Definition and transfer of a serological epitope specific for peptide-empty forms of MHC class I

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Abstract

Nascent class I molecules have been hypothesized to undergo a conformational change when they bind peptide based on the observation that most available antibodies only detect peptide-loaded class I. Furthermore recent evidence suggests that this peptide-facilitated conformational change induces the release of class I from association with transporter associated with antigen processing (TAP)/tapasin and other endoplasmic reticulum proteins facilitating class I assembly. To learn more about the structure of peptide-empty class I, we have studied mAb 64-3-7 that is specific for peptide-empty forms of Ld. We show here that mAb 64-3-7 detects a linear stretch of amino acids including principally residues 48Q and 50P. Furthermore, we demonstrate that the 64-3-7 epitope can be transferred to other class I molecules with limited mutagenesis. Interestingly, in the folded class I molecule residues 48 and 50 are on a loop connecting a β strand (under the bound peptide) with the α helix (rising above the ligand binding site). Thus it is attractive to propose that this loop is a hinge region. Importantly, the three-dimensional structure of this loop is strikingly conserved among class I molecules. Thus our findings suggest that all class I molecules undergo a similar conformational change in the loop around residues 48 and 50 when they associate with peptide.

Introduction

Class I molecules are thought to undergo a conformational change when peptide binds. More specifically, it has been speculated that peptide induces a change in the class I molecule from an open to a folded conformation. Evidence for this conjecture was initially based on the observation that peptide association with class I is required for detection by antibodies termed conformationally dependent (1–3). Indeed, most alloantibodies to mouse class I only detect a folded or peptide-associated conformation. However, we previously described an exceptional mAb called 64-3-7 that detects empty Ld molecules awaiting peptide (4–6). Evidence that 64-3-7+ Ld molecules are indeed empty includes (i) their early detection in pulse–chase experiments, (ii) their conversion to folded molecules with addition of peptide to cell lysates, and (iii) the lack of bound peptide in a 64-3-7 precipitate when cell lysates are incubated with labeled Ld ligands. Furthermore, peptide dissociation at the cell surface results in the acquisition of 64-3-7+ forms. Thus there appear to be two alternative conformations of Ld, i.e. an open one detected by 64-3-7 and a folded one detected by all other anti-Ld mAb. The fact that determinants surrounding the Ld ligand binding site are only detected after peptide binding suggests that the putative conformation change that occurs when peptide binds may be substantial (7). Because similar mAb recognizing only peptide-empty forms of other class I alleles are not available, it is unclear whether all class I undergo a similar conformational change when peptide binds.

In addition to its utility in studying the relationship of Ld with peptide, mAb 64-3-7 has also been efficacious in probing the interaction of Ld with endoplasmic reticulum (ER) proteins implicated in peptide loading (8–10). The reasons for this are that (i) many ER chaperones are preferentially associated

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with peptide-empty class I and (ii) the epitope detected by mAb 64-3-7 remains accessible when L^d is bound by these ER proteins. Nascent class I H chains are first detected in association with calnexin (11). After assembly with β2-microglobulin (β2m) the class I H chain is detected in physical association with the transporter associated with antigen processing (TAP) (8,12,13). Whilst in association with TAP, the H chain/β2m heterodimer is also associated with calreticulin, tapasin and Erp57 (9,14–20). Collectively the complex of TAP/tapasin/calreticulin/Erp57 has been referred to as the peptide assembly or peptide-loading complex. Furthermore, addition of peptide has been shown to induce the release of class I from TAP and presumably other members of the assembly complex (8,12,13). Although the precise roles of individual components of the peptide-loading complex are not defined, collectively they appear to function as molecular chaperones since they (i) assist in the optimal assembly of peptide-induced conformation. Furthermore, antibodies because most antibodies only detect class I after attaining a to do the reciprocal experiment of differences in their assembly and association with molecular chaperones.

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K^d R48Q molecules appear to be peptide-empty and are detected in physical association with TAP.

Methods

Cell lines, mutagenesis and transfection

L-L^d cells were made as described previously (3). The K^d cDNA was kindly provided by Dr Abastado (Pasteur Institute, Paris, France). It was subcloned into the mammalian expression vector RSVP.neo (29). Site-directed mutagenesis was performed using the Quik Change mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer’s instructions. Briefly, the reaction was allowed to cycle 18 times. The annealing step was performed at 50°C for 1 min and the extension was performed at 68°C for 12 min. The synthetic oligodeoxynucleotides used for the reaction were as follows: the forward oligodeoxynucleotide 5’-GCG ACG CGG GTA ATC CGA TAT TTG AGC CGC AGG CGC CCT GGA TGG-3’ and the reverse oligodeoxynucleotide 5’-CCA TCC AGG CCT TCC GCT CAA ATC TCG TAT CCG CGT CGC-3’. The XbaI–KpnI fragment spanning the mutation was sequenced completely and exchanged with the analogous fragment from the wild-type K^d construct to create the K^d R48Q mutant. The L cells were transfected with the cDNAs using LipoFectin and selected in 0.6–1.0 mg/ml geneticin, both from Life Technologies (Gaithersburg, MD). Transfections of LCL721.220 (16) were done by electroporation using the Gene Pulser II system from BioRad (Hercules, CA).

Peptides

The peptides were synthesized using Fmoc solid-phase chemistry (30) on an Applied Biosystems (Foster City, CA) model 432A peptide synthesizer. In each synthesis cycle the deprotection, activation and coupling steps were continuously monitored by changes in conductivity. At the end of the synthesis process the N-terminus amino acid was deprotected resulting in a free amine group. The completed peptides were separated from the resin, and the side chain protecting groups removed by cleavage with trifluoroacetic acid and appropriate ion scavengers. The resulting C-terminus of each peptide is a free acid. The crude peptide were separated by reverse-phase HPLC and mass spectrometry.

Peptide inhibition and flow cytometry

To test peptides for their ability to inhibit mAb 64-3-7 binding to L^d, peptides were diluted in 100 µl of HBSS containing 0.2% BSA and 0.1% sodium azide, and incubated at 4°C for 30 min in the wells of round-bottom microtiter plates with 20 µl of culture supernatant containing mAb 64-3-7 or control mAb. L-L^d cells (400,000/well) were then added and the peptide-mAb-cells incubation continued at 4°C for 1 h. The cells were then washed and incubated with FITC-conjugated, Fc-specific, affinity-purified F(ab')2 fragment of goat anti-mouse IgG (ICN, Cappel, Costa Mesa, CA). Viable cells, gated by forward and side scatter, were analyzed an a FACSCalibur (Becton Dickinson, San Jose, CA) equipped with an argon ion laser tuned to 488 nm and operating at
150 mW. The data are expressed as linear fluorescence values obtained from log-amplified data using CellQuest Software (Becton Dickinson). Cells incubated only with the fluorescent antibody were used as negative controls.

**Immunoprecipitation**

Cells were preincubated for 30–60 min at 37°C in culture media that lacked methionine, after which 125–250 µCi/ml of [35S]methionine was added and the cells radiolabeled for 15–30 min. The cells were then washed 3 times in PBS containing 20 mM iodoacetamide (Sigma, St Louis, MO) and lysed in buffer that contained 1% digitonin (Wako, Richmond, VA), 0.1 mM 7-amino-1-chloro-3- tosylamido-2-heptone and 0.5 mM freshly added PMSF. The lysis buffer was supplemented with a saturating amount of mAb or rabbit antibody before addition to pelleted cells. After incubation for 30 min on ice, nuclei were removed by centrifugation and lysates were incubated with Protein A-Sepharose beads (Pharmacia, Piscataway, NJ). The beads were washed 4 times with 0.1% digitonin in Tris-buffered saline, pH 7.4, and the samples were eluted by boiling in 0.125 M Tris, pH 6.8/2% SDS/12% glycerol/2% bromophenol blue. All immunoprecipitates were treated with Amplify (Amersham, Boston, MA), dried and exposed to BioMax MR film (Eastman Kodak, Rochester, NY) at -70°C for varied lengths of time. For Western blots, SDS–PAGE gels were transferred to Immobilon P membranes (Millipore, Bedford, MA). After overnight blocking, membranes were incubated in a dilution of antibody for 2 h, washed 3 times with PBS/0.05% Tween 20 and incubated for 1 h with biotin-conjugated goat anti-mouse or anti-rabbit IgG (Caltag, San Francisco, CA). Following three washes with PBS/0.05% Tween 20, membranes were incubated for 1 h with streptavidin-conjugated horseradish peroxidase (Zymed, San Francisco, CA), washed 3 times and incubated with Western blot developing reagents (Amersham). All immunoprecipitation and Western blot lanes within a figure are from a single experiment.

**Results**

**Definition of 64-3-7 epitope**

To learn more about the structure of peptide-empty class I molecules, we investigated the molecular basis of the 64-3-7 epitope that is specific for peptide-empty L<sup>d</sup> molecules. In a previous study of a panel of mutant L<sup>d</sup>H chains, we discovered that substitution of Q48R ablates mAb 64-3-7 binding to L<sup>d</sup> (31). Independently, we also observed that mAb 64-3-7 detects SDS-denatured L<sup>d</sup> by Western blotting (8). Based on these combined finding, we presumed that the 64-3-7 epitope was a linear determinant surrounding residue Q48. To further define this epitope, various peptides were tested for their ability to inhibit 64-3-7 binding to L<sup>d</sup> expressed on L-L<sup>d</sup> cells using cytofluorometry (Table 1). Specificity was determined by testing peptide inhibition of mAb to other epitopes on L<sup>d</sup> located in the α2 domain (mAb 30-5-7) and the α3 domain (mAb 28-14-8) (data not shown). As shown in Table 1, a 21 amino acid segment (21mer) derived from positions 35–55 of L<sup>d</sup> gave specific and potent inhibition of 64-3-7 binding to L<sup>d</sup> (IC<sub>90</sub> = 0.05 µM). By contrast, homologous peptides from the 64-3-7<sup>+</sup> mouse class I molecules K<sup>d</sup>, K<sup>b</sup>, D<sup>b</sup> and D<sup>d</sup> required 2–4 logs more peptide to inhibit 64-3-7 binding to L<sup>d</sup> (Table 1). Comparison of peptide sequence with inhibition potency clearly defined residues Q48 and P50 as important for 64-3-7 binding to L<sup>d</sup>. Position 48 is arginine in all mouse and human classical class I molecules except L<sup>L</sup> and L<sup>d</sup> (both 64-3-7<sup>+</sup>), where it is glutamine (32,33). By contrast, position 50 is dimorphic (proline or arginine) in the mouse and proline in humans (32,33). To further determine the importance of residues Q48 and P50 in the 64-3-7 epitope, peptides with substitutions were also tested. Substitutions of either Q48R or P50R in the L<sup>d</sup> 21mer impaired inhibitory potency 2–3 logs, whereas a peptide with the substitutions at both positions showed no inhibition at the highest concentration tested (Table 1). Furthermore, the K<sup>d</sup> 21mer was made 100-fold more potent with the single substitution of R48Q (note K<sup>d</sup> already has a P50). Indeed this single substitution made the K<sup>d</sup> R48Q 21mer as efficacious in the blocking assay as the L<sup>d</sup> 21mer, in spite of other differences. Thus, we conclude that the unique combination of polymorphic amino acids Q48 and P50 expressed on the L<sup>d</sup> and L<sup>L</sup> molecules determines their reactivity with mAb 64-3-7.

**Location of the 64-3-7 epitope in the folded class I H chain**

Residues 48 and 50 are located on a loop of the folded L<sup>d</sup> molecule connecting the end of the β pleated region coming out from under the ligand binding site with the beginning of the α helical region protruding above the ligand binding site. As shown in Fig. 1, the side chains of Q48 and P50 actually point down and away from the peptide binding groove of the folded L<sup>d</sup> molecule. This loop region of the H chain has a 3<sub>10</sub> helical structure (three residues per turn and 10 atoms in the ring closed by a hydrogen bond). The dipoles forming these hydrogen bonds are not aligned and thus are not at minimum energy. Furthermore, the side chain packing is rather unfavorable when in a 3<sub>10</sub> helix. Thus, residues in the vicinity of 48–50 may display considerable flexibility. Therefore, it is attractive to speculate that the 48–50 loop may constitute a hinge region such that mAb 64-3-7 is specific for a configuration of residues only aligned or exposed in the open conformation. Precisely how 64-3-7 differentiates open from folded L<sup>d</sup> will have to await high resolution of the three-dimensional structure of peptide-empty L<sup>d</sup>. Another question raised about the location of the 64-3-7 epitope is whether all class I alleles undergo a similar conformational change when peptide binds. To test the validity of this hypothesis, we sought to transfer the 64-3-7 epitope to other class I molecules.

**Transfer of the 64-3-7 epitope to K<sup>d</sup>**

The above peptide inhibition suggests that the introduction of the 64-3-7 epitope may simply require mutating residues of other class I molecules to 48Q and 50P if not already present. To initially test this conjecture, we first attempted to transfer the 64-3-7 epitope to the 64-3-7<sup>+</sup> K<sup>d</sup> molecule. Thus, we constructed and expressed a K<sup>d</sup> molecule with the single substitution of R48Q since K<sup>d</sup> already has a P50 residue. As shown in the middle panel of Fig. 2, this single mutation in K<sup>d</sup> was sufficient to render a subset of K<sup>d</sup> R48Q molecules 64-3-7<sup>+</sup> by cytofluorometry. Indeed, the 64-3-7<sup>+</sup> subset of K<sup>d</sup> R48Q molecules looks strikingly similar to the 64-3-7<sup>+</sup> L<sup>d</sup>
Table 1. Peptide inhibition of mAb 64-3-7 binding to L<sup>d</sup>

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>48</th>
<th>50</th>
<th>55</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>L&lt;sup&gt;d&lt;/sup&gt;</td>
<td>RFDSDAENPRYEPEQPAPWMEQE</td>
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<td></td>
<td></td>
<td>0.05</td>
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<tr>
<td>L&lt;sup&gt;d&lt;/sup&gt; Q48R</td>
<td>RFDSDAENPRYEPEQPAPAPE</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>L&lt;sup&gt;d&lt;/sup&gt; P50R</td>
<td>RFDSDAENPRYEPEQPAPAPA</td>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>L&lt;sup&gt;d&lt;/sup&gt; Q48R,P50R</td>
<td>RFDSDAENPRYEPEQPAPAPE</td>
<td></td>
<td></td>
<td></td>
<td>&gt;1000</td>
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<tr>
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<td>RFDSDAENPRYEPEQPAPAPE</td>
<td></td>
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<tr>
<td>K&lt;sup&gt;d&lt;/sup&gt; R48Q</td>
<td>RFDSDAENPRYEPEQPAPAPE</td>
<td></td>
<td></td>
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<td></td>
<td>&gt;1000</td>
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</tbody>
</table>

<sup>a</sup> Underlined residues represent differences from the L<sup>d</sup> sequence and residues at positions 48 and 50 are in bold.

<sup>b</sup> Concentration of peptide required to give 50% maximal inhibition of mAb 64-3-7 binding to L<sup>d</sup> as measured by cytofluorometry.

Epitope transfer to K<sup>d</sup> does not disrupt its peptide binding specificity

Based on the location of the 64-3-7 epitope, its transfer to other class I molecules would not be expected to alter peptide binding specificity. However, we considered this an important control to demonstrate that epitope transfer does not compromise the structure of the ligand binding groove. To compare peptide binding to K<sup>d</sup> versus epitope tagged K<sup>d</sup> (etK<sup>d</sup>), we expressed wild-type K<sup>d</sup> and etK<sup>d</sup> molecules in tapasin-deficient LCL721.220 cells (16). Both a 220-K<sup>d</sup> and a 220-etK<sup>d</sup> cell line were selected with significant levels of surface expression. Thus, tapasin is not an absolute requirement for K<sup>d</sup> expression. However, K<sup>d</sup> or etK<sup>d</sup> molecules expressed in the absence of tapasin were considerably more peptide accessible than when expressed in tapasin-positive cells (Figs 3 and 4, and data not shown). Thus the 220-K<sup>d</sup> and 220-etK<sup>d</sup> cells lines could be used for comparative peptide binding studies. Four different known K<sup>d</sup> ligands were used for this study, LLO 91–99 GYKDGNEYI (34) and p60 217–225 KYGVSVODI (35) from Listeria monocytogenes, HLA-Cw3 170–179-derived peptide RYLKNGKETL (36), tum<sup>P</sup> P198 14–22 KYQAVVTL (37) and Flu NP 147–155 TYQRTRALV (38). As a negative control peptide the P29 L<sup>d</sup> ligand YPNVNIHNF (39) was tested. Using a surface stabilization assay, cells were incubated overnight with peptide at various concentrations and K<sup>d</sup> expression was tested by FACS. As shown in Fig. 3, all four known K<sup>d</sup> ligands expression maximally induced K<sup>d</sup> or etK<sup>d</sup> ~2.6-fold compared with the negative controls (no peptide or irrelevant peptide). Furthermore, in a peptide titration experiment the rank order of ligand binding to K<sup>d</sup> versus etK<sup>d</sup> was identical and recognition of K<sup>d</sup> versus etK<sup>d</sup> by a T cell hybridoma specific for K<sup>d</sup>/Cw3 was comparable (data not shown). Thus the specificity of peptide binding to etK<sup>d</sup> and wild-type K<sup>d</sup> appeared indistinguishable in these comparisons, indicating that epitope transfer did not alter the structure of the ligand binding groove.

Surface expression of 64-3-7<sup>+</sup> etK<sup>d</sup> molecules is eliminated by culture of 220-etK<sup>d</sup> cells with exogenous peptide ligands

To determine whether the 64-3-7<sup>+</sup> forms of etK<sup>d</sup> were associated with peptide, 220 cells expressing etK<sup>d</sup> were incubated
with a known K\textsuperscript{d} peptide ligand (CW3) or a length-matched control peptide (YASNENMETM). As shown in Fig 4, the surface expression of etK\textsuperscript{d} as detected by two different K\textsuperscript{d} reactive mAb (SF1-1.1.1 and DO9) rose sharply following incubation with the CW3 peptide. Furthermore, this induction of epitope-tagged K\textsuperscript{d} was peptide dose dependent and only seen with the K\textsuperscript{d} binding peptide and not the control peptide. In striking contrast to these findings, the level of surface expression of 64-3-7\textsuperscript{/H11001} etK\textsuperscript{d} was not induced by the CW3 peptide, but rather declined in a dose-dependent manner (Fig 4, right panel). Indeed, this is exactly what happens to the expression of 64-3-7\textsuperscript{/H11001} L\textsuperscript{d} on live cells (5,6). The reciprocal effect of peptide incubation on L\textsuperscript{d} recognition by 30-5-7
increase in surface expression. By contrast, 64-3-7 is specific for folded class I molecules at the cell surface, resulting in an open H chain conformation that occurs after peptide dissociation (6). Our data further indicate that 64-3-7 detects peptide-empty forms of etK\(\beta\) molecules, whereas calnexin is associated with both folded and open forms of L\(\alpha\), K\(\alpha\) and etK\(\beta\). The association of calnexin with folded and assembled mouse class I results is consistent with published reports (8,70), and thus serves as a positive control for the Western blot analysis.

Intracellular etK\(\beta\) molecules are detected in association with TAP using mAb 64-3-7. L-L\(\alpha\), L-K\(\alpha\) and L-etK\(\beta\) cells were metabolically labeled and precipitated with mAb 64-3-7 (all three samples) or a mAb to folded H chains (30-5-7 for L\(\alpha\) and SF1-1.1.1 for K\(\alpha\) and etK\(\beta\)). Aliquots of these same immunoprecipitates were Western blotted with anti-mTAP or anti-calnexin (CXN) as shown in the lower two panels respectively. As shown TAP is associated predominantly with 64-3-7\(\alpha\) and 64-3-7\(\beta\), whereas calnexin is associated with both folded and open forms of L\(\alpha\), K\(\alpha\) and etK\(\beta\). The association of calnexin with folded and assembled mouse class I results is consistent with published reports (8,70), and thus serves as a positive control for the Western blot analysis.

Based on the unique ability of mAb 64-3-7 to detect peptide-empty forms of L\(\alpha\), we sought to define the structural basis of the 64-3-7 epitope. We report here that 64-3-7 detects predominantly the combination of residues Q48 and P50. Although this combination of amino acids is unique to L\(\alpha\) and L\(\alpha\), sequence in the 45–55 segment is quite conserved among all classical class I molecules. Furthermore, these residues are located on a loop of the folded class I H chain connecting the end of the \(\beta\) pleated region, coming out from under the ligand binding site, with the beginning of the \(\alpha\) helical region protruding above the ligand bind site (Fig. 1). Interestingly, Machold and Ploegh (40) recently characterized a mAb to K\(\beta\) that has striking similarities to 64-3-7. Their mAb, called KU1, was generated to denatured K\(\beta\) protein obtained from Escherichia coli expression system. mAb KU1 was found to be specific for a nascent folding intermediate of K\(\beta\) and it was mapped to the residues 49–54. Unfortunately, the affinity of mAb KU1 is insufficient to render it useful in Western blot or FACS analyses (40 and unpublished data). However, the striking similarities in the specificity and epitope mapping of KU1 and 64-3-7 suggest that both K\(\beta\) and L\(\alpha\) undergo similar conformational changes when they bind peptide. Furthermore, we report here that the 64-3-7 epitope can be transferred to other class I molecules and remain specific for their open conformation. These collective findings provide compelling serological evidence that all classical class I molecules undergo a similar conformational change when they bind peptide, and that this conformational change involves residues in the loop around residues 48 and 50.

46-3-7\(\alpha\) K\(\alpha\) molecules are detected in association with TAP

We and others have reported that most mAb to class I only detect peptide folded forms, and thus are incapable of detecting class I while in association with the TAP peptide-loading complex (8,12,13). Thus it was important to determine whether etK\(\beta\) molecules could be detected by mAb 64-3-7 while in association with the TAP complex. As shown in the immunoprecipitates in Fig. 5, a subset of etK\(\beta\) molecules, but not wild-type K\(\alpha\) molecules, was detected by mAb 64-3-7. It is important to note that the intracellular pool of 64-3-7\(\alpha\) etK\(\beta\) molecules in L-K\(\alpha\) R48Q cells is sizable and similar to the pool of 64-3-7\(\alpha\) L\(\alpha\) molecules seen in L-L\(\alpha\) cells. As expected both epitope-tagged and wild-type K\(\alpha\) molecules were precipitated with conformation-dependent mAb SF1-1.1.1. When these immunoprecipitates were Western blotted with an anti-TAP, 64-3-7 precipitates and not SF1-1.1.1 precipitates of etK\(\beta\) showed TAP association (Fig. 5). In data not shown, 64-3-7\(\alpha\) forms of etK\(\beta\) molecules were also found to be associated with tapasin and calreticulin. Thus mAb 64-3-7 detects open forms of etK\(\beta\) molecules while in association with the class I peptide-loading complex. Furthermore, based on their detection in physical association with TAP and their elimination on the surface in the presence of exogenous peptide, we conclude the 64-3-7\(\alpha\) etK\(\beta\) molecules are peptide-empty.

Discussion

Based on the unique ability of mAb 64-3-7 to detect peptide-empty forms of L\(\alpha\), we sought to define the structural basis of the 64-3-7 epitope. We report here that 64-3-7 detects predominantly the combination of residues Q48 and P50. Although this combination of amino acids is unique to L\(\alpha\) and L\(\alpha\), sequence in the 45–55 segment is quite conserved among all classical class I molecules. Furthermore, these residues are located on a loop of the folded class I H chain connecting the end of the \(\beta\) pleated region, coming out from under the ligand binding site, with the beginning of the \(\alpha\) helical region protruding above the ligand bind site (Fig. 1). Interestingly, Machold and Ploegh (40) recently characterized a mAb to K\(\beta\) that has striking similarities to 64-3-7. Their mAb, called KU1, was generated to denatured K\(\beta\) protein obtained from Escherichia coli expression system. mAb KU1 was found to be specific for a nascent folding intermediate of K\(\beta\) and it was mapped to the residues 49–54. Unfortunately, the affinity of mAb KU1 is insufficient to render it useful in Western blot or FACS analyses (40 and unpublished data). However, the striking similarities in the specificity and epitope mapping of KU1 and 64-3-7 suggest that both K\(\beta\) and L\(\alpha\) undergo similar conformational changes when they bind peptide. Furthermore, we report here that the 64-3-7 epitope can be transferred to other class I molecules and remain specific for their open conformation. These collective findings provide compelling serological evidence that all classical class I molecules undergo a similar conformational change when they bind peptide, and that this conformational change involves residues in the loop around residues 48 and 50.

Residues 48 and 50 are located on a loop of the folded class I molecule connecting \(\beta\) strand secondary structure with the \(\alpha\) helix of the \(\alpha1\) domain (Fig. 1). In the L\(\alpha\)-p29 (41), as well as most other class I molecules, residues 50–53 display \(\beta\)10 helical structure (42). Furthermore, the three-dimensional structure of the residues 50–53 is highly conserved among published structures of folded mouse and human classical class I molecules as indicated by their RMS displacements of corresponding \(\alpha\) atoms (43, 44). For example, the RMS deviation (for the \(\alpha\) atoms of residues 50–53) in comparison with L\(\alpha\)-p29 for K\(\beta\)-2vab (45) = 0.14, D\(\alpha\)-FluNp (46) = 0.28, L\(\alpha\)-QL9 (47) = 0.21, HLA-B27 (48) = 0.40, HLA-B35nef (49) = 0.35 and HLA-B53s6 (50) = 0.37. Interestingly, \(\beta10\) structures can represent one of the trapped \(\beta\)10 structures. For example, transition between \(\beta\)10 and \(\alpha\) helical conformations has been observed in domain motions in aspartate aminotransferase (54) and lactate dehydrogenase (55). Alternatively...
the class I residues 48–50 at the end of the β strand (residues 45–47) in the folded form may adapt an extended β strand conformation in the open form. Therefore, the interconversion between 310 helix and coil, α helix or β strand may play a key role in 64-3-7 recognition. In any case we propose that the H chain structure around residues 48 and 50 displays conformational fluidity, and that 64-3-7 detects a specific orientation of amino acid side chains only displayed when the H chain is not associated with peptide.

Structural resolution of peptide-empty class I has been difficult due to its inherent instability or perhaps its lack of a single defined conformation. However, biochemical support of an open class I conformation was recently published by Bouvier and Wiley (56). They characterized a peptide-empty class Iβ2m heterodimer using several different chemical assays including protease sensitivity and circular dichroism. These authors concluded that peptide-empty class I is partially folded and possesses many of the properties ascribed to the molten globule state (56). Furthermore, Catipovic et al. (57) used fluorescence resonance energy transfer to probe the structure of peptide-empty class I. They reported that empty Kβ molecules are in a relatively extended conformation that becomes more compact when peptide binds. Thus multiple lines of evidence suggest the existence of a distinct open conformation for peptide-empty class I. Resolution of the structure of ligand-free class I could greatly help our understanding of how empty class I molecules interact with ER proteins such as tapasin, TAP, calreticulin, calnexin and Erp57. Furthermore, definition of the structural changes that occur in class I when peptide binds could help define the signals received by these ER chaperones that release class I from ER retention after peptide binds.

Low levels of empty class I molecules also exists at the cell surface. Although this was first demonstrated with Ld, subsequent studies have shown that several different mouse and human alleles also have low levels of peptide-empty forms at the cell surface (e.g. 40,58). However, the mechanisms generating empty class I remain controversial with certain studies indicating that they are only expressed on activated and not on resting cells (59,60). Furthermore, allele-specific differences in the surface expression of peptide-empty class I have not been well characterized. The function of peptide-empty class I at the cell surface also remains controversial, but there have been several intriguing reports of their role in allograft rejection (61), as ligands for certain syngeneic NK receptors (62), in the induction of autoimmune disease in HLA-B27 transgenic mice (63) and finally in the presentation of endocytosed hepatitis B surface antigen particles (64). Thus peptide-empty class I may have important and diverse functions. However, the difficulty in many of these studies is in detecting class I molecules that are peptide-empty versus detecting class I molecules in merely a peptide-independent manner. The use of 64-3-7 as an epitope tag should help define the function of truly peptide-empty class I. Furthermore, epitope tagging peptide-empty forms of different class I alleles will permit more quantitative comparisons of their prevalence at the cell surface, and better mechanistic studies of their occurrence in physiological and pathological conditions.

In conclusion the definition and transfer of the 64-3-7 epitope represents compelling serological evidence that different class I alleles undergo a similar conformational change when peptide binds or dissociates. Furthermore the use of 64-3-7 as an epitope tag should greatly facilitate future studies comparing the folding and biosynthesis of various mouse and human class I alleles as well as the function of their peptide-empty surface conformers.

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Abbreviations

ER endoplasmic reticulum
et epitope tag
β2m β2-microglobulin
TAP transporter associated with antigen processing

References

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of empty and peptide-loaded MHC molecules at the cell surface. 