouse IgG, Fc-specific (CooperBiomedical, Inc., Malvern, PA) for 30 min at 4°C, washed with FMF medium three times, and finally resuspended in FMF medium containing 10 μg/ml propidium iodide. Fluorescein-labeled cells were analyzed using a FACS IV (Becton-Dickinson & Co., Mountain View, CA) equipped with an argon laser tuned to 488 nm and operating at 300 mW of power. Fluorescence histograms were generated with logarithmic amplification of fluorescence emitted by single variable cells. Each sample analyzed comprised a minimum of 2 × $10^4$ cells. Mean fluorescence was obtained with logarithmic amplification of fluorescence intensity expressed in channels, where 4 logs span 256 channels (64 channels per log). The FACS machine has been calibrated such that the relative increase is equal to $10^e$, where $e$ = Δ mean fluorescence (channels)/64 (channels/decade). According to this formula, an increase in fluorescence intensity of 40 channels represents a 4.2-fold increase in expression and 30 channels represents a 3-fold increase in expression.

*Immunoprecipitation.* Immunoprecipitation of class I molecules with specific mAbs was performed as previously described (16, 17). Briefly, cells were labeled with [$^{35}$S]methionine and then lysed with 0.5% NP-40 in Tris-buffer saline, pH 7.0, containing freshly added PMSF (0.2 mM; Sigma Chemical Co., St. Louis, MO) for 30 min on ice. Cell lysates were centrifuged at 100,000 *g* for 1 h and supernatants were purified by lentil lectin Sepharose-4B (Pharmacia Fine Chemicals, Piscataway, NJ) affinity chromatography using 0.5 M α-methylmannoside for elution to obtain the glycoprotein pool. The glycoprotein pools were precleared with an equal volume of 10% (vol/vol/) IgGSorb (The Enzyme Center, Malden, MA) and PMSF was added to a final concentration of 0.2 mM. Samples of these glycoprotein antigens were then incubated with 100 μl of individual mAb ascites for 30 min on ice, followed by incubation with 1 ml of 10% IgGSorb for 30 min on ice. The IgGSorb-mAb-antigen (Ag) complexes were washed three times in cold Tris-buffered saline containing 0.25% 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 the IgGSorb. SDS-PAGE was performed on a 10–15% linear polyacrylamide gradient Laemmli buffer system (18). Prestained molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD) were included on every gel. The gels were then treated with Enhance (NEN-Dupont, Boston, MA), dried, and exposed to X-Omat AR film at −70°C for 2–10 d.

*Generation of $L^d$-specific CTL and Analysis by $^{51}$Cr Release.* The in vitro generation of primary $L^d$-specific CTL and analysis of function by $^{51}$Cr release was performed as described (19). Briefly, $L^d$-specific CTL were generated by coculture of 7.5 × $10^6$

BALB/c-*H-2*$^{dm2}$ responder spleen cells with 3.5 × $10^6$, 2,000 rad-irradiated BALB/c stimulator spleen cells in 24-well tissue culture plates (Linbro; ICN Biomedicals, Costa Mesa, CA) containing 2 ml of medium. After 5 d cytotoxic activity was measured in a standard $^{51}$Cr-release assay. $^{51}$Cr-labeled target cells (R1.1-L$^d$) were added to the wells of round-bottomed microtiter plates. For antibody blocking, 50 μl of target cells were preincubated with 50 μl of antibody for 15 min at 37°C. 100 μl of effector cells were added to the wells and the plates were incubated in a humidified atmosphere at 37°C, 5% $CO_2$. At the end of 4 h, $^{51}$Cr released into the supernatant was measured. The percent-specific $^{51}$Cr release was calculated as 100 × [(experimental $^{51}$Cr release − spontaneous $^{51}$Cr release)/(maximum $^{51}$Cr release − spontaneous $^{51}$Cr release)]. The SEM percent specific lysis was <5% of the value of the mean.

*Peptide Labeling and Binding.* 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 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 (at 660 μM) 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 A6-1x8 ion exchange resin (BioRad 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 0.3 and 1 × $10^{16}$ cpm/mol. In the peptide binding assay, 3 × $10^7$ P815 cells (2 × $10^6$/ml) were cultured with labeled peptides (0.6–2 × $10^9$ cpm) for 6 h or 18 h at 37°C. After culture the cells were washed twice with PBS and lysed on ice with NP-40 lysis buffer. The lysate was kept at 4°C for 1–2 d, and the glycoprotein pools were then isolated from the lysate for immunoprecipitation. The radioactivities of class I precipitates (bound peptide) and supernatants (non-bound peptide) were determined using a gamma counter.

## Results

*Peptide Ligand-induced Surface Expression of 10 of 11 L$^d$ Serological Epitopes.* In an earlier study, we reported that culturing various L$^d$-positive cell types with peptide ligands led to a dramatic and specific induction of surface L$^d$ expression (9). For these initial experiments two different ligands previously shown to be recognized by L$^d$-restricted CTL were used. The sequence and derivation of these two peptides designated MCMV (pp89 168–176) and tum$^-$ (P91A$^-$ 12-24), as well as other peptides used for this study, are listed in Table 1. The L$^d$-specific surface induction was monitored in these initial studies using mAbs 30-5-7 and 28-14-8 which recognize epitopes in the class I α2 and α3 domains, respectively. Because the contribution of the peptide-ligand to antibody recognition of class I remains unclear, we extended our induction studies to include a panel of 11 different mAbs (Table 2) to L$^d$ and five different peptide ligands (Table 1). To test the various mAbs, L cells transfected with the *L$^d$* gene (L-L$^d$) were cultured with $10^{-4}$ M tum$^-$ peptide for 15 h at 37°C. These conditions were known to induce optimal surface expression of L$^d$ antigens as measured by indirect immunofluorescence. 10 of the 11 mAbs to L$^d$ showed a two- to fourfold increase in their staining on peptide-treated versus control cells. By contrast, one of the mAbs designated 64-3-7 showed no peptide ligand–increased expression. To extend this observation, L-L$^d$ or P815 cells were cultured with various concentrations of peptides. As shown in Fig. 1, expression of L$^d$ epitopes recognized by mAb 30-5-7, 28-14-8, or 66-3-5 increased in a dose-dependent manner in response to MCMV or tum$^-$ peptides. By contrast, treatment with these peptides resulted in no increase in the expression of the L$^d$ epitope detected by 64-3-7, or a K$^k$ epitope on the L cells or a K$^d$ epitope on the P815 cells. In data not shown, the lack of induction of the epitope recognized by 64-3-7 in contrast to other L$^d$ epitopes was also observed using the LCMV NP 118-126 peptide known to be a ligand for L$^d$. As specificity controls, two other immunogenic peptides, FLU NP 365-380 and FLU NP 147-158(R$^-$), known to use respectively the D$^b$ and K$^d$ class I molecules in CTL studies, were tested, and neither induced expression of L$^d$ antigens. It is important to note that the K$^d$-restricted peptide induced little if any K$^d$ antigen on P815 cells, and the D$^b$-restricted peptide induced little if any D$^b$ expression on EL4 cells in spite of the fact both are capable of eliciting CTL recogni-

**Table 1.** *Synthetic Peptides*

| Peptide source | Amino acid residues | Known sequence | H-2 restriction | Reference |
|---|---|---|---|---|
| MCMVpp89* | 168-176 | YPHFMPTNL | L$^d$ | 5 |
| tum$^-$P91A$^-$‡ | 12-24 | ISTQNHRALPLVA | L$^d$ | 6 |
| LCMV NP§ | 118-126 | RPQASGVYM | L$^d$ | 27 |
| FLU NP¶ | 365-380 | IASNENMETMESSTLE | D$^b$ | 8 |
| FLU NP¶ | 147-158(R$^-$) | TYQRTRALVTG | K$^d$ | 28 |

* Murine cytomegalovirus pp89.
‡ Tumor minus P815 variant P91A$^-$.
§ Lymphochoriomeningitis virus nucleoprotein.
¶ Influenza nucleoprotein.

**Table 2.** *Monoclonal Antibodies*

| mAb | Isotype | Class I specificity | Reference |
|---|---|---|---|
| 64-3-7 | IgG2b | $L^d$ ($\alpha$2) | 20 |
| 66-3-5 | IgG2a | $L^d$ ($\alpha$2) | 21 |
| 30-5-7 | IgG2a | $L^d$ ($\alpha$2) | 22 |
| 23-10-1 | IgM | $L^d$ ($\alpha$2) | 22 |
| 1634 | IgM | $L^d$ ($\alpha$2) | 20 |
| 66-2-4 | IgG3 | $L^d$ ($\alpha$1/$\alpha$2) | 21 |
| 28-14-8 | IgG2a | $L^d$ ($\alpha$3) | 22 |
| MA228 | IgG3 | $L^d$ ($\alpha$3) | 23 |
| 66-8-2 | IgG2b | $L^d$ ($\alpha$3) | 21 |
| 66-4-8 | IgG2b | $L^d$ | 21 |
| 66-13-5 | IgG2b | $L^d$ | 21 |
| 34-5-8 | IgG2a | $D^d$ ($\alpha$2) | 24 |
| MA215 | IgG2b | $K^d$ | 23 |
| SF1-1.1.1 | IgG2a | $K^d$ | ATCC HB 159 |
| DO4 | IgG3 | $K^d$ $D^d$ | 25 |
| 3-83 | IgG2a | $K^k$ $D^k$ | 22 |
| 11-4-1 | IgG2a | $K^k$ | 26 |

tion (data not shown). Thus, the inducibility of $L^d$ antigens is specific for certain peptides and $L^d$ appears unique among other class I molecules. Furthermore, this peptide-specific induction affects all $L^d$ epitopes except the one recognized by mAb 64-3-7.

To determine whether the expression of the 64-3-7 epitope was unique for certain cell types, various $L^d$-positive cell lines were compared by indirect immunofluorescence. L-$L^d$, R1.1-$L^d$, P815, and BALB/c splenocytes expressed the 64-3-7$^+$ $L^d$ epitope (Fig. 2), whereas the nontransfected L cells or R1.1 cells were negative (data not shown). To determine if recognition of $L^d$ by mAbs 30-5-7 and 64-3-7 is $\beta_2$m dependent, cells were also grown in serum-free medium. A previous study (16) showed that in serum-free medium $L^d$ molecules are detected as free heavy chains. Comparable levels of staining were observed on L-$L^d$ cells grown in serum-free medium (data not shown) or medium containing FCS (Fig. 2 *A*) using either mAb 64-3-7 or 30-5-7. Thus, both of these $L^d$ epitopes are independent of $\beta_2$m. To determine whether the $L^d$ epitopes defined by these various mAbs differ in their susceptibilities to proteolysis, L-$L^d$ cells were treated with 0.05% trypsin for various amounts of time. For this experiment, cells were also cultured in serum-free medium to avoid secondary effects attributable to $\beta_2$m association. In data not shown the $L^d$ epitope defined by mAb 64-3-7 showed a marked time-dependent reduction in expression on trypsinized cells. In fact, after only 30 min of treatment, fourfold less binding of 64-3-7 was observed. By contrast, the $L^d$ epitopes recognized by mAbs 30-5-7 and 28-14-8 were unaffected by this treatment. This latter finding is in agreement with earlier reports that showed trypsinization of class I molecules

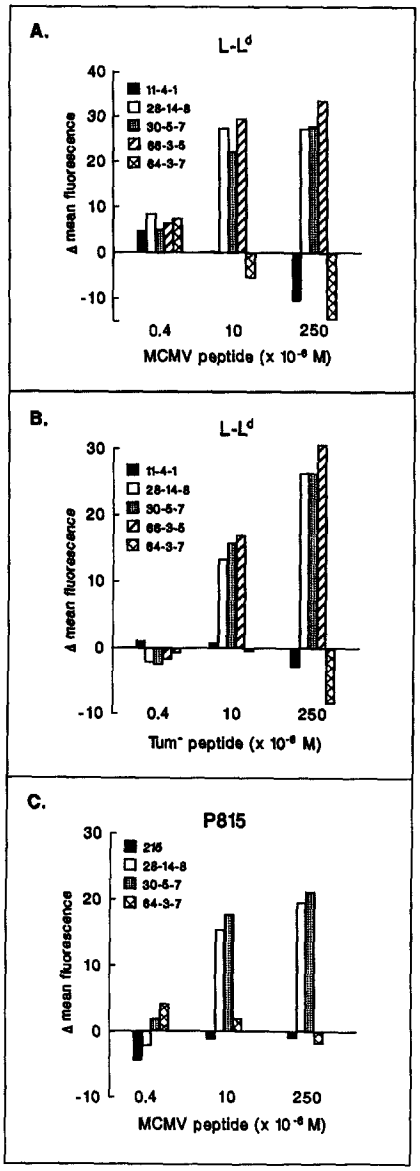


**Figure 1.** Specific surface induction of certain $L^d$ epitopes after culturing cells with $L^d$-specific peptide ligands. (*A*) Dose response of L-$L^d$ cells to MCMV peptide. (*B*) Dose response of L-$L^d$ to tum$^-$ peptide. (*C*) Dose response of P815 to MCMV peptide. $L^d$ gene L cell (H-2$^k$) transfectants (L-$L^d$) or P815 cells (H-2$^d$) were incubated with medium alone or with medium containing the indicated peptide at 0.4, 10, or 250 $\mu$M for 15 h at 37°C, 6.5% $CO_2$. Cells were then analyzed by indirect immunofluorescence using the mAbs indicated. mAbs 28-14-8, 30-5-7, 66-3-5, and 64-3-7 were used to detect $L^d$ expression and mAbs 11-4-1 and 215 were used to detect $K^k$ and $K^d$ antigens respectively. $\Delta$ mean fluorescence (channels) represents the increase in fluorescence intensity of cells treated with peptides. An increase in fluorescence intensity of 40 channels represents a 4.2-fold increase in expression as described in Materials and Methods.

requires significantly more enzyme and longer incubation times than that used here (29). Thus, the $L^d$ epitope recognized by 64-3-7 is highly and uniquely susceptible to proteolysis by trypsin.

*In BALB/c Lysates, mAbs 30-5-7 and 64-3-7 Detect Alternative Antigenic Forms of the $L^d$ Molecule.* The molecular

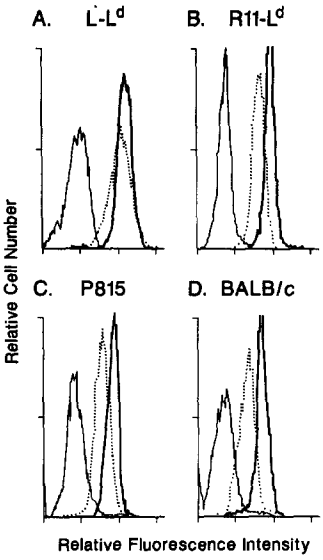


**Figure 2.** Surface expression of both 30-5-7 (—) and 64-3-7 (···) antigenic determinants on (*A*) L-L$^d$, (*B*) R1.1-L$^d$, (*C*) P815, and (*D*) BALB/c spleen cells. The cells were analyzed by indirect immunofluorescence using a FACS IV. The background histograms (–) represent fluorescence emitted from cells treated only with the fluorescein-conjugated developing antibody.

specificities of 64-3-7$^+$ L$^d$ and 30-5-7$^+$ L$^d$ were compared by immunoprecipitation analyses. BALB/c (K$^d$ D$^d$ L$^d$) spleen cells were biosynthetically labeled with [$^{35}$S]methionine for 4 h and the glycoprotein pool was purified by lentil lectin affinity chromatography. Aliquots of the BALB/c antigen preparation were precipitated using the L$^d$-reactive mAbs 30-5-7, 28-14-8, 64-3-7 and the K$^d$/D$^d$-reactive mAb Do4. As shown in Fig. 3 *A* a striking disparity in $\beta_2$m-association was observed among the class I precipitates. mAb 30-5-7 (lane *1*) or 28-14-8 (lane *2*) precipitated an L$^d$ heavy chain of ~45-kD associated with $\beta_2$m, whereas antibody 64-3-7 (lane *3*) precipitated L$^d$ with no detectable $\beta_2$m. Furthermore, the 28-14-8 and 30-5-7 precipitates contained less $\beta_2$m than did the Do4 K$^d$/D$^d$ precipitate (lane *4*) confirming that L$^d$ has a low avidity for $\beta_2$m. Another significant finding is that the 64-3-7 precipitate contained predominantly immature, endoglycosidase H-sensitive (Endo H$^s$), heavy chains in contrast to the 30-5-7 precipitate that contains approximately equal amounts of immature and mature, endoglycosidase H-resistant (Endo H$^r$), heavy chains. The basis of this difference between the 64-3-7 and 30-5-7 precipitates was explored further in a pulse-chase experiment described in the next section. To confirm that mAbs 64-3-7, 28-14-8, and 30-5-7 are all specific for L$^d$ in this gel system they were also tested on a lysate from the *L*$^d$-loss mouse strain BALB/c-*H-2*$^{dm2}$ (30). As shown in Fig. 3 *B*, all three of the aforementioned mAbs failed to precipitate class I molecules from the BALB/c-*H-2*$^{dm2}$ lysate, thus establishing their specificity for L$^d$. To determine whether mAbs 30-5-7 and 64-3-7 define separate antigenic forms of L$^d$, sequential immunoprecipitation experiments were performed using a $^{35}$S-labeled BALB/c lysate. The experiment in Fig. 4 *A* shows the result of preclearing with mAb 30-5-7. After complete clearance of all 30-5-7$^+$ L$^d$ molecules (lane *2*), both L$^d$ mAbs 28-14-8 (lane *3*) and 64-3-7 (lane *4*) detected residual, non-$\beta_2$m–associated heavy chains. Reciprocally, when a BALB/c lysate was precleared of 64-3-7$^+$ L$^d$, residual $\beta_2$m-associated heavy chains were detected by mAbs 30-5-7 and 28-14-8 (data not shown). Thus, mAbs 30-5-7 and 64-3-7 define two separate antigenic forms of L$^d$. As expected, when a BALB/c lysate was precleared

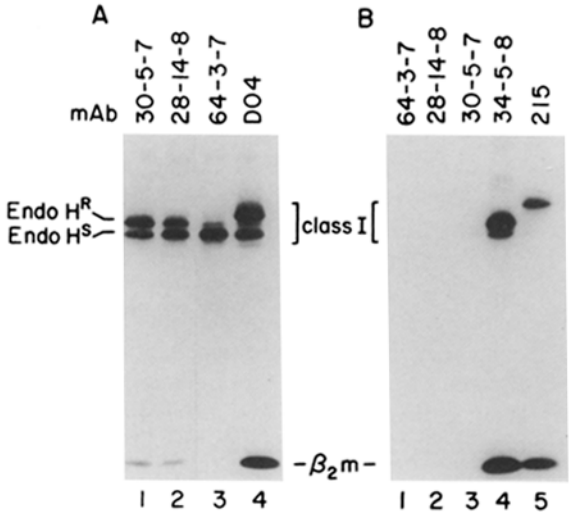


**Figure 3.** Immunoprecipitates of $^{35}$S-labeled spleen cell lysates using various anti-L$^d$ mAbs. On the autoradiograms of 10–15% gradient SDS-polyacrylamide gels shown, class I heavy chain molecules resolve into two distinct bands: Endo H$^r$, the slower migration band, and Endo H$^s$, the faster migration band. (*A*) Immunoprecipitation from $^{35}$S-labeled BALB/c (K$^d$D$^d$L$^d$) spleen cells. BALB/c lysate was immunoprecipitated using L$^d$-reactive mAbs 30-5-7 (lane *1*), 28-14-8 (lane *2*), or 64-3-7 (lane *3*) and K$^d$/D$^d$ reactive mAb Do4 (lane *4*). (*B*) Immunoprecipitation from $^{35}$S-labeled BALB/c-*H-2*$^{dm2}$ (K$^d$D$^d$) spleen cells. BALB/c-*H-2*$^{dm2}$ lysate was immunoprecipitated using L$^d$-reactive mAbs 64-3-7 (lane *1*), 28-14-8 (lane *2*), or 30-5-7 (lane *3*), and D$^d$ reactive mAb 34-5-8 (lane *4*), and K$^d$ reactive mAb 215 (lane *5*).

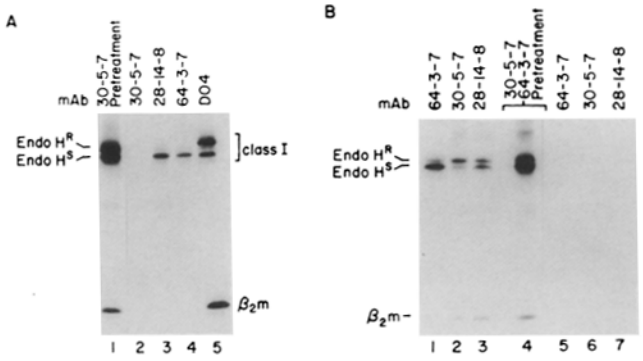


**Figure 4.** Sequential immunoprecipitation defines two antigenic forms of L$^d$, 30-5-7$^+$ 64-3-7$^-$ 28-14-8$^+$ L$^d$ and 30-5-7$^-$ 64-4-7$^+$, 28-14-8$^+$ L$^d$. (*A*) Sequential immunoprecipitations from $^{35}$S-labeled BALB/c spleen cells pretreated with mAb 30-5-7. The antigen preparation from lysates of $^{35}$S-labeled BALB/c spleen cells was pretreated with 30-5-7 (lane *1*). The supernatant precleared with 30-5-7 was then tested with 30-5-7 (lane *2*), 28-14-8 (lane *3*), 64-3-7 (lane *4*), or Do4 (lane *5*). (*B*) Sequential immunoprecipitation from $^{35}$S-labeled BALB/c spleen cells pretreated with both mAbs 30-5-7 and 64-3-7. Lanes *1*, *2*, and *3* contain test precipitates from half of the antigen preparation treated, respectively, with 64-3-7, 30-5-7, or 28-14-8. The other half of the antigen preparation was pretreated with a mixture of 30-5-7 and 64-3-7 (lane *4*), and then tested with 64-3-7 (lane *5*), 30-5-7 (lane *6*), and 28-14-8 (lane *7*).

with mAb 28-14-8 ($\alpha$3 domain), neither 30-5-7 nor 64-3-7 detected residual $L^d$ molecules (data not shown). Thus, both 30-5-7$^+$ $L^d$ and 64-3-7$^+$ $L^d$ are detected with mAb 28-14-8. To determine whether 64-3-7$^+$ $L^d$ plus 30-5-7$^+$ $L^d$ account for all $L^d$ molecules, a mixture of both mAbs was used for preclearance. For the experiment shown in Fig. 5 *B*, half of a BALB/c lysate was used for control precipitates of mAb 64-3-7 (lane *1*), 30-5-7 (lane *2*), or 28-14-8 (lane *3*), whereas the other half of the lysate was precleared with a mixture of mAbs 30-5-7 and 64-3-7 (lane 4). This latter preparation was then tested and found to contain no residual 64-3-7$^+$ (lane *5*), 30-5-7$^+$ (lane *6*), or 28-14-8$^+$ (lane 7) molecules. Because 28-14-8 recognizes an epitope in the $\alpha$3 domain, it should detect all $L^d$ molecules. Thus, mAbs 30-5-7 and 64-3-7 define alternative serological forms of $L^d$ and the combined pool of 30-5-7$^+$ plus 64-3-7$^+$ molecules includes all $L^d$ molecules.

*Disparate Rates of Oligosaccharide Maturation of 64-3-7$^+$ $L^d$ versus 30-5-7$^+$ $L^d$ Molecules.* To compare the relative rates of intracellular transport of the alternative forms of $L^d$, a pulse-chase experiment was performed using metabolically labeled BALB/c spleen cells. Cells were pulsed with [$^{35}$S]methionine for 15 min followed by a chase period of 15, 60, or 120 min. The antigen preparation from cells from each chase period was precipitated with an excess amount of mAb 30-5-7 or 64-3-7. As shown in Fig. 5 *A* both immature (Endo H$^s$) and mature (Endo H$^r$) forms of 30-5-7$^+$ $L^d$ were detected at each time point. However, comparison of the two glycan forms indicate a steady but slow rate of maturation of 30-5-7$^+$ $L^d$ molecules (31). By comparison, 64-3-7$^+$ $L^d$ molecules were detected exclusively with immature glycans at the 15- and 60-min time points, and even after 120 min only trace amounts of mature 64-3-7$^+$ $L^d$ molecules were detected. Thus, the alternative serological forms of $L^d$ clearly have disparate rates of oligosaccharide maturation, implying a disparate rate of intracellular transport. As also shown in Fig. 5 *A* both 30-5-7$^+$ $L^d$ and 64-3-7$^+$ $L^d$ were detected at each time point and their ratio remained relatively constant throughout the experiment. Thus, it is difficult to discern any precursor/product relationships between these antigenic variants. It is clear, however, from this and other pulse/chase experiments that mature 64-3-7$^+$ $L^d$ can be detected after longer chase periods. This finding is consistent with the detection of 64-3-7$^+$ $L^d$ on the cell surface (Fig. 2). Interestingly, surface 64-3-7$^+$ $L^d$ molecules also show weak if any $\beta_2$m association, even when cells are grown in medium containing bovine $\beta_2$m that is known to avidly bind $L^d$ (16).

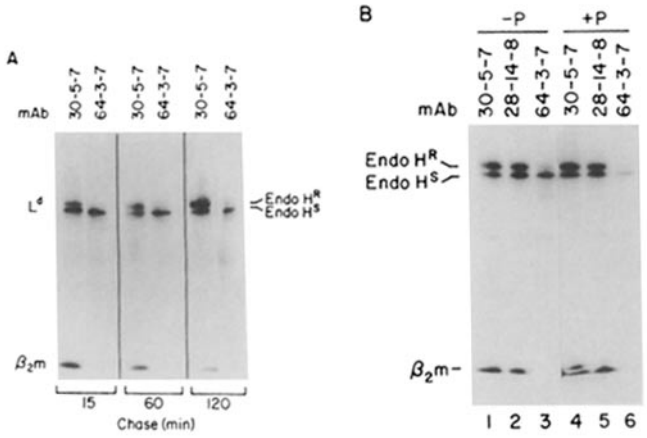


**Figure 5.** Chemical comparisons of the alternative antigenic forms of $L^d$. (*A*) Pulse-chase experiment comparing the relative rate of oligosaccharide maturation of the 30-5-7$^+$ $L^d$ versus 64-3-7$^+$ $L^d$ molecules. BALB/c spleen cells were pulsed with [$^{35}$S]methionine for 15 min followed by a chase period of either 15, 60, or 120 min. The antigen preparation from cells from each respective time point was precipitated with either mAb 30-5-7 or 64-3-7. (*B*) Immunoprecipitation analysis comparing the two antigenic forms of $L^d$ molecules (30-5-7$^+$ $L^d$ versus 64-3-7$^+$ $L^d$) from $^{35}$S-labeled BALB/c spleen cells cultured with or without MCMV peptide. BALB/c spleen cells were incubated in the presence of $10^{-4}$ M MCMV peptide or medium alone for 3 h at 37°C, 6.5% $CO_2$. [$^{35}$S]methionine was included during the last 2 h of culture. Lysates from the cells were then treated with mAb 30-5-7 (lanes *1* and *4*), 28-14-8 (lanes *2* and *5*), or 64-3-7 (lanes *3* and *6*). Lanes *1*, *2*, and *3* contain precipitates from cells cultured without peptide. Lanes *4*, *5*, and *6* contain precipitates from cells cultured with peptide. From each lysate, the ratio of 30-5-7$^+$ $L^d$ form to 64-3-7$^+$ $L^d$ can be observed by comparing precipitate bands in lanes *1* with *3* for control cells and lanes *4* and *6* for cells grown with MCMV peptide.

As mentioned above, surface 64-3-7$^+$ $L^d$ molecules were originally detected by flow cytometry due to their lack of inducibility after culturing cells with $L^d$-specific peptide ligands. To extend this observation in a chemical analysis, we immunoprecipitated the alternative antigenic forms of $L^d$ from BALB/c spleen cells grown with or without peptide ligand. In this experiment cells were grown either in the presence of $10^{-4}$ M MCMV peptide or medium alone for 3 h. During the last 2 h of culture, [$^{35}$S]methionine was added and after lysis, precipitates were obtained using mAbs 30-5-7, 28-14-8, or 64-3-7. As shown in Fig. 5 *B*, culturing with peptide ligand significantly altered the ratio of the serological forms of $L^d$ detected. Whereas in precipitates from untreated cells (lanes *1–3*) both antigenic forms of $L^d$ were clearly detectable, in precipitates from peptide-treated cells (lane *4–6*) little 64-3-7$^+$ $L^d$ were detected. The 28-14-8 precipitate was comparable in both preparations (lanes *2* and *4*), thus serving as an internal control representing total $L^d$. The effect of peptide treatment on the oligosaccharide maturation of the alternative $L^d$ forms can also be implied from the data shown in Fig. 5 *B*. Even though the 30-5-7 precipitate of both treated and untreated cells showed both immature and mature forms, their ratio appeared unaffected. This result implies peptide treatment does not affect the rate of oligosaccharide maturation. By contrast, 64-3-7$^+$ $L^d$ molecules were detected almost exclusively with immature oligosaccharides from both treated and untreated cells (lanes *3* and *6*). Thus, culturing cells with $L^d$-specific ligand dramatically affects the ratio of antigenic forms of $L^d$ detected, but not their respective rates of oligosaccharide maturation.

*The Alternative Serological Forms of $L^d$ Are Distinguished by Their Physical Interaction with Peptide Ligand as well as their Recognition by Alloreactive CTL.* The aforementioned inducibility of $L^d$ antigens by certain peptides suggests that their binding

**Table 3.** *Binding of $^{125}I$ Peptide to $L^d$ Antigen*

| Exp. | Culture time | mAb | Class I molecule | Pellet* | Sup‡ cpm | Precipitable counts§ |
|---|---|---|---|---|---|---|
| | *h* | | | *cpm* | *cpm* | *%* |
| 1 | 6 | 30-3-7 | $L^d$ | 10,303 | 441 | 98 |
| | 6 | 64-3-7 | $L^d$alt | 49 | 10,084 | <1 |
| | 6 | 34-5-8 | $D^d$ | 151 | 8,845 | <2 |
| | 6 | 3-83 | $K^k$ $D^k$ | 43 | 10,906 | <1 |
| 2 | 18 | 30-5-7 | $L^d$ | 7,631 | 1,936 | 80 |
| | 18 | 64-3-7 | $L^d$alt | 54 | 9,388 | <1 |
| | 18 | 34-5-8 | $D^d$ | 121 | 7,824 | <2 |
| | 18 | 3-83 | $K^k$ $D^k$ | 43 | 8,581 | <1 |
| 3 | 6 | 30-5-7 | $L^d$ | 38,721 | 1,526 | 98 |
| | 6 | 34-5-8 | $D^d$ | 886 | 38,463 | 2 |
| | 6 | SP1-1.1.1 | $K^d$ | 218 | 218 | <1 |

The binding assay was performed by immunoprecipitation from lysates of P815 cells cultured with $^{125}I$-MCMV peptide using class I reactive mAbs as indicated (see Materials and Methods for details).
* Radioactivity of precipitates (bound peptide) in cpm.
‡ Radioactivity of supernatants (non-bound peptide) in cpm.
§ Percent precipitable counts = 100 × [pellet cpm/(pellet cpm + sup cpm)].

sites are accessible to appropriate ligands. To substantiate this conclusion, P815 cells were cultured for either 6 or 18 h at 37°C with $^{125}I$-labeled MCMV peptide. After culture the cells were lysed and their glycoproteins were purified by lentil lectin affinity chromotography. Aliquots of these glycoprotein pools were then precipitated with various mAbs as indicated in Table 3 and radioactivity in the supernatant (unbound) versus the precipitate (bound) was determined. In Exps. 1 and 2, the 30-5-7 precipitate was found to contain 98% and 80% of the precipitable counts, respectively. Background counts were obtained with mAb 64-3-7 ($L^d$), 34-5-8 ($D^d$ $\alpha 2$ domain), or 3/83 (negative control). Similarly, in Exp. 3, 98% of the counts were precipitable by mAb 30-5-7, in contrast to mAbs 34-5-8 ($D^d$) and SF1-1.1.1 ($K^d$), each of which precipitated <2% of the counts. Assuming 1-to-1 stoichiometry and the specific counts shown in Table 3 would suggest as many as $10^6$ $L^d$ molecules per cell contain the labeled peptide. For example, in Exp. 3, $0.58 \times 10^9$ cpm of labeled peptide (300 $\mu$g) was added to $3 \times 10^7$ P815 cells and $10^7$ cell equivalents were precipitated by mAb 30-5-7. Thus, the 38,000 cpm precipitated by mAb 30-5-7 would represent $8.0 \times 10^{12}$ bound peptides, implying $8.0 \times 10^5$ peptides are bound per P815 cell. To compare the relative amounts of the class I molecules precipitated by various mAbs used in these experiments, parallel cultures of P815 cells were labeled with [$^{35}S$]methionine instead of $^{125}I$-peptide. As expected these control precipitates detected significantly more $K^d$, $D^d$, and $L^d$ molecules than alternative forms of $L^d$ molecules (data not shown). Using this same protocol we have also observed specific binding of the LCMV peptide to 30-5-7$^+$ $L^d$ molecules (data not shown). Furthermore, mAb 28-14-8 ($\alpha 3$ domain) was found to precipitate counts comparable to mAb 30-5-7 (data not shown), thus indicating that the detection of specific binding to $L^d$ is not dependent upon using a mAb to the $\alpha 1/\alpha 2$ domains. To test the peptide specificity of the binding to $L^d$, the $K^d$-specific FLU NP 147-158($R^-$) peptide was also tested. Even though this peptide labeled with comparable specific activity as the MCMV and LCMV peptides, no binding was observed to either $L^d$ or $K^d$ molecules. These findings thus substantiate the induction studies and confirm that $L^d$ molecules have accessible binding sites that specifically bind certain peptide ligands.

The above comparisons indicate that 64-3-7$^+$ $L^d$ molecules differ from 30-5-7$^+$ $L^d$ molecules in their associations with peptide and $\beta_2$m. In spite of their deficiencies, however, 64-3-7$^+$ $L^d$ are expressed on the cell surface at relatively high levels. It was thus considered relevant to determine whether 64-3-7$^+$ $L^d$ molecules could be recognized by alloreactive CTL. To address this issue, primary cultures of CTL were generated in the $L^d$-specific strain combination BALB/c-*H-2*$^{dm2}$ anti-BALB/c. These effector cells were then tested for cytotoxicity on $^{51}Cr$-labeled R1.1-$L^d$ target cells. To selectively assess recognition of 64-3-7$^+$ $L^d$ versus 30-5-7$^+$ $L^d$, their respective mAbs were used to block the $L^d$-specific CTL response. The results in Fig. 6 *A* show data obtained at a fixed concentration of mAb and various E/T cell ratios. Alternatively, the results shown in Fig. 6 *B* show a fixed E/T cell ratio and various concentrations of mAb. As clearly shown in both analyses, mAb 30-5-7 was a potent blocker of the anti-$L^d$ CTL response in contrast to mAb 64-3-7 that did not block. The lack of alloreactive CTL recognition of 64-3-7$^+$ $L^d$ could be attributable to its lower surface expres-

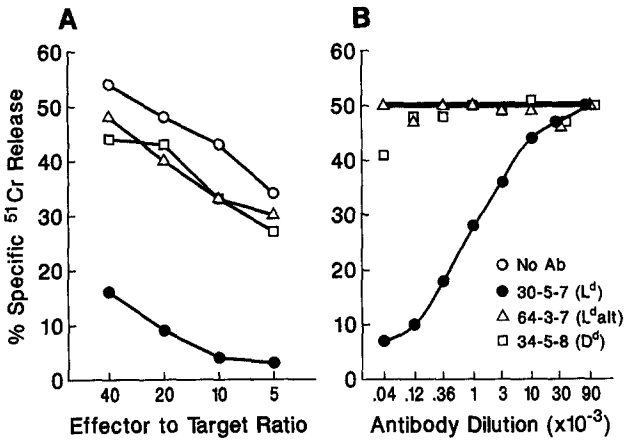


**Figure 6.** (*A*) Lysis by L$^d$-specific CTLs at various E/T ratios in the presence of mAbs (ascites) used at 1:100 dilution. (*B*) Lysis by L$^d$-specific CTLs in the presence of various concentrations of mAbs at 20:1 E/T ratio. The antibodies included in the CTL assay were 30-5-7 (●), 64-3-7 (△), 34-5-8 negative control (□), and no antibody (○).

sion; however, fluorometric analyses demonstrate that the endogenous K$^k$ and D$^k$ antigens on R1.1-L$^d$ cells are expressed at levels comparable to 64-3-7$^+$ L$^d$, yet anti-K$^k$/D$^k$ CTL effectively lyse R1.1-L$^d$ target cells (data not shown). The lack of 64-3-7 blocking might also be explained by its recognition of an epitope sterically distinct from the site recognized by the TCR. Two observations suggest that this is not the case: (*a*) all other mAbs to L$^d$ $\alpha1/\alpha2$ epitopes have been found to block the anti-L$^d$ CTL responses (data not shown); and (*b*) mAbs such as 30-5-7 (Fig. 6) show complete blocking of this CTL response. If 64-3-7 L$^d$alt and 30-5-7 L$^d$ molecules both function as target antigens, neither mAb should show complete blocking. These findings thus suggest that 64-3-7$^+$ L$^d$ are qualitatively nonfunctional in allogeneic CTL responses. Similarity, mAb 30-5-7 and not 64-3-7 has been found to block peptide-specific syngeneic CTL responses (data not shown), thus confirming the aforementioned binding studies that indicated 64-3-7$^+$ L$^d$ molecules lack peptide ligand.

## Discussion

Recent studies suggest that the binding of peptide ligand to the class I MHC molecule influences its surface expression. Various cell types have been cultured with known antigenic peptide ligands, resulting in the specific induction of surface expression of their respective class I molecules. The initial studies of peptide-induced class I expression used the RMA.S cell line (8). This variant cell line was obtained by mutagenesis followed by immunoselection of a class I–deficient phenotype. Characterization of RMA.S cells showed them to have low levels of K$^b$ and D$^b$ molecules on the surface, in spite of the detection of normal levels of class I– and $\beta_2$m-specific message. Furthermore, most of the D$^b$ molecules detected in biosynthetic lysates of RMA.S cells were sensitive to Endo H, suggesting residency in the ER, and most of RMA.S-derived D$^b$ molecules could only be detected with a mAb to the $\alpha3$ domain suggesting aberrant folding or assembly. Townsend et al. (8) reported that RMA.S cells specifically increased their surface expression of K$^b$ and D$^b$ molecules when cultured with high concentrations of peptide ligands for either K$^b$ or D$^b$ molecules. They also noted an increase in the $\beta_2$m association of RMA.S-derived D$^b$ molecules after peptide induction. These findings were interpreted as evidence that the binding of ligand to class I molecules facilitates its folding and assembly with $\beta_2$m. It could then be inferred that ligand binding and/or ligand-induced folding and/or $\beta_2$m assembly was required for optimal surface expression of class I molecules. Although this conclusion assumes that RMA.S cells have defective transport of endogenous peptides, the precise lesion(s) in these cells has yet to be elucidated.

Previous studies of the L$^d$ class I molecule of the mouse suggest that it bears several unique features, including a weak avidity for $\beta_2$m, a slower rate of intracellular transport, and a lower cell surface expression (31–33). Furthermore, each of these deficiencies was found to be approximately threefold, suggesting they are interdependent. To determine whether the weak avidity for $\beta_2$m caused the slower transport and lower expression of L$^d$, we overproduced $\beta_2$m by transfection. Unexpectedly, the increased expression of $\beta_2$m was found not to result in a higher surface expression of L$^d$ (unpublished data). Since $\beta_2$m appeared not to be the limiting factor controlling L$^d$ expression, experiments were performed to test whether peptide ligand influenced L$^d$ expression. Using the peptide feeding protocol reported with RMA.S cells, we have now tested three different peptides known to be recognized by L$^d$-restricted CTL. All three peptides were found to significantly and specifically induce surface expression of L$^d$ on L cell *L$^d$* gene transfectants as well as P815 mastocytoma cells. These findings suggest that aberrant expression of L$^d$ results from an impairment in binding peptide ligands. Either there is an insufficient pool of self-peptides capable of binding L$^d$ or the L$^d$ molecule is more selective in its ligand binding than other class I molecules. Alternatively, the structure of L$^d$ may render it more dependent upon peptide to retain its conformation and prevent its denaturation. In any case these findings indicate that there is not an exhaustive supply of self peptides necessary for maximal expression of L$^d$, thus leaving their ligand binding site available for foreign peptides. Perhaps this property of L$^d$ is a contributing factor to why several virus-specific CTL responses are L$^d$ restricted (34–36). It will be interesting to determine whether other class I molecules with suboptimal expression such as D$^k$ (37) and HLA.C (38–40) antigens share this property with L$^d$.

Another peculiar feature of L$^d$ is its detection in all cell types in two distinct antigenic forms or conformational variants. Originally these alternative forms of L$^d$ were distinguished by their reactivity with mAb 30-5-7. Thus, 30-5-7$^-$ L$^d$ molecules could only be detected by preclearance of all 30-5-7$^+$ L$^d$ molecules followed by precipitation with mAb 28-14-8 that detects an $\alpha3$ domain epitope (16). In this paper we show that 30-5-7$^-$ L$^d$ molecules can be directly detected

using antibody 64-3-7, and 64-3-7$^+$ $L^d$ molecules have been designated $L^d$alt (for alternative form). Comparisons of $L^d$ and $L^d$alt have shown they have disparate avidity for $\beta_2$m. In serum-free medium neither surface $L^d$ nor $L^d$alt is associated with $\beta_2$m, demonstrating that their antigenic difference reflects a conformational difference in the $L^d$ heavy chain. In pulse-chase experiments the rate of oligosaccharide maturation of $L^d$ is clearly faster than $L^d$alt, indicating a preferential transport of $L^d$. After culture with specific peptide ligands, the surface expression only of $L^d$ and not $L^d$alt molecules was increased. In addition, culturing L-$L^d$ transfected fibroblasts (9) or BALB/c spleen cells (Fig. 6 *B*) with specific ligand resulted in a significant decrease in the amount of $L^d$alt versus $L^d$ detected in cell lysates. Finally, in binding assays, only 30-5-7$^+$ $L^d$ molecules bound the MCMV peptide, in contrast to 64-3-7$^+$ $L^d$alt molecules that showed no binding. Thus, all three of the above comparisons indicated that the alternative antigenic forms of $L^d$ are distinguished by their interaction with peptide ligand. Our data also suggest that 64-3-7$^+$ $L^d$alt molecules have empty binding sites. If $L^d$alt molecules merely bound a separate pool of ligands, then they would not have shown the deficient $\beta_2$m assembly or the slower intracellular transport. Sequential immunoprecipitation experiments indicate that the combined 30-5-7$^+$ $L^d$ plus 64-3-7$^+$ $L^d$ precipitates account for all $L^d$ molecules. Furthermore, culturing with peptide ligands was found to induce the expression of all $L^d$ epitopes tested except for the 64-3-7 epitope. These findings suggest that the mAbs detecting inducible $L^d$ epitopes cannot discriminate which peptide is bound and that they detect a common antigenic subpopulation of $L^d$ molecules. Given that $L^d$ and $L^d$alt are distinguished by their interaction with peptide, it could be proposed that ligand sterically blocks only the 64-3-7 epitope. Thus, 64-3-7$^+$ molecules would simply be $L^d$ molecules with empty binding sites, whereas 64-3-7$^-$ $L^d$ molecules would contain a bound ligand. However, this model would not explain why 64-3-7$^+$ molecules are negative for all other $\alpha1/\alpha2$ $L^d$ epitopes tested. It is important to note that the lack of 30-5-7 recognition of the $L^d$alt molecule cannot be attributed to its lack of $\beta_2$m association, since 30-5-7 is capable of recognizing free $L^d$ heavy chains. An alternative model supported by both the serological and proteolysis data is that the 64-3-7 epitope is exposed, and other $L^d$ epitopes are lost by partially denatured $L^d$ molecules. Thus, $L^d$alt molecules could be ER-retained class I molecules awaiting components required for folding or post-ER class I molecules that underwent denaturation. Regardless of which model is correct, the results reported here clearly demonstrate that peptide ligand influences the antigenic structure of the class I molecule. Furthermore, the availability of the unique mAb 64-3-7 should be invaluable for detecting and isolating a homogeneous population of non–ligand-associated class I molecules for structural (crystallographic) and cell biological studies.

An unexpectedly high level of $L^d$alt molecules was detected on the surface of all cell types tested including spleen, mastocytoma, and $L^d$ gene–transfected cells. This finding implies that non–ligand-associated class I molecules are expressed on the cell surface at relatively high levels. In addition to their failure to be ligand-associated, surface $L^d$alt molecules bind little if any mouse or bovine $\beta_2$m and are not recognized by alloreactive CTL. Thus, $L^d$alt molecules are clearly nonfunctional, reflecting either a lack of native conformation or a requirement of CTL for recognition of $\beta_2$m or peptide ligand. We can envisage two nonmutually exclusive models that would explain the occurrence of $L^d$alt molecules on the cell surface. If culturing cells with peptide ligand only affects class I molecules in the ER, then it could be proposed that $L^d$alt molecules accumulate in the ER until appropriate peptide ligand becomes available. Once $L^d$alt molecules interact with peptide, correct folding occurs, resulting in the acquisition of several $L^d$ epitopes and the loss of the 64-3-7 epitope. It could then be proposed that the accumulation of $L^d$alt molecules permits them to escape ER retention, thus allowing them to be transported to the cell surface. Consistent with this model, no peptide-induced expression of surface $L^d$ molecules was observed at 15°C, suggesting that transport of newly synthesized $L^d$ molecules is required for induction (data not shown). Alternatively, if culturing cells with peptides affects post-ER class I molecules then it could be proposed that specific ligand can stabilize the $L^d$ structure and prevent denaturation. In this model, peptide ligand, $\beta_2$m, and/or other intracellular factors could control proper folding, and proper folding could be a prerequisite for egress from the ER. $L^d$ molecules without a bound ligand might be more susceptible to denaturation. Supplying an exogenous source of a high concentration of appropriate ligand would then decrease the turnover of $L^d$ molecules and thus increase their surface expression. Consistent with this model BFA preferentially affects surface expression of $L^d$ relative to $K^d$ or $D^d$ molecules, suggesting $L^d$ molecules have a higher turnover (manuscript in preparation). Regardless of the validity of each of these models, our studies clearly indicate that the interaction of peptide with $L^d$ influences folding, serology, assembly, and function of class I molecules. Whether each of these effects are the direct result of peptide ligand engagement remains to be established.

An adjunct to the study reported here is the demonstration that the binding of peptide ligand to $L^d$ is exquisitely specific. Exploiting the fact that $L^d$ molecules have accessible binding sites, P815 cells were cultured with radiolabeled peptides using conditions analogous to our aforementioned surface induction studies. Using this assay we found that $L^d$ molecules account for as much as 98% of glycoprotein-bound peptide from the cell lysate, whereas $D^d$ and $K^d$ molecules showed no detectable binding. Furthermore, two different known $L^d$ ligands showed specific binding, whereas a known $K^d$ ligand showed no detectable binding to either $L^d$ or $K^d$ molecules. Thus, these results substantiate the peptide-induced expression studies and demonstrate that the relationship of $L^d$ with peptide ligand is unique and highly specific. Three relevant reports have just been published that employ RMA/RMA.S cells to study class I ligand interaction (41–43). In one of these studies, RMA.S cells grown at 19–33°C were found to express high levels of surface class I-$\beta_2$m complexes (41). These complexes did not present en-

dogenous antigen and were thus presumed to have empty ligand-binding sites. In support of this conclusion, direct binding of peptide was observed to RMA.S (and to a lesser extent RMA) cells cultured at 26°C (42). Because the empty class I molecules on the surface of RMA.S cells were found to be thermolabile at 37°C, these authors concluded that peptide ligand is important for maintaining the class I structure (42). However, our detection of high levels of non–ligand-associated $L^d$alt molecules on the surface would appear to contradict this conclusion. In another study, lysates of RMA.S/RMA cells (grown at 37°C) were combined with peptide ligand at 4°C overnight and ligand binding, assembly, and the gain of antigenic epitopes, were observed (43). In agreement with the results reported here, this latter report presented evidence that the peptide ligand association with class I is exquisitely specific (43). It will be very important in future investigations to determine the relationship between the alternative forms of $L^d$ reported here and the temperature-dependent antigenic forms of class I molecules detected in RMA.S cells.

We thank Drs. Benjamin D. Schwartz, Neal Nygard, Susan E. Cullen, and Janice Blum for helpful discussions. We also thank David W. McCourt for sequence and amino acid analyses of synthetic peptides; Jan M. Bradgon and Gary Elliott for FACS analysis; Karen Perks, Melinda Becker, and Linda Pawelek for excellent secretarial assistance.

This work was supported by grants from the National Institutes of Health.

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

*Received for publication 31 August 1990 and in revised form 2 November 1990.*

## References

1. Bjorkman, P.J., M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, and D.C. Wiley. 1987a. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature (Lond.).* 329:506.
2. Bjorkman, P.J., M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, and D.C. Wiley. 1987b. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature (Lond.).* 329:512.
3. Garrett, T.P.J., M.A. Saper, P.J. Bjorkman, J.L. Strominger, and D.C. Wiley. 1989. Specificity pockets for the side chains of peptide antigens in HLA-AW68. *Nature (Lond.).* 342:692.
4. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I-restricted T lymphocytes. *Annu. Rev. Immunol.* 7:601.
5. 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.
6. Lurquin, C., A. Van Pel, B. Mariame, E. De Plaen, 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.
7. Chen, B.P., and P. Parham. 1989. Direct binding of influenza peptides to class I HLA molecules. *Nature (Lond.).* 337:743.
8. 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.
9. 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.
10. 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.
11. 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.
12. 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.
13. Merrifield, R.B. 1963. Solid phase peptide synthesis I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 85:2149.
14. 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.
15. Lee, D.R., R.J. Rubocki, W.-R. Lie, and T.H. Hansen. 1988. The murine MHC class I genes, H-2D$^q$ and H-2L$^q$, are strikingly homologous to each other, H-2L$^d$ and two genes reported to encode tumor specific antigens. *J. Exp. Med.* 168:1719.
16. Myers, N.B., W.-R. Lie, M. Nett, R.J. Rubocki, and T.H. Hansen. 1989. The conformation of $L^d$ induced by $\beta$2-microglobulin is fixed during de novo synthesis and irreversible by exchange or dissociation. *J. Immunol.* 142:2751.
17. 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.
18. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
19. Connolly, J.M., T.A. Potter, E.-M. Wormstall, and T.H.

Hansen. 1988. The Lyt-2 molecule recognizes residues in the class I $\alpha$ 3 domain in allogeneic cytotoxic T cell response. *J. Exp. Med.* 168:325.
20. 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.
21. Chatterjee-Das, S., E.P. Lillehoj, D.M. Hernandez, J.E. Coligan, and D.H. Sachs. 1987. Analysis of the *D*-region products of *H-2*$^q$ using monoclonal antibodies reveals the expression of a new class I-like molecule. *Immunogenetics.* 25:7.
22. Ozato, K., T.H. Hansen, and D.H. Sachs. 1980. Monoclonal antibodies to mouse MHC antigens. II. Antibodies to the H-2L$^d$ antigen, the product of a third polymorphic locus of the mouse major histocompatibility complex. *J. Immunol.* 125:2473.
23. Hasenkurg, K.J., J.M. Cory, and J.H. Stimpfling. 1987. Monoclonal antibodies defining mouse tissue antigens encoded by the H-2 region. *Immunogenetics.* 25:136.
24. 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-2$^d$ antibodies and an examination of expression of H-2$^d$ antigens on the surface of these cells. *Transplantation (Baltimore).* 34:113.
25. Hauptfeld, V., M. Nahm, M. Hauptfeld, and D.C. Shreffler. 1984. Monoclonal antibodies to mouse MHC antigens. I. Serological characterization of ten anti-H-2 and anti-Ia reagents. *Immunogenetics.* 19:169.
26. 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.
27. 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.
28. Bodmer, H.C., R.M. Pemberton, J.B. Rothbard, and B.A. Askonas. 1988. Enhanced recognition of a modified peptide antigen by cytotoxic T cells specific for influenza nucleoprotein. *Cell.* 52:253.
29. Lillehoj, E.P., N.B. Myers, D.R. Lee, T.H. Hansen, and J.E. Coligan. 1985. Structural definition of a family of L$^d$-like molecules distributed among four of seven haplotypes compared. *J. Immunol.* 135:1271.
30. Rubocki, R.J., T.H. Hansen, and D.R. Lee. 1986. Molecular studies of murine mutant BALB/c-*H-2*$^{dm2}$ define a deletion of several class I genes including the entire *H-2L*$^d$ gene. *Proc. Natl. Acad. Sci. USA.* 83:9606.
31. Beck, J.C., T.H. Hansen, S.E. Cullen, and D.R. Lee. 1986. Slower processing, weaker $\beta$2-M association, and lower surface expression of H-2L$^d$ are influenced by its amino terminus. *J. Immunol.* 137:916.
32. Weis, J.H., and C. Murre. 1985. Differential expression of H-2D$^d$ and H-2L$^d$ histocompatibility antigens. *J. Exp. Med.* 161:356.
33. Weis, J.H., and J.G. Seidman. 1985. The expression of major histocompatibility antigens under metallothionein gene promoter control. *J. Immunol.* 134:1999.
34. Ciavarra, R., and J. Forman. 1982. *H-2L*-restricted recognition of viral antigens: In the *H-2*$^d$ haplotype, anti-vascular stomatitis virus cytotoxic T cells are restricted solely by *H-2L*. *J. Exp. Med.* 156:778.
35. 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.
36. 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.
37. Williams, D.B., S.J. Swiedler, and G.W. Hart. 1985. Intracellular transport of membrane glycoproteins: two closely related histocompatibility antigens differ in their rates of transit to the cell surface. *J. Cell Biol.* 101:725.
38. Gussow, D., R.S. Rein, I. Meijer, W. de Hoog, G.H.A. Seemann, F.M. Hochstenbach, and H.L. Ploegh. 1987. Isolation, expression, and the primary structure of *HLA-Cw1* and *HLA-Cw2* genes: evolutionary aspects. *Immunogenetics.* 25:313.
39. Snary, D., C.J. Barnstable, W.F. Bodmer, and M.J. Crumpton. 1977. Molecular structure of human histocompatibility antigens: the HLA-C series. *Eur. J. Immunol.* 8:580.
40. Sodoyer, R., M. Damotte, T.L. Delovitch, J. Trucy, B.R. Jordan, and T. Strachan. 1984. Complete nucleotide sequence of a gene encoding a functional human class I histocompatibility antigen (HLA-Cw3). *EMBO (Eur. Mol. Biol. Organ.) J.* 3:879.
41. Ljunggren, H.-G., N.J. Stam, C. Ohlen, J.J. Neffjes, 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.
42. 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.
43. 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.