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Crystal Structures of Two Closely Related but Antigenically Distinct HLA-A2/Melanocyte-Melanoma Tumor-Antigen Peptide Complexes

Piotr Sliz, Olivier Michelin, Jean-Charles Cerottini, Immanuel Luescher, Pedro Romero, Martin Karplus, and Don C. Wiley

We have determined high-resolution crystal structures of the complexes of HLA-A2 molecules with two modified immunodominant peptides from the melanoma tumor-associated protein Melan-A/Melanoma Ag recognized by T cells-1. The two peptides, a decamer and nonamer with overlapping sequences (ELAGIGILTV and ALGIGILTV), are modified in the second residue to increase their affinity for HLA-A2. The modified decamer is more immunogenic than the natural peptide and a candidate for peptide-based melanoma immunotherapy. The crystal structures at 1.8 and 2.15 Å resolution define the differences in binding modes of the modified peptides, including different clusters of water molecules that appear to stabilize the peptide-HLA interaction. The structures suggest both how the wild-type peptides would bind and how three categories of cytotoxic T lymphocytes with differing fine specificity might recognize the two peptides.

The isolation of tumor-reactive CTL clones from either tumor-infiltrating lymphocytes or circulating lymphocytes from cancer patients has led to the identification of a number of tumor-associated Ags (1, 2). Structural properties of those Ags are poorly understood, and currently only a single structure of tumor-associated peptide/MHC complex has been reported (3). Because tumor-infiltrating lymphocytes can have potent antitumor activity (4), tumor-associated Ags may be used to induce cancer regression by vaccination. A detailed understanding of peptide-Ag/MHC binding could lead to the development of more efficient modified peptides and potentially improved vaccines.

Melan-A/melanoma Ag recognized by T cells-1 (Melan-A/MART-1) is one of the most frequently recognized tumor Ags in melanoma patients expressing HLA-A2 (5, 6). Initially, two parental peptides were identified that mimic the protein Ag, the nonapeptide Melan-A/MART-127–35 AAGIGILTV and the decapeptide Melan-A/MART-126–35 EAAGIGILTV, with which contains an additional residue at the amino terminus (6). Discovery of those peptides led to the synthesis of a variety of modified Melan-A/MART-1 peptides that are now used in phase I vaccination trials in patients with high-risk stage III-IV malignant melanoma (7).

All CTL clones obtained from patients against the Melan-A/MART-1 gene product recognize both wild-type nonamer and decamer peptides bound to HLA-A2. Indeed, target cells sensitized with saturating concentrations of either peptide are lysed with equal efficiency by all CTL clones. However, peptide titrations reveal that the wild-type decamer is generally recognized more efficiently than the natural nonamer peptide (12 of 18 CTL analyzed) (8). Although the natural decamer binds to HLA-A2 three to five times more efficiently than the natural nonamer, this difference does not fully account for the higher efficiency of decamer Ag recognition. A smaller, second category of CTL clones recognizes the two natural peptides with similar efficiency (4 of 18 CTL analyzed). A rare, third category of CTL recognizes the nonamer more efficiently than the decamer peptide (2 of 18 CTL clones analyzed) (6, 8). The responses of these different categories of CTL suggest that the HLA-A2-bound natural nonamer and decamer exhibit some surfaces that are similar and some that are different to CTL.

Both Melan-A/MART-1 parental peptides display an intermediate binding affinity for the HLA-A2 molecule, because they have Ala rather than a large anchor residue at peptide position two (P2) (Table I). Replacement of P2 by residues such as Leu or Thr leads to detectable increases in HLA-A2 binding, as measured in functional assays (9). Although the complexes with either parental peptide have very short t1/2 of <1 h, the introduction of the better P2 anchor residues prolongs the stability of both complexes to t1/2 of >6 h. Curiously, however, while the modified decamer led to an improvement in the efficiency of Ag recognition by most CTL clones tested, the modified nonamer led to a strong reduction in Ag recognition, in the same population of CTL clones, even at very high peptide concentrations. This loss of CTL reactivity suggests that the modified nonamer peptide binds to HLA-A2 differently than the natural nonamer and differently than the natural and modified decamer (10, 11).
In this study, we report the crystallographic comparison of modified decapetide (ELAIGILTV, referred to as ELA hereafter) and modified nonapeptide (ALGIGILTV, referred to as ALG hereafter) from the Melan-A/MART-1 protein, both complexed with HLA-A2 (HLA-A2/ELA and HLA-A2/ALG). Complexes with the natural peptides were not studied because they were too unstable to purify and crystallize. The high resolution of the structures, 1.8 and 2.15 Å, allows detailed comparisons of the HLA-A2/peptide interactions, including the roles of bound waters. The modified nonamer binds in a mode that is unreactive with CTL specific for the protein Ag, and therefore, probably not like the natural nonamer. The structures suggest both how the wild-type peptides would bind and how the three categories of CTL with differing fine specificity might recognize the two natural peptides and the modified decamers.

Materials and Methods

Synthetic peptides

Both peptides, ELA and ALG, were synthesized and purified using HPLC, as described previously (9).

Folding and purification of HLA-A2 complexes

Soluble HLA-A2 was refolded in vitro in the presence of peptide after expression in bacteria, as described previously (12). In brief, the HLA-A2 heavy chain (truncated at Glu275) and β2-microglobulin (β2m), isolated from Escherichia coli inclusion bodies, were refolded by dilution to unstable to purify and crystallize. The high resolution of the structures, 1.8 and 2.15 Å, allows detailed comparisons of the HLA-A2/peptide interactions, including the roles of bound waters. The modified nonamer binds in a mode that is unreactive with CTL specific for the protein Ag, and therefore, probably not like the natural nonamer. The structures suggest both how the wild-type peptides would bind and how the three categories of CTL with differing fine specificity might recognize the two natural peptides and the modified decamers.

Table I. Summary of relative HLA-A2 binding and antigenic activities of natural and modified Melan-A/MART-1 peptides

<table>
<thead>
<tr>
<th>Position</th>
<th>Substitution</th>
<th>Amino Acid Sequence</th>
<th>A2 Binding</th>
<th>Antigenic Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>27–35</td>
<td>A28L</td>
<td>AAGIGILTV</td>
<td>+/−</td>
<td>+</td>
</tr>
<tr>
<td>27–35</td>
<td>A28L</td>
<td>ALGIGILTV</td>
<td>+</td>
<td>Reduced</td>
</tr>
<tr>
<td>26–35</td>
<td>A28L</td>
<td>EAAGIGILTV</td>
<td>+/−</td>
<td>++</td>
</tr>
<tr>
<td>26–35</td>
<td>A28L</td>
<td>ELAIGILTV</td>
<td>+</td>
<td>++ or more</td>
</tr>
</tbody>
</table>

* Position of the peptide sequence in the Melan-A/MART-1 gene product.

Materials and Methods

In this study, we report the crystallographic comparison of modified decapetide (ELAIGILTV, referred to as ELA hereafter) and modified nonapeptide (ALGIGILTV, referred to as ALG hereafter) from the Melan-A/MART-1 protein, both complexed with HLA-A2 (HLA-A2/ELA and HLA-A2/ALG). Complexes with the natural peptides were not studied because they were too unstable to purify and crystallize. The high resolution of the structures, 1.8 and 2.15 Å, allows detailed comparisons of the HLA-A2/peptide interactions, including the roles of bound waters. The modified nonamer binds in a mode that is unreactive with CTL specific for the protein Ag, and therefore, probably not like the natural nonamer. The structures suggest both how the wild-type peptides would bind and how the three categories of CTL with differing fine specificity might recognize the two natural peptides and the modified decamers.

Table II. X-ray crystallographic data collection

<table>
<thead>
<tr>
<th>HLA-A2/ALG</th>
<th>HLA-A2/ELA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P2₁2₁2₁</td>
</tr>
<tr>
<td>Molecules in AU</td>
<td>1</td>
</tr>
<tr>
<td>Cell parameters (Å)</td>
<td>a = 49.45 b = 74.72 c = 123.72</td>
</tr>
<tr>
<td>X-ray source/detector</td>
<td>APS 14-BM-C (Chicago, IL)/Quantum 4 CCD</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>30–2.15</td>
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<tr>
<td>Mosaicity</td>
<td>0.35</td>
</tr>
<tr>
<td>Total reflections</td>
<td>190647/19192</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>25511/30855</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>7.46.2</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.0/96.4</td>
</tr>
<tr>
<td>Rmerge⁵</td>
<td>3.9/12.4</td>
</tr>
<tr>
<td>I/σ</td>
<td>368.2</td>
</tr>
</tbody>
</table>

* Highest resolution shell.

* Rmerge = Σ |h–(h)|/Σ h, where (h) is the average intensity over symmetry equivalents.
Both complexes were refined using CNS version 1.0. Model building was performed with O (19) using 2Fo-Fc and simulated annealing composite omit-maps. The final refinement statistics for both structures are presented in Table III.

Figures were generated using SPOCK (20) and InsightII (Molecular Simulations, San Diego, CA).

Metal ion identification

Strong 13.5Å electron density was observed in the composite omit-map of the HLA-A2/ELA complex and was assigned to a metal ion. The distances between the metal ion and the coordinating atoms are 2.01, 2.07, 2.06, and 2.06 Å. Based on these distances and the preferences of metals for the tetrahedral coordination (21), it is likely that the metal binding site is occupied by either Mg\(^{2+}\), Zn\(^{2+}\), or Cu\(^{2+}\) (minimum metal-ligand oxygen distances 2.07, 1.91, and 1.82 Å, respectively; Ref. 21). B factor refinement with all three metals eliminated Mg\(^{2+}\) as a candidate. The B factor for Mg\(^{2+}\) refined to below 1 Å\(^2\), whereas the B factors for Zn\(^{2+}\) and Cu\(^{2+}\) both refined to 22.4 Å\(^2\), in agreement with the B factors of the atoms coordinating the metal, which are in the 19–25 Å\(^2\) range.

Results

The crystal structures of HLA-A2/ELA and HLA-A2/ALG complexes have been determined to a resolution of 1.8 and 2.15 Å, respectively, and show clear, unambiguous density for the peptide ligands (Fig. 1, A and B). The structures were refined to a reasonable agreement between observed and calculated structure factors, as well as good stereochemistry and geometry (Table III). The 1.8 Å resolution of HLA-A2/ELA complex allowed incorporation of additional water molecules (Table III), but the high resolution and quality of both structures permitted a detailed interpretation of the peptide binding.

HLA-A2 structure

The HLA-A2 structures in the HLA-A2/ELA and HLA-A2/ALG complexes are very similar to the highest resolution, 1.8 Å MHC class I (MHC-I)/peptide structure in the Protein Data Bank, HLA-A2/Tax peptide (1DUZ; Ref. 17), with root-mean-square difference (rmsd) of 0.79 Å\(^2\) and 0.86 Å\(^2\) for the heavy chain and 0.55 Å\(^2\) and 0.63 Å\(^2\) for β2m, respectively. The few differences include disorder of Gln\(^{226}\) and Asp\(^{227}\) in the α3 domain of the melanoma-peptide complexes. In addition, the HLA-A2/ELA complex contains a crystallographic metal binding site (Fig. 1C) that has not been observed in any other MHC-I/peptide crystal structure. The tetrahedral metal is coordinated by His\(^{192}\) from the heavy chain of HLA-A2 and Asp\(^{98}\) from the β2m of the same molecule, as well as by His\(^{153}\) and Glu\(^{154}\) from the heavy chain of a symmetry-related molecule. Although no metal ions were added to the solutions during the purification or crystallization procedures, it is possible that even trace amounts of contaminants would be enough to become incorporated. Metal binding sites can stabilize the oligomerization of proteins in solution, and therefore it would be expected that the presence of metal might have stabilized the crystal packing and increased the diffraction limit of the HLA-A2/ELA crystals (full data set is 1.8 Å, but some weak reflections were observed at 1.5 Å). Although the crystals of the two complexes are isomorphous, the metal is not present in the HLA-A2/ALG complex, and one of the metal ligands, Glu\(^{154}\), is in a different conformation, rotated away from the position of the metal in the other crystal (see discussion of Gln\(^{155}\) in Results).

Modified melanoma-peptide structures

The Melan-A/MART-1 nonamer and decamer peptides bind to HLA-A2 in modes similar to that of five viral peptides, including nonamers and a decamer previously observed bound to HLA-A2 (22). To facilitate comparison, we divide the peptides into three regions (Fig. 2A): an N-terminal region, which includes three N-terminal residues P1-P3; a C-terminal region, which includes four C-terminal residues (P6-P9); and the central region consisting of a three-residue bulge in the decamer peptide (P4, P*, P5) and a two-residue link in a nonamer peptide (P4-P5). Arrows in Fig. 2A show the directions of the peptide side chains (up, out of the groove; down, into the groove; right, toward the α2 helix; or left, toward the α1 helix).

Despite the sequence variability, the N-terminal regions of each peptide (ELA in decamer and ALG in nonamer) are similar (Fig. 2, B and C). All the atoms that are common to both peptides (all atoms of the ALG peptide form a subset of those in the ELA peptide) are in the same position in both structures. The Cα atoms can be superimposed with rmsd of 0.03 Å\(^2\), and common atoms (excluding the carboxyl group of P3) can be superimposed with the rmsd of 0.07 Å\(^2\). The N-terminal motif contains an anchor residue at position P2 (Leu in both peptides), which occupies the B pocket of HLA-A2. This confirms that the increased binding of Ala→Leu-substituted peptides can be attributed to the introduction of the P2 anchor. The “additional” atoms of the ELA peptide, which are not present in the ALG peptide, include the carboxylate group of P1 Glu, which forms a secondary anchor for the peptide and points toward the surface of the complex, and the Cβ carbon of Ala P3, which points down into the groove. In the observed conformation, the side chain of Glu\(^{29}\)/P1 could participate in the interaction with TCR and hence affect the immunogenicity of the decapetide.

### Table III. Refinement statistics

<table>
<thead>
<tr>
<th></th>
<th>HLA-A2/ALG</th>
<th>HLA-A2/ELA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Å)</td>
<td>2.15</td>
<td>1.8</td>
</tr>
<tr>
<td>R_free (test set size/count)</td>
<td>0.247 (9.9%/2510)</td>
<td>0.225 (4.8%/1852)</td>
</tr>
<tr>
<td>R_work(^a)</td>
<td>0.201</td>
<td>0.181</td>
</tr>
<tr>
<td>No. of HLA-A2 atoms</td>
<td>3067</td>
<td>3067</td>
</tr>
<tr>
<td>No. of peptide atoms</td>
<td>60</td>
<td>69</td>
</tr>
<tr>
<td>No. of solvent molecules</td>
<td>265</td>
<td>341</td>
</tr>
<tr>
<td>No. of metal atoms</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>rmsd bonds (Å)</td>
<td>0.008</td>
<td>0.013</td>
</tr>
<tr>
<td>rmsd angles (°)</td>
<td>1.4</td>
<td>1.6</td>
</tr>
<tr>
<td>rmsd dihedral (°)</td>
<td>25.3</td>
<td>25.3</td>
</tr>
<tr>
<td>(\langle B\rangle) (Å(^2)) (chain A, B, C, solvent, metal)</td>
<td>32.4, 27.0, 23.7, 35.4, N/A</td>
<td>25.7, 21.8, 22.3, 36.2, 21.7</td>
</tr>
<tr>
<td>Ramachandran plot (%) (favorable, additional, generous, disallowed)</td>
<td>90.1/9.3/0.6/0.0</td>
<td>92.5/7.2/0.3/0.0</td>
</tr>
</tbody>
</table>

\(^a\) R_work and R_free = \(\sum|F_o| - |F_c|/\sum|F_o|\), where F_o and F_c are the observed and calculated structure factor amplitudes. R_free was calculated with 10% of the reflections not used in refinement for HLA-ALG and 5% of reflections for HLA-ELA.
The central region (GIG(EAL) vs IG(ALG)) is drastically different in the two complexes, which might explain the differences in CTL recognition of the peptides. When compared with the two-residue link in the nonamer, the first and the last residues of the decamer bulge are shifted in opposite directions, creating an opening for the extra residue, P*, and a zigzag when viewed from above the binding site (at P* in Fig. 2C). The first residue of the bulge, Gly 29(EAL)/P4, is shifted toward the N terminus and up and to the left toward the α1 domain α-helix (decamer, yellow; nonamer, blue). B, Side view (TCR above, MHC below) of the bound structures of the two modified peptides where superimposed using Ca atoms of three N-terminal and four C-terminal residues. RMS deviation of the superimposed Ca positions is 0.32 Å. The red dashed line surrounds the Ile 30 side chain that bulges from the center of the decamer (yellow). Solid red line shows displacement of P3 positions in the two peptides. Waters, W1 and W2, involved in the hydrogen-bonding network connecting Glu26 and Gly29 are colored red. HLA-A2-binding pockets at P2, P6, and P9 are indicated by black arcs. Colors as in Fig. 1. C, Top view (looking down on the MHC) of the same superimposed peptides in Fig. 1A, showing the zigzag at P* in the decamer (yellow).

The central (GIC(EAL) vs IG(ALG)) is drastically different in the two complexes, which might explain the differences in CTL recognition of the peptides. When compared with
and last residue of the peptide). The P4 and P5 residues are elevated by ~5.7 Å, while the rest of the peptide is displaced from the axis by between 0 and 2.7 Å. In the ALG peptide link, the Ile^{30}/P4 is displaced upward by 4.8 Å and Gly^{31}/P5 by 6.2 Å. The large nonpolar side chain of the Ile^{30} of the decamer peptide not only protrudes more extensively from the molecular surface, but its conformation is also very different from that of the nonamer (dotted red lines in Fig. 2, B and C). This centrally

**FIGURE 3.** Water molecules in the peptide binding site. A, Stereographic drawing of the HLA-A2 (gray) binding site with the bound modified decamer Melan-A/MART-1 peptide (yellow) (atom colors as in Fig. 1). Colored spheres are bound solvent molecules, color coded as explained in the text. B, As A, except modified nonamer Melan-A/MART-1 peptide (blue). C, The hydrogen-bonding network (dashed lines) between the central part of modified nonamer peptide and the α1 domain α-helix of HLA-A2 (gray). Waters are stabilized by the peptide backbone atoms and the side chains of Arg^{65} and Thr^{73} from HLA-A2. Several interactions between water molecules are observed creating a different environment than in the decamer peptide (D). D, The hydrogen-bonding network (dashed lines) between the central part of modified decamer peptide (yellow) and the α1 domain α-helix of HLA-A2 (gray). Waters are stabilized by the peptide backbone atoms and the side chains of Arg^{65} and Thr^{73} from HLA-A2. E, Three water molecules in decamer Melan-A/MART-1 peptide/HLA-A2 complex (blue spheres: 25, 79, and 91) form a hydrogen-bonding network linking the side chains of buried His^{114} and the surface-exposed Gln^{155} (blue side chains). Yellow side chains show the HLA-A2 conformation when the modified decamer peptide is bound. The difference in the conformation of the His^{114} creates an empty cavity in the binding groove underneath the peptide and a change in the positions of Gln^{155} and Glu^{154}, creating a different surface for TCR binding.
located difference could account for the differences in the T cell recognition of these peptides.

The C-terminal regions of both peptides have identical sequences (ILTV) and similar conformations. Because this is the only region of the peptide that is invariant, its structure might be required for recognition of HLA-A2/melanoma complexes by all three categories of the CTL clones. There are only two minor differences between the two complexes that we can observe. When the structures of the peptides are superimposed, the Cα atoms of Ile32/P6 anchor are positioned 0.8 Å apart and their side chains are rotated in the opposite direction (Fig. 3B). In addition, the surface-exposed, hydrophobic side chain of Leu33/P7 has two alternate conformations in the decamer, but only one conformation in the nonamer. Nevertheless, the general direction of Leu33/P7 side chain in both peptides is identical, and in fact the orientation of that side chain in the nonamer corresponds to the average of the two conformations observed in the decamer (see Leu33 in Figs. 1, A and B and 2, A and C).

**Water molecules involved in peptide/MHCI interactions**

In all MHCI/peptide structures, solvent molecules appear to stabilize the interaction of the melanoma peptides in five locations (corresponding water molecules in HLA-A2/ELA and HLA-A2/ALG complexes are color coded similarly in Fig. 3, A and B): at the peptide N terminus and C terminus (yellow), below the peptide near P7 (green), between the peptide and the α helix (red) and between peptide and the α2 helix (light blue). The hydrogen-bonding networks involving water molecules at both the N- and C-terminal regions of the peptides are very similar in all MHCI/peptide complexes (23, 24). The water molecules underneath the peptide are likely to be similar in the subset of HLA-A2/peptide complexes, where residues Arg97 and Tyr116 of the HLA-A2 are oriented toward the peptide C terminus (see discussion in Ref. 24). The interactions with bound water molecules on the sides of the peptides (Fig. 3, C, D, and E) appear to be distinctive.

Between the peptide and the α2 helix, the hydrogen-bonding pattern differs between the two complexes investigated in this study. The decapeptide carbonyl group from Ile30/P* and the side chain of Ala28/P3 are positioned too close to the α2 helix to allow water entry into this volume. In the nonamer, there is an opening between the peptide chain and α2 helix, which contains three water molecules (Fig. 3E). The water molecules interact with the peptide main chain and with two residues from the adjacent α-helix, His114 and Gln155 (blue spheres in Fig. 3E). To accommodate this interaction, Gln155 is rotated in the nonapeptide complex toward the water cluster (toward the N-terminal region of ALG peptide, blue in Fig. 3E). Gln155 in the HLA-A2/ELA complex and other MHCI peptide complexes (22) in absence of the TCR is exposed on the surface and points in the direction of the central region of the peptide (yellow in Fig. 3E). The conformation of Gln155 is altered in the complexes of HLA/Tax with the human A6 and B7 TCRs, in which Gln155 points upward toward the TCR (1AO7, Ref. 25, 1BD2, Ref. 26).

Although there are five water molecules involved in a hydrogen-bonding network on the α1 domain side of both peptides, the hydrogen-bonding patterns are different in the two complexes (Fig. 3, C and D). In the nonapeptide complex, the waters fill the gap between the peptide and the α1 helix and create a tightly packed network that connects the peptide backbone to Thr73 and Arg65. Three water molecules (34, 51, and 95) interact directly with the peptide backbone, water 189 connects waters, and water 206 bridges water 34 to Arg65 (Fig. 3C). In the decapeptide complex, water molecules also fill the gap between the peptide and α1 domain α helix, but they are packed more loosely. Two water molecules interact directly with peptide backbone, water 225 interacts with Thr73, and water 279 with Arg65 (Fig. 3D). However, there is no water molecule that bridges them together and, in general, the bonds between water molecules and peptide atoms are longer than in the nonapeptide complex. This results in higher B factors for all these water molecules. This difference in water structure might influence TCR binding in this region because the positions of the water molecules, the adjacent protein atoms, or both may be more easily altered upon TCR binding in the decamer peptide complex.

**Potential peptide flexibility**

The effect of deformability on TCR binding has been considered previously in a poorly binding HER-2/new epitope GP2 bound to HLA-A2. Based on the x-ray structure (the rigidity of the peptide in the binding groove can be judged by the quality of the corresponding electron density map), the weak interaction of the GP2 peptide with HLA-A2 has been attributed to increased flexibility in the center of the peptide (3).

Electron density maps for Melan-A/MART-1-modified peptides, calculated without the peptide atoms or any atoms within 5 Å of the peptide to avoid model bias, indicate that the central residues of the nonapeptide are less well defined than those of the decapeptide (Fig. 1). In the nonapeptide, the electron density for two residues from the central region, Ile30/P4 and Gly31/P5 (Fig. 1B), is weak (contoured at 1.4σ, although present at 0.7σ). In the decapeptide, by contrast, only the distal side chain atom C8 of Ile30/P* and the side chain atoms of Leu33/P7 are missing at the same contour levels. When the electron density map is contoured at 0.7σ, electron density is observed for the C8 atom of Ile30/P*, and two alternate conformations of Leu33 are visible (Fig. 1A). The observation that the central region of decamer peptide may be somewhat more ordered than the nonamer is consistent with the peptide-to-MHC contacts observed in the structures. In the decamer peptide, in addition to the P6 anchor, the central region of the peptide is stabilized by two bound water molecules (W1 and W2 in Fig. 2B, orange in Fig. 3A) bridging the Glu29/P1 side chain and the Gly29/P3 and additional constraints created by the zigzagging of the peptide. Sparse water packing on the α1 domain side of the decamer peptide, by reducing constraints, might permit the peptide to fold into a more energetically stable conformation.

Relative to the major conformation differences between the modified Melan-A/MART-1 nonamer and decamer described in previous sections, the minor differences in peptide flexibility may be less likely to play a prominent role in T cell recognition.

**Discussion**

The x-ray crystal structures of HLA-A2 complexes with the modified nonamer and decamer peptides, Melan-A/MART-127–35 (ALG) and Melan-A/MART-126–35 (ELA), show how the P2 peptide substitutions determine the binding mode of the nonamer and stabilize the binding of the decamer. The different conformations of the bound peptides also suggest structural explanations for the fine specificities observed by T cell recognition experiments (8, 10).

**Improving ligands for generating tumor-reactive CTL**

Modifying the natural decamer sequence of the Melan-A/MART-126–35 peptide by changing the P2 anchor from Ala to Leu stabilized the peptide/HLA-A2 complex, increased the efficiency of T cell recognition by a number of clones, and improved the in vitro
generation of tumor-reactive CTLs by stimulation of PBMC from HLA-A2 melanoma patients (9, 27). Substitution of P1 Glu to Ala increases HLA affinity, reactivity by tumor-infiltrated lymph node 6- to 60-fold, and reactivity by Melan-A-specific CTL clones by 4- to 1000-fold (10). These improved properties suggest that modified peptides might be better candidates for peptide-based vaccine trials and/or for improving the generation of tumor-reactive CTL ex vivo for adoptive transfer therapy (9, 27). Improvements in peptide affinity and HLA-A2 complex stability of the gp100 154–162 epitope have been reported with a substitution at a nonanomer peptide position, P8 (28).

In some cases, even very subtle peptide modifications can have profound effects on T cell recognition and signaling, switching from agonists to antagonists (reviewed in Ref. 29; for structural examples, see Ref. 30), indicating that modifying peptides can have unpredictable consequences that need to be tested experimentally. Nevertheless, the array of water molecules around both the modified peptides investigated in this study (Fig. 3) suggests atomic positions in which other atoms might be added to the peptide or MHC molecule to replace waters and provide novel ligands of potential therapeutic value.

**T cell reactivity data and the observed binding modes of the modified peptides suggest how the wild-type peptides bind**

T cell reactivity data suggest that the binding mode in the HLA-A2 complex of the wild-type decamer is very likely to be the same as the zigzag binding mode observed in the modified decamer, but the wild-type nonamer is more likely to bind with a zigzag like the modified decamer than stretched out like the modified nonamer observed in this study. The observation that decamer peptides modified with P2 substitutions, which are more suitable anchors for HLA-A2 than the wild-type P2 Ala, are universally recognized by the same T cells as the wild-type peptide, but at lower peptide concentrations (9), indicates that the wild-type and modified decamers share the same zigzag binding mode. Increasing the peptide affinity for HLA-A2 by substituting a more suitable P2 anchor apparently increases the number of HLA-A2 molecules loaded with the decamer, which then present essentially the same antigenic surface to T cells specific for the Melan-A/MART-1 protein.

The observation that P1 substitutions that replace the P1 Glu with Ala increase HLA affinity, reactivity by tumor-infiltrated lymph node 6- to 60-fold, and reactivity by Melan-A-specific CTL clones by 4- to 1000-fold (10) suggests that the same antigenic surface is being presented by wild-type and these P1-substituted peptide/HLA-A2 complexes, and that the P1 glutamic acid side chain is either not optimal for HLA-A2 binding or constrains the peptide conformation (see Fig. 2B) away from a conformation that TCR prefers.

Decamers with the large aromatic residues, Tyr or Phe, substituted at P1 are more T cell reactive than wild-type peptide (8), possibly indicating better interaction with HLA-A2 than P1 Glu, but they are selectively recognized by only some CTL clones, suggesting that these large P1 side chains may interact with the TCR as well.

CTL reactivity data indicate that the substitution of Leu, a good P2 anchor residue, for P2 Ala in the nonapeptide significantly increased its binding to HLA-A2, as expected, but strongly reduced its reactivity with Melan-A-specific T cells (9, 11). This loss of reactivity suggests that the wild-type nonamer binds differently than the P2 Leu-substituted nonamer, observed in this study (10, 11). A possibility consistent with the cross-reactivity of all the Melan-A-specific T cells with the wild-type nonamer and decamer peptides is that the wild-type nonamer binds predominately in the zigzag mode like the modified decamer observed in this study, but with the first pocket, P1, empty, rather than like the modified nonamer that binds in the stretched-out mode typical on nonamers with strong P2 anchor residues. This possibility has been suggested before, based on the cell reactivity (9, 31). The observation that nonamers with P1 substitutions to Leu or Met, which are good anchors for the P2 pocket, have increased CTL reactivity (9, 31) is consistent with this possibility, as those modifications are expected to cause P1 to bind in the P2 pocket, making those modified nonamers bind in the zigzag decamer mode. Although there are no examples of crystal structures, as yet, of nonamer peptides that bind with the P1 pocket empty and in a zigzag mode characteristic of decamers, an octamer of the human T cell leukemia virus-1 Tax peptide has been observed bound to HLA-A2, leaving the P1 pocket empty (17). In that Tax-8/HLA-A2 complex, a number of water molecules are observed in place of a peptide residue, linking the peptide to HLA-A2 and forming hydrogen bonds with the HLA-A2 residues that normally form hydrogen bonds with the main chain polar atoms of the first peptide residue (17).

Because it seems likely that both wild-type nonamer and decamer Melan-A/Mart-1 peptides bind predominately in the similar conformation, like that of the modified decamer observed in this study, the structure of the modified decamer bound to HLA-A2 can be used as a model for further studies involving mutational analysis of this peptide family.

The fine specificity of T cell responses to Melan-A/MART-1 peptides

All T cells tested recognize both the wild-type nonamer and decamer Melan-A/MART-1 peptides, as indicated by the lysis of target cells sensitized with high concentrations of either peptide. But fine specificities are revealed in peptide titration studies: most clones are more reactive with the decamer than the nonamer, although a few clones react to both equally well, and a rare clone favors the nonamer (6, 8, 10). These T cell reactivity data suggest that the antigenic surface presented by the wild-type nonamer and decamer peptides and HLA-A2 has some common features contacted by the clones that recognize both peptides equally well and at least one structural difference contacted by clones that discriminate in favor of either the decamer or nonamer. If, as suggested by the data discussed above, the wild-type nonamer binds in the zigzag mode like the decamer, most of the center and C-terminal sections of the peptide/HLA-A2 complex should have the same structure in the nonamer and decamer complexes as that observed in this study in the modified decamer complex (Figs. 2 and 3A) providing the basis for cross-reactivity. The x-ray crystal structure of a TCR/MHC-peptide complex of an alloreaction has been described in which the TCR makes no contacts to the N-terminal half of a bound peptide, indicating that such focused interactions can exist and can initiate strong T cell signals (32). Another example of CTL cross-reactivity between different lengths of peptides from one antigenic epitope has been documented in the case of H2-Kb-restricted CTL directed against the Plasmodium berghei peptide nonamer 252–260, in which most clones also recognized the octamer 253–260 efficiently (33).

The structures of the wild-type nonamer and decamer Melan-A/MART-1 peptide/HLA-A2 complexes would be expected to diverge toward the N terminus of the peptides. Clones that distinguish the nonamer from the decamer may make direct contacts near the P1 pocket, which would be filled with a peptide amino acid in the decamer complexes, but empty or more likely water filled in the wild-type nonamer complex, offering a structural basis
for distinguishing the complexes. Because the two waters, W1 and W2 (red, Fig. 2B), that form a hydrogen-bonding network connecting P1 Gly26 to P4 Gly29 in the modified decamer complex would be absent in a wild-type nonamer complex missing P1 Gly, the peptide conformations in the P4-P5 may differ in structure, as the nonamer complex would lack the stabilization of this hydrogen-bonding network. Structural differences resulting from the absence of the P1 Gly residue and its stabilizing interactions via waters to P4 could also affect residues on the surface of HLA-A2, for example at Glu155 and Glu154 (Fig. 3E), creating a different surface for TCR recognition. The absence of a good P2 anchor residue in the wild-type nonamer might also have structural consequences in peptide and MHC residues in its vicinity that could be distinguished by T cells, although such structural differences are difficult to predict.

It is possible that some wild-type nonamers bind in the stretched-out mode observed in this study for the modified nonamer, but if that were the case, some T cell clones might have been expected to recognize the modified nonamer even better as a result of its higher affinity to HLA-A2 than the wild-type nonamer, but no clones of that type have been observed.

Alanine-scanning mutagenesis of the decamer peptide has established that both the P7 Ile12 and P10 Val15 anchors, critical for stabilizing the C-terminal region of the bound peptides, are crucial for recognition by all CTL clones, regardless of their fine specificity (10). Neither of the two glycines in either peptide have affected residues on the surface of HLA-A2, as deduced from competition experiments (10). Because both residues have been previously shown to be crucial for peptide binding, it is possible that the presence of Gly at positions P4 and P6 is required for the formation of the zigzag binding mode of these peptides, as previously suggested (10).

A complete structural description of the fine specificity of T cell reactivity for the immunodominant nonamer and decamer peptides from the melanoma tumor-associated protein Melan-A/MART-1 would require crystallization of TCR complexes with these peptide/HLA-A2 complexes. The instability of both wild-type peptide complexes with HLA-A2 has to date prevented their purification. We have determined high resolution crystal structures of the complexes of HLA-A2 molecules with two modified Melan-A/MART-1 peptides, with increased HLA-A2 affinity. Our conclusions can be summarized as follows: 1) Differences in the structures of the modified peptides, including in clusters of bound water molecules, readily explain the differences in their recognition by T cells: the modified nonamer binds stretched out, the modified decamer in a zigzag mode. 2) T cell reactivity and structural data suggest that the wild-type nonamer binds predominately like the modified decamer, and that the fine specificity of T cell reactivity of the wild-type peptides results from the similarities between the nonamer and decamer binding in the middle and C terminus of the peptide, while the differences in reactivity are caused by differences in the peptide and MHC surfaces near the N-terminal end of the peptides. 3) Modifications in the decamer peptide to increase its affinity for HLA-A2 and possibly to form better Ags for therapy might include chemical modifications to place atoms in the location of many bound waters observed clustered in five locations around the peptide. 4) A metal, probably zinc, found at a crystal lattice contact in the decamer complex, may have stabilized that crystal, resulting in observable x-ray diffraction to a Bragg spacing beyond 1.8 Å.

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References


