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

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Summary

To determine the mechanism and structural consequences of peptide binding to class I molecules, we have studied the Ld molecule of the mouse. Previous studies have shown that a significant proportion of surface and intracellular Lα molecules can be detected in an alternative conformation designated Lαalt. Lαalt molecules are non-ligand associated and show weak if any β2-microglobulin (β2m) association. We report here that Ld molecules have a relatively rapid surface turnover compared with other class I molecules and that exogenous peptide dramatically prolongs Ld surface half-life. By contrast, Lαalt molecules are stably expressed on the surface and their half-life is unaffected by exogenous peptide. To study the surface interaction of peptide with Ld, live cells were incubated with iodinated peptides and Ld molecules were precipitated from cells precoated with monoclonal antibody before lysis. Using this assay, peptide binding to surface Ld molecules was found not to depend upon exchange with exogenous β2m, but did correlate with the level of β2m association. To study the intracellular interaction of peptide with Ld, cell lysates were used. In cell lysates, peptide was found to convert Lαalt molecules to properly folded Ld. This peptide-induced folding was almost complete at earlier but not later time points in pulse-chase analyses. Furthermore, conversion of Lαalt to Ld was found to affect almost exclusively immature (Endo H+) class I molecules. Thus intrinsic properties of immature Lαalt molecules or their associated chaperonins are maintained in cell lysates that allow them to undergo de novo folding in vitro. These combined results demonstrate that immature Lαalt molecules are precursors awaiting constituents such as peptide and β2m that influence folding, whereas surface Lαalt molecules appear refractory to association with peptide, β2m, and consequent folding.

Class I MHC molecules are membrane-bound glycoproteins that function as receptors for peptide ligands which are presented to CTL. For example, after infection, cells process virus-derived proteins into short peptides of eight to nine amino acids that bind self class I molecules (1-3). When expressed on the cell surface, peptide-class I complexes can identify virally infected cells for destruction by host immune CTL. Studies using the drug brefeldin A (Bfa)1 suggest that the initial binding of peptide by class I occurs in a pre-Golgi compart-
In regard to the intracellular interactions of peptide with class I, recent studies suggest that specific ligands play an integral role in the folding of the nascent class I molecule. The initial support for this hypothesis came from studies of the RMA.S cell line, a mutagenized cell line immunoselected to be class I deficient (11). Indeed, there is considerable indirect evidence indicating that RMA.S has a defect in the transport of intracellular peptides (6, 12). At 37°C most of the RMA.S class I molecules are sensitive to endoglycosidase H (Endo H), implying that they are ER retained, and most cannot be detected by conformation-dependent (i.e., α1/α2 domain) antibodies, implying that they are misfolded or unassembled. Townsend and colleagues (13) reported that when specific peptide ligands were added to RMA.S cell lysates, properly folded and assembled class I molecules were detected. In agreement with this observation, peptide ligands were found to specifically facilitate the folding of HLA.B27 molecules produced in a cell-free translation system (14). Even though these studies provide strong evidence that peptide influences de novo folding of class I, other findings suggest that class I molecules can attain functional conformation in the absence of peptide. For example, untreated lysates of RMA.S cells grown at 37°C contain significant amounts of conformed class I molecules and RMA.S cells grown at 25°C express high levels of class I molecules recognized by conformation-dependent mAbs. Thus, peptide may not be an absolute requirement for de novo folding. Besides peptide ligand, the other known participant in class I folding is β2-microglobulin (β2m). Studies of the β2m-deficient cell lines DAUDI (15) and RIE (16) suggested that intracellular β2m is a requisite for folding and transport of most class I heavy chains. An exception to this rule came from studies of RIE.D b, a β2m - cell line transfected with the D b gene under a strong viral promoter (17). Surface D b molecules are expressed by RIE.D b cells but remain unassembled and are not recognized by conformation-dependent antibodies. This observation suggests that β2m association is not an absolute requirement for intracellular transport, a conclusion also supported by recent studies of L a (18) and D D 196 (19) molecules. However, studies of RIE.D b would suggest that intracellular β2m is required for proper folding. In apparent contradiction of this conclusion, Townsend and colleagues (20) reported that the addition of either peptide ligand or β2m to an RIE.D b lysate resulted in the detection of conformed D b molecules. Thus, even though both peptide and β2m clearly influence the folding of class I molecules, their separate contributions under physiological conditions have yet to be defined and very well may differ significantly among class I molecules.

In regard to the consequences and mechanisms of peptide binding to surface class I molecules, studies of RMA.S indicate that ligand association is required for stable class I expression. RMA.S cells grown at 25°C express what appears to be empty class I molecules that, when switched to 37°C, rapidly become undetected by conformation-dependent mAbs (6, 12). Furthermore, either specific peptide (11) or mAb (21) was found to dramatically and specifically increase the half-life of class I molecules expressed by RMA.S. In studies of unselected live cells cultured at 37°C with labeled peptide ligands, specific binding was observed to L d (9), K d (10), B27 (7), and D b (8) molecules. These findings were interpreted as evidence that a significant proportion of these class I molecules are expressed at 37°C with empty ligand binding sites (7, 10). In murine systems evidence has been provided that β2m exchange may influence peptide binding at the cell surface. Clearly, the addition of human β2m can facilitate peptide binding to murine class I molecules (22-24). However, certain mouse class I molecules show a strong propensity to exchange their own β2m for either human or bovine β2m, and this exchange may facilitate peptide binding at the surface. It is therefore unclear whether peptide binds to surface class I molecules previously conformed by intracellular peptide, to open folded class I molecules that have not yet encountered peptide, or to unfolded class I molecules that can fold at the cell surface. Thus, the mechanism and physiological significance of peptide binding to surface class I is unclear and again may differ significantly among class I molecules.

Our approach to investigating the structural and functional consequences of the interaction of peptide with class I has focused on the L a molecule. The uncharacteristically low level of surface expression of L a relative to K d or D d was found to be the result of, and specifically increased by cultivating H-2 d haplotype cells with known L a ligands (25). This result indicated that a high percentage of L a molecules have readily accessible binding sites. Exploiting this feature of L a, a binding assay was developed whereby live cells were grown with 125I-labeled peptides (9). In these assays, we discovered a unique mAb, designated 64-3-7, that detects a non-peptide associated conformational variant of L a. This alternative form of L a, designated L a-alt, showed weak if any β2m association and was detected both intracellularly and at the cell surface (9). In this communication we present data indicating that when cells are cultured with peptide, binding occurs to properly folded surface L a molecules and significantly prolongs their half-life. By contrast, when cell lysates are incubated with peptide, binding occurs to immature or nascent L a-alt molecules and facilitates their conversion to proper conformation. These distinctions between the effect of peptide on immature vs. surface L a molecules are discussed in the context of other recent studies of class I-ligand interaction.

Materials and Methods

Cell Lines. L-L d cells were generated by introducing the L d gene into murine Ltk-DAP-3 (H-2 k) fibroblastic cells. All of the cell lines used were maintained at 37°C, 6.5% CO2 in DME (Gibco Laboratories, Grand Island, NY) containing 10% FCS (HyClone Laboratories Inc., Logan, UT), 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin (DME-FCS). In certain experiments, L-L d cells were grown in serum-free DME medium supplemented with 1% Nutridoma SP (Boehringer Mannheim Corp., Indianapolis, IN) for 3 d before the assays. In other assays cells were treated with Bfa (Epicentre Technologies, Madison, WI) to block new surface class I molecules are expressed at 37°C with empty ligand binding sites (7, 10). In murine systems evidence has been provided that β2m exchange may influence peptide binding at the cell surface. Clearly, the addition of human β2m can facilitate peptide binding to murine class I molecules (22-24). However, certain mouse class I molecules show a strong propensity to exchange their own β2m for either human or bovine β2m, and this exchange may facilitate peptide binding at the surface. It is therefore unclear whether peptide binds to surface class I molecules previously conformed by intracellular peptide, to open folded class I molecules that have not yet encountered peptide, or to unfolded class I molecules that can fold at the cell surface. Thus, the mechanism and physiological significance of peptide binding to surface class I is unclear and again may differ significantly among class I molecules.

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mAbs. For detection of L d molecules mAb 30-5-7 (α2 domain) was used. Additionally, for detection of L d molecules mAb 50-5-7 (α2 domain) was used.
Immunoprecipitation. Immunoprecipitation of class I molecules with specific mAbs was performed as previously described (31). To monitor Lα antigenic conformation in biosynthetic lysates cells were labeled with [35S]methionine and then lysed with 0.5% NP-40 in Tris-buffered saline, pH 7.0, containing freshly added PMSF (0.2 mM; Sigma Chemical Co., St. Louis, MO) and supplemented plus or minus βm and/or peptide for 30 min on ice. Cell lysates were incubated overnight at 4°C. After overnight incubation, lysates were centrifuged at 100,000 g for 1 h and supernatants were precleared with an equal volume of 10% (vol/vol) IgGsorb (The Enzyme Center, Malden, MA). Samples of these glycoproteins were next incubated with 0.1 vol of individual mAb ascites for 30 min on ice, followed by incubation with an equal volume of Protein A-Sepharose (PAS) beads for 30 min on ice. The IgGsorb-mAb-antigen complexes were washed three times in cold Tris-buffered saline containing 1% NP-40 and 0.2 mM PMSF. The mAb-Ag complexes were eluted by incubation of the samples in 0.125 M Tris-HCl, pH 6.8, 12.5% glycerol, 2% SDS, and 1% 2-ME (SDS-PAGE sample buffer) at 90°C for 2 min, followed by centrifugation to remove PAS. SDS-PAGE was performed on a 10–15% linear polyacrylamide gradient Laemmli buffer system (32). Prestained molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD) were included on every gel. The gels were then treated with ENHANCE (NEN-Dupont, Boston, MA), dried, and exposed to X-Omat AR film at −70°C for 1–10 d.

For measurements of surface binding of radiiodinated peptide, L11c cells were detergent lysed for 4 h at 37°C with [125I]-labeled peptide. Cells were harvested and washed five times with DME (unsupplemented). Each cell sample was resuspended in 500 µl PBS and incubated 30 min on ice with 0.1 vol antibody. After the incubation, cells were washed three times with DME (unsupplemented) and lysed in 0.5% NP-40/0.2 mM PMSF. Peptide-class I complexes were precipitated with an equal volume of 10% IgGsorb.

In lysate binding experiments, L11c cells were detergent lysed in 0.5% NP-40/0.2 mM PMSF supplemented plus or minus radiolabeled peptide ligand. Lysates were clarified by 1 h, 100,000 g, 4°C centrifugation and incubated overnight at 4°C. Glycoproteins from these lysates were purified by lentil lectin Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) affinity chromatography using 0.5 M α-methylmannoside for elution. Samples were incubated with 0.1 vol mAb for 30 min on ice. IgGsorb was used to precipitate class I complexes.

Flow Cytometry. Flow cytometry was performed as previously described (9). Briefly, 2–4 × 10^6 cells were placed in the wells of round-bottomed microtiter plates, washed once with HBSS (lacking phenol red) containing 0.2% BSA/0.1% sodium azide (FACS® medium), and incubated with a saturating concentration of mAb or with FACS® medium alone for 30 min at 4°C. The cells were washed three times with FACS® medium and then incubated with a saturating concentration of fluorescein-conjugated P(ab')2 fragment of goat anti-mouse IgG, Fc-specific (Organon-Teknika-Cappel, Durham, NC) for 30 min at 4°C, washed with FACS® medium three times, and finally resuspended in FACS® medium containing 10 µg/ml propidium iodide. Fluorescein-labeled cells were analyzed using a FACSScan® (Becton Dickinson & Co., Mountain View, CA) equipped with an argon laser tuned to 488 nm and operating at 150 mW of power. Fluorescence histograms were generated with logarithmic amplification of fluorescence emitted by single viable cells. Each sample analyzed comprised a minimum of 1 × 10^4 cells. Mean fluorescence values were converted from logarithmic amplification of fluorescence intensity by linear regression analysis using the CONSORT 30 computer software.

Peptide Synthesis. Peptides were synthesized using Merrifield's solid-phase method (33) on a peptide synthesizer (model 431A; Applied Biosystems, Inc., Foster City, CA). All peptide synthesis reagents were of high purity (>99%) and supplied by Applied Biosystems, Inc. The resin used for peptide synthesis was phenylacetamide and was preloaded with 0.5 mM of required amino acid. All amino acids used were β-Boc protected at the NH2 terminus and their side chains were protected with the standard groups recommended by Applied Biosystems, Inc. for β-Boc synthesis, with the exception of histidine. The histidine derivative used was N,N-β,β,β-benzylaminomethyl-t-histidine supplied by Bachem (Torrance, CA). The β-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 diethyl ether to remove organic by-products generated during hydrogen fluoride cleavage. The peptide was then extracted from the resin with 30% acetic acid. The acetic acid was removed by rotary evaporation, and the remaining aqueous peptide solution was diluted fourfold with H2O, shell frozen, and lyophilized. Peptides were purified (>90%) by reverse-phase HPLC and subjected to purity assessment techniques as previously described (34).

Peptide Labeling. Peptides were iodinated using the Iodo-Beads (Pierce Chemical Co., Rockford, IL) method. Briefly, Iodo-Beads were washed twice with iodination buffer (100 mM sodium phosphate, pH 7.4). Two Iodo-Beads were mixed with 1–5 µCi of Na[125I] (New England Nuclear, Boston, MA) in 200 µl iodination buffer at room temperature for 5 min. A solution of 300 µl of peptide in iodination buffer was added to the Iodo-Beads reaction mixture. The iodination reaction was allowed to proceed for 15 min at room temperature and terminated by removing the reaction mixture from the Iodo-Beads. The radiolabeled peptide was recovered by transferring the reaction mixture to a 1-ml packed AG1-X8 ion exchange resin (Bio-Rad Laboratories, Richmond, CA) column to bind the free [125I]. After centrifugation (2,500 g, 5 min) the radiolabeled peptide-containing fluid forced through the column was collected and stored at 4°C before use. Peptides were labeled with specific activities between 0.2 and 1 × 10^6 cpm/mol.

Results

To define the parameters involved in the association of ligand with class I MHC molecules, we have studied the Ld molecule of the mouse. Previous studies of peptide-induced surface expression of Ld identified a unique mAb designated 64-3-7. Whereas expression of other epitopes was increased two- to fivefold after treatment with different known peptide ligands, the epitope recognized by 64-3-7 was unaffected (9).

Immunoprecipitation studies showed that mAb 64-3-7 defined an alternative form of Ld (Ldalt) as compared with other Ld molecules. Ldalt molecules were distinguished by their minimal, if any, βm association and their slower rate of oligosaccharide maturation. The intracellular ratio of 64-3-7+ to 30-5-7+ Ld molecules was found to be variable and generally higher in cells that overexpress Ld, such as the Ld-transfected cell line. In an earlier report (9) we measured ligand binding to Ld by culturing Ld cells or
P815 cells with $^{125}$I-labeled murine CMV peptide (pp89, 168-176) (35). After 4-6 h culture, cells were lysed in NP-40 and class I molecules were immunoprecipitated. Whereas 30-5-7 precipitates contained high levels of labeled CMV peptide, 64-3-7 precipitates contained no specific radioactivity (9). This result indicated that Ldalt molecules are not ligand associated, in contrast to 30-5-7+ Ld molecules that demonstrated considerable ligand association. To quantitate what proportion of the binding in this assay occurred to surface Ld molecules, the experiment shown in Fig. 1 A was performed. Ld cells were incubated with $^{125}$I-CMV peptide for 4 h, after which time they were treated with an excess amount of mAb 30-5-7 (anti-Ld), 64-3-7 (anti-Ldalt), or 11-4-1 (anti-Kk). After extensive washing, half of each of these mAb-coated cells were lysed in NP-40 and Ab-class I complexes were precipitated. Precipitates were then counted to determine the amount of peptide bound to surface class I molecules. Under these conditions no Ab/Ld/peptide dissociation was observed in the lysate as determined by using excess competitors (data not shown). As shown in the upper panel of Fig. 1A, of the three mAbs only 30-5-7 precipitated significant peptide-specific counts. The other half of the antibody-coated Ld cells were lysed and each lysate was treated again with the same respective mAb. Thus, precipitates from these samples should include surface and intracellular class I molecules with bound peptide. As shown in the lower panel of Fig. 1 A, only 30-5-7 precipitates again contained peptide-specific counts. Comparisons of the amount of peptide binding observed by surface versus surface plus lysate precipitate indicate that 80% of the peptide binding can be accounted for by surface 30-5-7+ Ld molecules. The remaining 20% of the peptide binding could either have occurred intracellularly or in the lysate. To quantitate the amount of binding that can occur in a lysate, the mixed-lysate experiment shown in Fig. 1 B was performed. As a positive control a lysate from Ld cells that were fed $^{125}$I-peptide was mixed with a lysate of Ltk- cells. As expected, significant specific peptide binding to 30-5-7+ Ld was observed (Fig. 1 B, upper panel). In a reciprocal experiment, a lysate of unfed Ld cells was mixed with a lysate of CMV-fed Ltk- cells. In this mixture, binding to Ld could only occur in the lysate because Ltk- cells are Ld negative. As shown in the lower panel of Fig. 1 B, a small but specific amount of lysate binding to Ld was detected. Comparison of the amount of peptide binding measured in these reciprocal experiments (Fig. 1 B) indicates that only 5% of the binding to Ld occurred in the lysate when Ld cells was cultured with labeled peptide. Thus, using this assay system, most of the binding clearly occurs to surface Ld molecules before cell lysis.

Although the above experiments indicate that culturing cells with peptide results in binding to predominantly surface Ld molecules, they also suggest that binding to Ld can occur in cell lysates (Fig. 1 B). To better characterize this latter binding, a lysate of Ld cells was incubated with $^{125}$I-labeled CMV peptide. As shown in Fig. 1 C, anti-Ld mAbs 30-5-7 and 28-14-8 precipitated significant peptide-bound molecules in contrast to 64-3-7 precipitates that contained no counts above background. In data not shown, comparable results were obtained with another known ligand for Ld derived from the lymphochoriomeningitis virus (LCMV) nucleoprotein (NP 118-126) (36). To define the relationship between alternative forms of Ld, various concentrations of peptide ligand plus or minus human $\beta_{2m}$ (h$\beta_{2m}$) were incubated with aliquots of biosynthetically labeled Ld lysates. Peptide treated lysates exhibited a dramatic, dose-dependent increase in 30-5-7+ Ld molecules in conjunction with a proportional decrease in 64-3-7+ Ld molecules (Fig. 2 A).
This crisscross relationship between 64-3-7+ Ld and 30-5-7+ Ld molecules is graphically represented in Fig. 2 B. In the same experiment, addition of purified hB2m alone or together with peptide was found to shift the ratio of the two forms toward 30-5-7+ Ld (Fig. 2 A). The addition of the LCMV peptide to an Ld cell lysate was also found to increase 30-5-7+ Ld and decrease 64-3-7+ Ld, whereas an irrelevant peptide had no effect (data not shown). To directly test whether 64-3-7+ Ld molecules could be converted to 30-5-7+ Ld molecules, a lysate was selectively precleared of all 30-5-7+ Ld molecules and subsequently treated with hB2m/peptide. New 30-5-7+ Ld molecules were detected in hB2m/peptide-treated lysates but not untreated controls (Fig. 2 C). This experiment formally established the
Figure 3. Pulse-chase experiment demonstrating that predominantly immature (Endo H') forms of Ld glycoproteins are susceptible to peptide-induced conformational change. (A) Biosynthetically labeled LdLa cells were pulsed for 15 min and chased for 0, 4, or 6 h as indicated along the bottom of the figure. Lysates (2 × 10⁷ LdLa, 500 μl, 0.5% NP-40) from each chase period were incubated overnight (4°C) with or without hB2m (3 μM) and CMV peptide (40 μg). Class I molecules were precipitated with mAbs 64-3-7 and 30-5-7 and resolved by SDS-PAGE. Using this gel system, mature (Endo H') and immature (Endo H+) class I molecules resolve into two separate bands as indicated. (B) Densitometric tracing of the class I precipitin bands from the 6-h chase period shown in Fig. 3 A. For comparison, the profiles of the control (——) and hB2m/peptide-treated (· · ·) are superimposed.

precursor–product relationship between 64-3-7* Ld and 30-5-7* Ld. In conjunction with the aforementioned properties of Ldalt, these findings demonstrate that 64-3-7 uniquely detects nonconformed Ld molecules capable of binding peptide ligand. To better characterize the Ld molecules susceptible to antigenic conversion, pulse/chase experiments were performed. Even though 64-3-7* Ld molecules are transported more slowly than 30-5-7* Ld molecules, after a 4-h chase time mature (Endo H') forms of both are detected. As shown in Fig. 3 A, Ld molecules were increasingly susceptible to peptide/hB2m-induced conversion with increased time of chase. At the 0 time point >80% of the 64-3-7* Ld molecules were convertible to 30-5-7* Ld; after 4 and 6 h that percentage dropped to 50 and 40%, respectively. Furthermore, Endo H' forms were preferentially affected by peptide/hB2m treatment as observed in both the loss of im-

Figure 4. Pulse-chase experiment demonstrating that peptide alone is as effective as peptide and hB2m in inducing Endo H' forms of 30-5-7* Ld. (A) Biosynthetically labeled LdLa cells were pulsed for 15 min and chased for 0 or 4 h as indicated. Respective lysates (1.25 × 10⁷ LdLa, 500 μl, 0.5% NP-40) were divided and treated overnight with CMV peptide (40 μg), hB2m (3 μM) + CMV (40 μg) peptide, or untreated control. Ld molecules were precipitated with mAb 30-5-7 and half of each precipitate was digested with Endo H (++) (1 mU/ml; ICN ImmunoBiologicals, Lisle, IL), whereas the other half was mock digested (−). Precipitates were resolved by SDS-PAGE. (B) Densitometric tracing of the precipitin bands from the 4-h chase period shown in A. For comparison, the profiles of the control (——) and peptide-treated (· · ·) are superimposed.
mature 64-3-7$^+$ L$^d_{alt}$ and the gain of immature 30-5-7$^+$ L$^d$
(Fig. 3 B). A second important feature of this experiment is
that nearly all 30-5-7$^+$ L$^d$ molecules exchange mouse for h$\beta$2m. Compared with control lysates, h$\beta$2m/CMV-treated
lysates undergo significant or complete exchange of exogenous cold h$\beta$2m for endogenous-labeled $\beta$2m (Fig. 3 A).
This result was also obtained with h$\beta$2m treatment alone
(data not shown). To substantiate the observation that
immature 64-3-7$^+$ L$^d$ molecules are preferentially affected by
peptide/h$\beta$2m treatment, a pulse-chase experiment was
performed using Endo H digestion to formally define immature
molecules (Fig. 4 A). In this experiment peptide or peptide/h$\beta$2m resulted in the comparable induction of 30-5-7$^+$
L$^d$ molecules (a 90% increase at time 0 versus a 50% in-
crease at time 4 h), implying that h$\beta$2m exchange is not re-
quired for peptide-induced folding of L$^d$ in cell lysates. Fur-
thermore, the affected molecules are clearly sensitive to Endo
H digestion. As shown in Fig. 4 B densitometric tracings
of 30-5-7 precipitates from the 4-h time point indicate that
the peptide-induced 30-5-7$^+$ L$^d$ molecules were exclusively
immature (Endo H$^+$) forms. Thus, this in vitro antigenic
conversion of L$^d$ molecules accurately mimics in vivo folding
of nascent L$^d$ molecules, demonstrating that intrinsic prop-
ties of immature class I molecules or their associated
chaperonins are maintained in vitro in cell lysates.

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

Figure 5. Comparison of the peptide inducibility and surface turnover of alternative forms of L$^d$ on L$^d$-IcL$^d$ cells. L$^d$-IcL$^d$ cells ($5 \times 10^9$/ml) were in-
cubated with $10^{-4}$ M CMV peptide (○), medium (X), Bfa (△), or Bfa plus $10^{-4}$ M CMV peptide (□) for a period of 0, 2, 4, or 6 h. After peptide
and/or Bfa treatment L$^d$ cells were stained with mAb against L$^d$
(30-5-7) (A), L$^d_{alt}$ (64-3-7) (B), or K$^k$ (11-4-1) (C), respectively.
Quantification of class I surface expression on these cells was analyzed
by cytofluorometry on a FACScan®. Mean fluorescence values were con-
verted from logarithmic amplification of fluorescence intensity by linear
regression analysis.

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

To better quantify the factors controlling the extracellular
binding of peptide, direct binding of $^{125}$I-labeled CMV to
surface L$^d$ was measured after cells were cultured under vari-
ous conditions. In the experiment shown in Fig. 6 A, L$^d$
cells were tested in a 4-h assay for their ability to bind pep-
tide in media containing FCS, FCS + Bfa, serum free (SF),
or SF + h$\beta$2m. Specific binding to surface L$^d$ was deter-
mined by precoating cells with mAb before lysis (method
detailed in the legend of Fig. 1). Precipitates of 30-5-7$^+$ L$^d$
from cells grown with Bfa were found to contain about half
as many peptide-specific counts as precipitates from cells grown
in FCS. This reduced binding could clearly be accounted for
by the reduced L$^d$ expression after 4 h in Bfa (data not
shown). Thus, peptide binding to L$^d$ is not dependent upon
new arrival of L$^d$ molecules to the cell surface. When cells
were incubated in SF media with labeled-CMV peptide. Significant binding to surface L^d was observed (85% of the FCS control). This result indicates that exchange for exogenous β2m appears not to be the major factor in determining surface binding to L^d. It should be noted that the cells used for the assay shown in Fig. 6 A were grown in media containing 10% FCS before their 4-h incubation with labeled peptide under indicated conditions. Thus, entering the peptide binding assay the surface L^d molecules would be expected to be heavily β2m associated. Interestingly, the purified hβ2m augmented the binding of peptide in SF media, probably by maintaining a higher level of β2m-associated L^d during the 4-h assay.

To further explore the role of β2m association in peptide binding to L^d, L-L^d cells were grown for 3 d in either 10% FCS, 1% FCS, or 1% normal mouse serum (NMS). In our previous immunoprecipitation studies (18), surface 30-5-7^+ L^d molecules were detected to be predominantly free heavy chains when cells were grown in NMS as compared to heterodimers with bovine β2m when grown in FCS. In spite of the differences in β2m association, the amount of 30-5-7^+ L^d and the ratio of 30-5-7^+ L^d to 64-3-7^+ L^d was comparable on cells grown in 10% FCS, 1% FCS or 1% NMS (fluorometric data not shown). However, the ability of cells under each of these conditions to bind peptide was clearly different (Fig. 6 B). Correlating with their expected level of β2m association (18), peptide binding to surface L^d was highest on cells grown in 10% FCS, next highest on cells grown in 1% FCS, and lowest on cells grown in 1% NMS. As an alternative method to assess the role of β2m association, L-L^d cells were grown in SF medium for 3 d before the binding assay in order to maximize β2m dissociation and to eliminate the potential to bind bovine β2m. After growth in SF, cells were treated with Bfa to prevent new β2m expression during the binding assay. During the 4-h incubation with peptide, aliquots of these cells were grown with no β2m, 0.5 μg/ml hβ2m, or 5 μg/ml hβ2m as indicated in Fig. 6 C. Precipitates from the control cells with no exogenous β2m showed a small but significant amount of peptide binding to 30-5-7^+ L^d. This binding could be attributed to either surface expression of intracellular L^d-β2m complexes beyond the Bfa block or the failure of all L^d molecules to dissociate in SF media. In any case, the addition of purified hβ2m to SF media was found to greatly enhance the specific peptide binding to L^d (Fig. 6 C). In total, these experiments measuring direct peptide binding indicate that new surface expression of L^d and exchange for exogenous β2m are not required for peptide binding (Fig. 6 A). However, when new expression is blocked and cells are grown for an extended period without serum, peptide binding in the absence of exogenous β2m was ~22% that seen in the presence of hβ2m (Fig. 6 C). It is noteworthy that this 22% binding did not occur in the lysate since all the assays shown in Fig. 6 were performed using Ab-coated cells to detect exclusively surface class I molecules. What is strikingly apparent from each of the experiments shown in Fig. 6 is that peptide binding correlates precisely with the amount of β2m associated with L^d, implying that mainly class I:β2m heterodimers bind peptide.

Discussion

Several recent publications have studied the association of class I molecules with peptide by culturing live cells in media containing known peptide ligands (6–10). The initial experiments monitored peptide association indirectly by quantitating the peptide-induced increase in surface class I expression. Culturing cells with peptide resulted in the specific increase of class I molecules on immunoselected cells such as RMA.S (11) or LBL721.174 (37) or of L^d (25) molecules on unselected cells. The mechanism of this induction was origi-
nally thought to result from retrograde peptide transport to the ER. Therein, the fed-peptide would facilitate the folding, assembly, and/or transport of the class I H chain. Consistent with this notion, immature (Endo H sensitive) class I H chains were affected by peptide feeding. However, a subsequent experiment by Townsend and colleagues (13) showed that peptide-induced unfolding of class I could have occurred in the cell lysate and not in the intact live cells. Thus, there was no need to hypothesize retrograde peptide transport. Furthermore, the observation that peptide stabilized class I expression on RMA.S cells provided a viable mechanism to explain the surface increase in class I expression by peptide. In accordance with this conclusion, we report here that peptide ligands significantly prolong the half-life of the uncharacteristically labile surface Ld molecule. Therefore, peptide-induced class I expression on live cells certainly affects surface molecules and needs not implicate retrograde peptide transport.

Inspired by these earlier peptide-feeding protocols, several groups have incubated various cell types with labeled peptide ligands for different class I molecules. Specific binding was then measured by immunoprecipitating the class I molecules from the lysates of the peptide-fed cells (6-10). Although each of these reports provides direct evidence for specific peptide binding to class I, in none of these studies was the mechanism elucidated. Mechanistic dilemmas were encountered due to two findings: first, cell inhibitory drugs such as Bfa, cyclohexamide, or sodium azide have frequently yielded equivocal results; and second, the aforementioned findings of Townsend raised questions of whether binding occurs to intact cells or in the cell lysate. Two experiments reported here are relevant to this issue. Reciprocal lysate mixtures from peptide-fed versus unfed, Ld-positive versus La-negative cells were used to quantify the amount of binding occurring in a lysate. This experiment indicated that at most 5% of the peptide binding occurred exclusively in the lysate of peptide-fed cells. This finding is in agreement with similar results obtained in studies of B27 (7). As an alternative approach, cells previously incubated with iodinated peptide were coated with mAb before lysis and thus only surface Ld molecules were detected. Using this approach, surface Ld molecules could be shown to account for at least 80% of the total (surface + lysate) peptide binding. We therefore feel confident that at least in our system most of the peptide ligand binding occurs to surface Ld molecules on live intact cells. Using this assay system we go on to show that the binding of ligand to surface Ld does not require new expression of class I nor exchange for heterologous B2m. However, without both new expression of Ld and exogenous B2m, the peptide binding to Ld falls off precipitously. Furthermore, this binding capability can be restored by adding purified hB2m. The reduced but significant peptide binding to Ld in the presence of Bfa agrees with a recent study of B27 (7), and the observation of significant peptide binding to Ld in the absence of exogenous B2m is consistent with studies of Db (8) and B27 (7). It should be noted, however, that these latter studies did not quantitate ligand binding with or without exogenous B2m. Perhaps the most striking feature of our data is that there is a precise quantitative correlation between the amount of B2m association and the ability to bind peptide. We thus conclude that B2m exchange is not a prerequisite for peptide binding to surface class I, but that B2m association promotes binding.

It has been speculated that ligand binding is to empty class I molecules in these assays using live cells. Indeed, there is good suggestive evidence supporting this conclusion from studies using RMA.S. In functional studies, RMA.S cells grown at 25°C were found to express high levels of surface class I molecules incapable of presenting endogenous antigens to CTL (12). Furthermore, in structural studies these class I molecules expressed by RMA.S at 25°C were found to bind labeled-exogenous peptide ligands and were found to be thermo-labile at 37°C in the absence of exogenous peptide (12). Thus, these studies provide structural and functional evidence consistent with the notion that class I molecules expressed by RMA.S cells grown at 25°C are empty. Given the validity of this conclusion, it is tempting to speculate that Ld molecules may also have a propensity to be expressed with empty ligand binding sites. Like RMA.S-expressed class I molecules, surface binding of peptide to Ld can be readily demonstrated and exogenous peptide dramatically enhances the surface stability of Ld. In addition, culturing cells at 23-26°C substantially increases 30-5-7+ Ld expression 1.5 to 4-fold, in contrast to other class I molecules that showed modest if any increases (W-R. Lie, unpublished observations). However, under SF conditions 30-5-7+ Ld are stably expressed and at levels equal to normal serum conditions. This result is in direct contrast to the extremely labile Kb/Dd molecules expressed by RMA.S cells at 25°C under SF conditions (38). Therefore our data favor the contention that at 37°C, 30-5-7+ Ld are occupied by endogenous peptides. The unique inducibility of Ld at 25°C or with peptide ligand could imply that there is a smaller pool of endogenous peptides capable of binding Ld compared with other class I molecules. It should be noted that in functional studies the Ld molecule has been found to be the restriction element for several CTL responses (35, 36, 39-42). Thus, these putative differences in the ability of Ld to bind peptide do not impair its physiological function. Perhaps the weak affinity of Ld for mouse B2m causes it to be more selective in ligand binding than other class I molecules. In support of the uniqueness of the interaction of nascent Ld molecules with B2m, we report here that Ld-peptide complexes readily exchange mouse B2m for hB2m in cell lysates. By contrast, Townsend et al. (13) found that Db-peptide complexes retain mouse B2m in RMA.S cells lysates. In agreement with their studies, we found that the de novo folding of Ld molecules in cell lysates was highly influenced by peptide ligand. Given this result it is possible that all 30-5-7+ Ld molecules synthesized at 37°C initially contain (or require) a peptide to attain proper folding. However, once folded the weak affinity for B2m could render Ld more vulnerable for peptide exchange.

Perhaps the most intriguing result presented in this study is the ambivalent nature of the 64-3-7+ form of Ld. Our studies of cell lysates show that peptide converts immature 64-3-7+ Ld to 30-5-7+ Ld, clearly establishing their precursor/product relationship. Furthermore, this conversion is
extensive if not complete for nascent 64-3-7+ Ld molecules. By contrast, our attempts to convert mature or surface forms of 64-3-7+ Ld to 30-5-7+ Ld have been unsuccessful. These failures to see conversion were observed in: (a) fluorometric comparisons of surface Ld forms on cells treated with Bfa +/- peptide (Fig. 5), (b) fluorometric analysis of sodium azide-treated cells +/- peptide ligand (data not shown), (c) cells grown in SF media +/- purified c~3 (Fig. 6, data not shown), and (d) immunoprecipitates of surface 125I-labeled Ld forms treated with peptide in the cell lysate (data not shown). No evidence for conversion was observed in each of the above experiments, suggesting that unlike immature 64-3-7+ Ld, mature forms are refractory to peptide-induced folding. Furthermore, direct evidence showing that only immature forms of Ld are peptide convertible was shown in pulse-chase experiments (Figs. 3 and 4).

These characterizations of the 64-3-7+ Ld molecules present some interesting comparisons in regard to previous reports of denatured heavy chains detected in/on cells. In studies using lysates of RMA.S, nascent heavy chains were detected by either a mAb to the c~3 domain (e.g., 28-14-8, [c~3] DPs), or a heteroantiserum to a cytoplasmic domain of Kp (43). These latter reagents, in contrast to 64-3-7, detect both non-conformed and conformed heavy chains. Carrying this analogy with RMA.S further, one might predict that surface 64-3-7 is equivalent to melted class I molecules on RMA.S. However, this appears not to be the case. Melted surface class I molecules on RMA.S can only be stably detected with the aforementioned heteroantiserum and not c~3 domain antibodies, whereas 64-3-7+ Ld molecules stably express c~3 serological epitopes (e.g., 28-14-8). More importantly, there is considerable evidence that conformed class I molecules on RMA.S melt and become detectable with this heteroantiserum (21), whereas we have no evidence suggesting that conformed Ld molecules melt and become detectable with mAb 64-3-7. If 64-3-7+ Ld were melted 30-5-7+ Ld, then adding peptide to Bfa-treated cells, adding hB2m to cells in SF media, or growing cells at 25°C would be expected to retard melting, leading to decreased amounts of surface 64-3-7+ Ld. However, no reductions were seen in these experiments. Perhaps the implication of the studies of RMA.S is that the truly melted form of surface Ld cannot be detected by either 64-3-7 or 28-14-8, but only by reagents like the heteroantiserum to the cytoplasmic domain. The better analogy for surface 64-3-7+ Ld is the Db molecule expressed by the B2m+ cell line RIE. RIE.Db and 64-3-7+ Ld share several properties including: (a) their detection with c~3 domain mAb, (b) their lack of B2m association, and (c) an undefined function. In addition, we found the RIE.Db molecules like 64-3-7+ Ld are stably expressed on the surface (t1/2 ~6 h) and are not readily convertible to proper conformation with exogenous B2m and/or peptide ligand (data not shown). These findings imply that the fates of both 64-3-7+ Ld and Db expressed by RIE were determined intracellularly.

Based on the findings reported here and these comparisons to studies of RMA.S or RIE.Db cells, we propose that structural distinctions between the alternative forms of Ld are determined by whether or not peptide/B2m-induced folding occurs. Furthermore, there is a critical time frame in which this folding must occur, as indicated by our pulse-chase experiments. The results imply either that the ER microenvironment is required for proper folding or that structural features of nascent Ld molecules, or their associated proteins, render them more susceptible to peptide/B2m-induced folding. What is remarkable is that these properties are maintained in cell lysates. Once 64-3-7+ Ld molecules are expressed on the cell surface they appear structurally inert. In contrast, surface 30-5-7+ Ld molecules that are B2m associated readily bind exogenous peptide ligand.
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