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Increased Immunogenicity of an Anchor-Modified Tumor-Associated Antigen Is Due to the Enhanced Stability of the Peptide/MHC Complex: Implications for Vaccine Design

Oleg Y. Borbulevych,* Tiffany K. Baxter,* Zhiya Yu, ‡ Nicholas P. Restifo, ‡ and Brian M. Baker**†

The use of “anchor-fixed” altered peptide ligands is of considerable interest in the development of therapeutic vaccines for cancer and infectious diseases, but the mechanism by which successful altered peptide ligands elicit enhanced immunity is unclear. In this study, we have determined the crystallographic structure of a major tumor rejection Ag, gp100_{209–217}, in complex with the HLA-A^*0201 (HLA-A2) molecule, as well as the structure of a modified version of the peptide which substitutes methionine for threonine at position 2 (T2M; gp100_{209–217}). The T2M-modified peptide, which is more immunogenic in vitro and in vivo, binds HLA-A2 with a 9-fold greater affinity and has a 7-fold slower dissociation rate at physiological temperature. Within the limit of the crystallographic data, the T2M substitution does not alter the structure of the peptide/HLA-A2 complex. Consistent with this finding, in peripheral blood from 95 human subjects, we were unable to identify higher frequencies of T cells specific for either the native or modified peptide. These data strongly support the conclusion that the greater immunogenicity of the gp100_{209–217} peptide is due to the enhanced stability of the peptide/MHC complex, validating the anchor-fixing approach for generating therapeutic vaccine candidates. Thermodynamic data suggest that the enhanced stability of the T2M-modified peptide/HLA-A2 complex is attributable to the increased hydrophobicity of the modified peptide, but the gain due to hydrophobicity is offset considerably by the loss of a hydrogen bond made by the native peptide to the HLA-A2 molecule. Our findings have broad implications for the optimization of current vaccine-design strategies.

The development of therapeutic vaccines based on tumor-associated Ags (TAA)s presented by class I MHC molecules is one of the goals of cancer immunotherapy. As most TAA are self-Ags (1), the Ag-specific CTL repertoires may be significantly reduced during the processes of negative selection, leaving a T cell repertoire that is poorly effective at mounting a productive antitumor immune response. Efforts to use unmodified forms of peptide immunogens to elicit antitumor responses in vivo have been largely unsuccessful, leading investigators to modify naturally occurring tumor Ags. Indeed, a number of altered peptide ligands (APL) capable of eliciting enhanced immunity to tumor-associated epitopes have been developed (e.g., Refs. 2–4). The majority of these APL are “anchor-fixed” variants, in which sub-optimal primary anchors have been replaced with more optimal amino acids.

The mechanisms underlying the enhanced immunogenicity of these APL have remained incompletely elucidated. Speculations on how APL elicit enhanced immune responses have included the possibility that APL present a different peptide conformation to T cells, thus stimulating a different set of TCRs which are capable of cross-reacting with the native epitope. Indeed, anchor-fixing does not always result in enhanced immunogenicity (e.g., Ref. 5), and in some cases this has been shown to result from structural alterations (6, 7). TCRs can be exquisitely sensitive to changes in the structure of the peptide, in some cases sensing alterations resulting from removal or addition of a single methylene group (6, 8).

Others have suggested that native TAA function as partial agonists or antagonists, stimulating anergy in responding cells, whereas APL trigger T cells to signal in a more productive fashion (9). A third hypothesis is that the CTL repertoire specific for TAA remains intact and inducible, but weak peptide affinities or fast peptide dissociation rates preclude the formation of a stable immunological synapse, preventing sufficient signaling through the TCR to convert naive precursor TAA-specific CTLs into differentiated CTLs capable of lysing TAA-presenting tumor cells (10).

In this study, we focus on the 209–217 epitope of the gp100 membrane glycoprotein (gp100_{209}; sequence IMDQVPFSV), for which the anchor-fixing substitution of the threonine at position 2 with methionine has previously been described (5). The modified peptide (IMDQVPFSV; gp100_{209–217}) is considerably more immunogenic in vitro and in vivo (5, 11), and its use in clinical trials is correlated with a higher incidence of objective antitumor immunity (2, 12). Using a human-mouse chimera in which the gp100_{209} epitope was knocked out, we recently found that absence of the target epitope did not substantially increase the ability of the native peptide to immunize, whereas the T2M modification to gp100_{209} dramatically increased its immunogenicity (10), demonstrating that the poor immunogenicity of the native peptide is not due to negative selection of gp100_{209}-specific T cells.

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3 Abbreviations used in this paper: TAA, tumor-associated Ag; APL, altered peptide ligand; P2, peptide position 2; RMSD, root mean square deviation; β2m, β2-microglobulin.

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Materials and Methods

Proteins and peptides

Protein was produced by the standard technique of refolding bacterially expressed HLA-A2 (truncated at the transmembrane region) and β2-microglobulin (β2m) inclusion bodies in the presence of excess peptide (13). Refolded protein was purified via ion-exchange and size-exclusion chromatography. Peptides were synthesized and purified to >95% commercially (SynPep). Extinction coefficients at 280 nM (units of M⁻¹ cm⁻¹) were 93439 for peptide/HLA-A2 complexes with unlabeled peptide and 103200 for complexes with labeled peptide.

X-ray crystallography

Peptide/HLA-A2 crystals were grown from 25 mM MES, pH 6.5, 24% polyethylene glycol 3350, 20% glycerol. Diffraction data were collected at the Argonne Structural Biology Center using the 19BM beamline. Data reduction was performed with HKL2000 (14). Structures were solved using molecular replacement with MOLREP from CCP4 (15). The search model was Protein Data Bank entry 1B0R (16), using only the coordinates for the H chain and β2m. Rigid body refinement, TLS refinement, and restrained refinement were performed with Refmac5 (17). Anisotropic and bulk solvent corrections were performed in all steps of refinement. TLS groups were chosen for residues 1–182 and 183–275 of the H chain, 0–99 of β2m, and 1–9 of the peptides. After TLS refinement, it was possible to unambiguously position the peptide in 2F₂−F₁ maps. Waters were added using ARP/wARP (18). Graphical evaluation of the model and fitting to maps was performed using XtalView (19). Procheck (20) and the template structure check in the WHATIF server (21) were used to evaluate the quality of the structure during and after refinement. Structure factors and coordinates have been submitted to the Protein Data Bank (entries 1TVB and 1TVH). Cavity calculations were performed with SURFNET (22).

**IFN-γ ELISPOT assays**

PBMC from 95 HLA-A*0201⁺ patients at high risk for recurrence of melanoma were collected using apheresis and cryopreserved during their course of immunization with the gp100209–2M peptide in protocols reviewed and approved by the Institutional Review Board of the National Institutes of Health. All patients signed an Institutional Review Board-approved consent form and had histologically confirmed melanoma. A total of 106 PBMC samples from these patients were assayed for peptide-specific IFN-γ ELISPOT. The PBMC were collected at various times during the course of immunization, but assays of these samples with the native and modified peptides were always done at the same time. Cryopreserved PBMC were thawed and rested in culture medium overnight and then were added to 1 µM peptide-pulsed C1R-A2 target cells at a 1:1 ratio (10⁶ of each effector and target cells) in triplicates of 96-well ELISPOT plates ( precoated with anti-human IFN-γ Abs from BioSource). After a 24-h incubation, the plates were washed, coated with biotinylated anti-IFN-γ Abs (BD Pharmingen), followed by alkaline phosphatase-conjugated avidin incubation. The spots were developed with alkaline phosphatase substrate. Numbers of spots were corrected by subtracting background spots caused by PBMC incubation with C1R-A2 cells pulsed with an irrelevant control peptide (gp100256; YLEPGPVTA). Nonspecific spots were between 0 and 14/10⁵ cells.

**Peptide dissociation kinetics**

Dissociation kinetics were measured using fluorescence anisotropy with a Beacon 2000 polarization instrument (Invitrogen Life Technologies) as previously described (23). Briefly, 7.5 nM peptide/HLA-A2 loaded with a fluorescently labeled peptide was mixed with 7.5 µM unlabeled peptide, and the decrease in anisotropy was measured as a function of time. Data were fit to single or biphasic functions of the form

\[
y(t) = y_0 + \sum A_i e^{-k_i t}
\]

where \(y_0\) is the baseline offset, the summation is over the number of phases \(i\) (1 or 2), \(A_i\) is the amplitude for phase \(i\), \(k_i\) is the rate constant for phase \(i\), and \(t\) is the time. Data were analyzed using the program Origin (OriginLab). Error analysis was performed using standard error propagation techniques (24). All measurements were performed in 10 mM HEPES, 150 mM NaCl, pH 7.4 or 6.5, as indicated.

**Results**

The crystal structures of the gp100209 and gp100209–2M peptides bound to HLA-A2 are indistinguishable

The crystal structures of the native gp100209 and anchor-modified gp100209–2M peptides bound to HLA-A2 were determined at 1.8 Å resolution via molecular replacement. Both complexes crystallized in space group P2₁, with two molecules per asymmetric unit (molecules “A” and “B”). For both complexes, unambiguous electron density was observed for the peptides (Fig. 1). Crystallographic data and refinement statistics are provided in Table I.

In both the native and T2M-modified gp100209/HLA-A2 crystal structures, both molecules in the asymmetric unit are nearly identical. There are, however, differences in the positioning of Gln, Phe, and Ser of the peptide. As shown in Fig. 2, A and B, though, these differences are common to both the native and T2M-modified structures. Comparison of the two sets of peptides thus reveals that the T2M substitution has little, if any, effect on peptide conformation. For the A molecules, all common atoms in the peptides superimpose to a root mean square deviation (RMSD) of 0.20 Å. For the B molecules, the superimposition is 0.21 Å (a detailed comparison of the two structures is provided in Table II). The largest differences in the peptides are the side chains of positions 4, 7, and 8, the same positions which show differences between the A and B molecules (Fig. 3). Thus we cannot attribute the small differences...
between the native and modified peptides to the T2M substitution, as the differences are in the same positions and are of lower magnitude than the differences observed between the A and B molecules in both structures. The temperature factors for the atoms of these side chains do not differ significantly from the average in either structure. Thus, these differences may reflect different side chain conformations that interconvert on a slow timescale, captured by crystallization, or alternatively, the influence crystal packing has on otherwise mobile side chains. We note that similar differences in exposed side chains are seen elsewhere in the structures, for example Lys19 in β2m, a full 42 Å away from the site of the T2M substitution. The side chain differences are thus reminiscent of the kind of differences one observes when comparing different structures of the same protein. For example, when comparing the original structure of the Tax peptide bound to HLA-A2 reported in 1993 (25) with a higher resolution structure reported in 2000 (26), all atoms of the peptides superimpose to an RMSD of 0.21 Å, identical to the values obtained for the superimposition of the gp100_{209} and gp100_{209–2M} peptides reported in this study (Table II).

Comparing the backbones of the gp100_{209} and gp100_{209–2M} peptides in the two structures, for both the A and B molecules, the backbone of the gp100_{209} peptide superimposes on that of the gp100_{209–2M} peptide with an RMSD of ~0.1 Å, near the estimated coordinate error (Table I). Thus, within the limit of the x-ray data, the T2M substitution has no effect on the structure of the peptide in the HLA-A2 binding groove.

Comparison of the HLA-A2 α1 and α2 helices in the two structures indicates that the T2M substitution does not induce changes into the structure of the peptide binding domain. The backbones of these helices superimpose to RMSDs of 0.09 and 0.11 Å, respectively. The side chains of HLA-A2 amino acids that contact TCRs in known TCR/peptide/HLA-A2 structures likewise show no significant differences. For example, the side chains of Lys66 and Gln155, implicated as important positions for most HLA-A2restricted TCRs (27, 28), are virtually indistinguishable, with equivalent atoms differing by no more than 0.45 Å when the peptide binding grooves are superimposed. As with the peptides, the positions of some side chains differ slightly when comparing the A and B molecules. Again though, as summarized in Table II, these

### Table I. X-ray data and refinement statistics

<table>
<thead>
<tr>
<th>Complex</th>
<th>gp100_{209}/HLA-A2</th>
<th>gp100_{209–2M}/HLA-A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>APS 19BM</td>
<td>APS 19BM</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁</td>
<td>P2₁</td>
</tr>
<tr>
<td>a (Å)</td>
<td>58.39</td>
<td>58.41</td>
</tr>
<tr>
<td>b (Å)</td>
<td>84.45</td>
<td>84.49</td>
</tr>
<tr>
<td>c (Å)</td>
<td>84.02</td>
<td>84.01</td>
</tr>
<tr>
<td>β</td>
<td>90.01°</td>
<td>90.11°</td>
</tr>
<tr>
<td>Molecules/a.u.</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Resolution, Å</td>
<td>20–1.8</td>
<td>20–1.8</td>
</tr>
<tr>
<td>Total number of reflections</td>
<td>74684</td>
<td>74189</td>
</tr>
<tr>
<td>Mosaicity</td>
<td>0.36°</td>
<td>0.60°</td>
</tr>
<tr>
<td>Completeness, %</td>
<td>99 (98.2)</td>
<td>98.7 (97.0)</td>
</tr>
<tr>
<td>I/σ</td>
<td>16.8 (3.7)</td>
<td>16.3 (2.7)</td>
</tr>
<tr>
<td>R_{merge}, %</td>
<td>8.5 (34)</td>
<td>6.5 (40)</td>
</tr>
<tr>
<td>Average redundancy</td>
<td>3.9 (3.6)</td>
<td>2.8 (2.6)</td>
</tr>
<tr>
<td>R_{work}, %</td>
<td>16.5</td>
<td>18.5</td>
</tr>
<tr>
<td>R_{free}, % (no. of reflections)</td>
<td>21.6 (3748)</td>
<td>24.7 (3703)</td>
</tr>
<tr>
<td>Average B factor, Å²</td>
<td>12.7</td>
<td>14.5</td>
</tr>
<tr>
<td>Ramachandran plot</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Most favored, %</td>
<td>92.0</td>
<td>91.9</td>
</tr>
<tr>
<td>Allowed, %</td>
<td>7.7</td>
<td>7.8</td>
</tr>
<tr>
<td>Generously allowed, %</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Disallowed, %</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>RMS deviations from ideality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bonds, Å</td>
<td>0.018</td>
<td>0.022</td>
</tr>
<tr>
<td>Angles</td>
<td>1.946°</td>
<td>2.140°</td>
</tr>
<tr>
<td>Coordinate error, Å</td>
<td>0.08°</td>
<td>0.11</td>
</tr>
</tbody>
</table>

*Numbers in parenthesis refer to the highest resolution shell.

*Mean estimate based on maximum likelihood methods.
differences tend to be equal to or greater than any differences observed between the structures with the native and modified peptides. The structure with the native peptide reveals how the polar side chain of Thr2 is accommodated in the apolar HLA-A2 P2 pocket, inserting itself into a network of hydrogen bonds strongly conserved in peptide/HLA-A2 structures (7, 16, 25, 26, 29–35). As shown in Fig. 4, the threonine at position 2 hydrogen bonds with Glu63 of the HLA-A2 H chain. Glu63 also hydrogen bonds with the conserved water molecule near the peptide amino terminus and forms a salt-bridge with Lys66. Lys66 in turn hydrogen bonds to the carbonyl oxygen of Thr2. Substitution of Thr2 with methionine removes the hydrogen bond between the side chain and Glu63, but Glu63, Lys66, and the position 2 carbonyl oxygen are undisturbed.

As the two structures are so similar, one expectation is that replacement of the smaller threonine with the larger methionine should alter packing in and around the HLA-A2 P2 pocket. In the structure with the native peptide, cavity calculations (22) between the side chain of Thr2 and HLA-A2 reveal the presence of cavities in and around the P2 pocket, shown in Fig. 4A. The same calculation on the modified structure confirms that substitution of Thr2 with Met reduces total cavity volume. The larger cavity shown in Fig. 4A increases in volume from 15.4 to 25.6 Å³ in response to removal of the threonine hydroxyl and repositioning of the γ carbon (Fig. 4B). However, the smaller cavity (volume of 14.8 Å³) is not present in the modified structure, as this space is occupied by the -S-CH₃ moiety of the methionine side chain. The total reduction in cavity volume upon making the T2M substitution is thus 4.6 Å³. The H chain atoms defining the cavities shown in Fig. 4 superimpose to an RMSD of 0.14 Å, indicating that the overall size and shape of the P2 pocket is not altered due to the T2M substitution.

It is informative to compare our findings with other structural studies of native and modified peptides presented by MHC molecules. In studying the Hb peptide presented by the murine class II MHC I-Eβ, Kersh et al. (6) found that changing the P6 anchor from glutamic to aspartic acid resulted in a subtle (0.4 – 0.6 Å) but crystallographically significant shift in the position of the peptide backbone (6). Although the change was maximal at the site of the substitution, the effects propagated from the site of the substitution all the way to the peptide C terminus. No such trends are observed in our data; indeed, the backbone differences tend to be at or below the estimated coordinated error. Sharma et al. (7) obtained similar results with variants of the HER-2/neu peptide bound to HLA-A2: with the leucine at position 9 changed to valine, a significant change in the conformation of the peptide backbone in the center of the peptide was observed (7). Again, no such changes are detected in this study.

Other structural studies have revealed conformational changes in peptide/MHC complexes when the peptides are modified (e.g., Refs. 36 and 37), but our findings of no conformational changes

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**Table II. Differences between the native and T2M-modified peptide/MHC structures are smaller than the differences between the two molecules in the asymmetric units**

<table>
<thead>
<tr>
<th>Region Compared</th>
<th>Comparison of the Two Molecules in the Asymmetric Units</th>
<th>Comparison of Native and T2M-Modified Structures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native A onto native B</td>
<td>T2M-modified A onto T2M-modified B</td>
</tr>
<tr>
<td>Whole complex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All common atoms</td>
<td>0.76</td>
<td>0.76</td>
</tr>
<tr>
<td>Backbone</td>
<td>0.35</td>
<td>0.29</td>
</tr>
<tr>
<td>Peptide binding domain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All atoms</td>
<td>0.80</td>
<td>0.78</td>
</tr>
<tr>
<td>Backbone</td>
<td>0.46</td>
<td>0.33</td>
</tr>
<tr>
<td>Peptide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All common atoms</td>
<td>1.10</td>
<td>1.05</td>
</tr>
<tr>
<td>Backbone</td>
<td>0.10</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* RMSD in angstroms.
occurring upon modifying the gp100\textsubscript{209} peptide are not without precedent. With the RT309–317 peptide bound to HLA-A2, Kirksey et al. (32) found that changing the P1 residue from isoleucine to either phenylalanine or tyrosine did not alter the structure of the peptide/HLA-A2 complex (32), despite large changes in peptide/HLA-A2 stability and immunogenicity. Likewise, with the NY-ESO157–165 peptide bound to HLA-A2, modifications to P9 did not alter the structure (38). Finally, in a ternary complex between the A6 TCR and the Tax peptide presented by HLA-A2, the conformation of the Tax peptide was found to be identical whether a proline or an alanine was present at position 6, despite the fact that the P6A substitution changes the peptide from a strong agonist to an antagonist (8, 39).

T cells from vaccinated patients are unable to distinguish between the native and T2M-modified peptides

To investigate whether T cells can distinguish between the native and T2M-modified gp100\textsubscript{209} peptides, 106 PBMC samples from 95 patients at high risk for recurrence of melanoma and who had been vaccinated with the T2M-modified peptide were assayed for peptide-specific IFN-\gamma release using ELISPOT. As shown in Fig. 5, across this large sample size, the number of cells activated by the native peptide is highly correlated with the number of cells activated by the T2M-modified peptide. The slope of the regression line, 1.1 \pm 0.1, indicates that there is little, if any, trend indicating the existence of T cells that can discriminate between the two peptides. The slightly positive value of the slope in favor of the T2M-modified peptide may reflect its improved binding affinity and slower dissociation rate (5, 10).

Because ELISPOT only measures the frequencies of Ag-specific T cells, we could not confirm whether a gp100\textsubscript{209–2M}-reactive T cell was indeed gp100\textsubscript{209}-reactive. However, if some or all of the T cell subsets tested were capable of specifically recognizing only the T2M peptide, against which these patients were immunized, in Fig. 5 we would expect to see a large number of points "pinned" to the y-axis, with greatly diminished recognition of the native peptide. However, in 106 samples from 95 patients, the majority had nearly identical frequencies of gp100\textsubscript{209} and gp100\textsubscript{209–2M}-specific T cells. This strongly suggests that T cells specific for gp100\textsubscript{209} presented by HLA-A2 cannot distinguish between the native and T2M-modified peptide, in accordance with the crystallographic data. Very recent data indicate that the T cell reactivity against these peptides seen in ELISPOT is also a measure of their
reactivity toward tumors, which likely present much lower amounts of the native peptide than is achieved with the 1 μM pulse to cultured APCs performed in this study (N. P. Restifo, manuscript in preparation).

**Activation thermodynamics for dissociation indicate hydrophobicity as the mechanism for the improved stability of the gp100<sub>209–217</sub>/HLA-A2 complex**

The crystallographic structures described above do not directly indicate the physical mechanism behind the increased stability of the gp100<sub>209–217</sub>/HLA-A2 complex. Filling cavity space is an established way of enhancing protein stability, but at 24–33 cal/molÅ<sup>3</sup> of cavity space (40), the net decrease in volume of only 4.6 Å<sup>3</sup> (Fig. 4) provides for very little gain.

To gain insight into how substitution of Thr<sup>2</sup> with Met enhances peptide/HLA-A2 stability, we examined peptide dissociation kinetics as a function of temperature, determining activation thermodynamics for peptide dissociation via transition state analysis. Peptide dissociation measurements were performed with soluble, purified peptide/HLA-A2 molecules using steady-state fluorescence anisotropy. We used fluorescent derivatives of the gp100<sub>209–217</sub> and gp100<sub>209–217</sub>M peptides with a single amino acid substituted with a fluorescein-conjugated lysine. Data were collected over the range of 4–37°C in the presence of a large excess of unlabeled peptide. Fig. 6 shows dissociation data for peptides modified at Val<sup>5</sup>. The decreased dissociation rate for the T2M-modified peptide is evident at all temperatures.

Consistent with similar quantitative analyses of peptide dissociation from class I MHC molecules (23, 41–43), the majority of the data in Fig. 6 were best fit with a double-exponential decay function, consisting of a slow phase accounting for the bulk of the data and a minor, faster phase typically accounting for much smaller (5–10%) of the total amplitude. In similar measurements, the minor phase has been attributed to the presence of a small amount of peptide/H chain heterodimer, from which peptide more rapidly dissociates (23, 41). The major phase thus represents the more physiologically relevant dissociation of peptide from the complete class I MHC complex.

Table III summarizes complete data for measurements at 37°C, as well as measurements for peptides with fluorescein-conjugated lysine substitutions at Gln<sup>4</sup> and Phe<sup>7</sup>. From Table III, it is clear that the position of the fluorescein label can influence the measured off-rates, with substitutions at Phe<sup>7</sup> differing substantially from those at Val<sup>5</sup> and Gln<sup>4</sup>. However, the effect of the T2M substitution is independent of the position of the fluorescein label, as the rate of peptide dissociation from the fully assembled complex at physiological temperature (k<sub>1</sub> in Table III) is a relatively constant 6- to 8-fold slower when methionine is present at position 2 compared with threonine.

Fig. 7 shows Eyring plots for the data in Fig. 6. Via transition state theory, analysis of kinetic data in this fashion permits determination of activation thermodynamics, with the slope of the line proportional to the activation enthalpy (∆H<sup>‡</sup>) and the intercept proportional to the activation entropy (∆S<sup>‡</sup>) (see Ref. 23). The thermodynamic parameters derived from this analysis are shown in Table IV. The most important parameters are the differences between the native and T2M-modified peptides (∆ΔH<sup>‡</sup> and ∆ΔS<sup>‡</sup>). The values indicate that the slower dissociation of the T2M-modified peptide results from a more unfavorable activation entropy change (∆ΔS<sup>‡</sup> < 0), but this is offset by a favorable activation enthalpy change (∆ΔH<sup>‡</sup> < 0). As discussed further below, these data are most consistent with the increased hydrophobicity of the T2M-modified peptide (resulting in the unfavorable ∆ΔS<sup>‡</sup>) and the loss of a stabilizing hydrogen bond between Thr<sup>2</sup> and Glu<sup>63</sup> (resulting in the favorable ∆ΔH<sup>‡</sup>).
The crystals of the two complexes were grown from drops at pH 6.5, yet the measurements above were performed at pH 7.4. To control for the effect of pH, we repeated the measurements with the two peptides labeled at position 5 at pH 6.5, 37°C (data not shown). Under these conditions, the half-life of the T2M-modified peptide/MHC complex is ~5-fold longer than the half-life of the native peptide/MHC complex, compared with ~7-fold longer at pH 7.4. These results indicate that while pH does influence peptide dissociation rates, the slower dissociation of the T2M-modified gp100209 peptide is unlikely to arise from differences in the two structures at pH 7.4 that are not visible at pH 6.5, where the two complexes were crystallized.

**Discussion**

*The improved immunogenicity of gp100_{209–2M} correlates with the enhanced stability of the peptide/HLA-A2 complex*

The crystallographic structures of the native and T2M-modified gp100_{209} peptides bound to HLA-A2 indicate that the T2M substitution in gp100_{209} does not alter the structure of the peptide/HLA-A2 complex. In accordance with the structures, in PBMC from 95 individuals, we were unable to determine different frequencies of T cells reactive toward either the native or T2M-modified peptides. Our findings strongly support the interpretation that the enhanced immunogenicity of the T2M-modified peptide is attributable to its enhanced affinity for HLA-A2, rather than TCR cross-reactivity or qualitatively different T cell responses (e.g., partial agonism or antagonism) resulting from different peptide conformations (note that as the T2M substitution in gp100_{209} does not change the surface that is presented to TCRs, our data imply that Ag-specific TCRs would be unable to distinguish between the native and modified gp100_{209}/HLA-A2 complexes, i.e., TCR binding affinity and kinetics for the gp100_{209}/HLA-A2 complex should be no different from the values for the gp100_{209–2M}/HLA-A2 complex).

As the ~9-fold enhancement in peptide binding affinity is composed of a ~7-fold decrease in peptide dissociation rate, our data are most consistent with the increase in immunogenicity resulting from the slower dissociation rate of the T2M-modified peptide. Although the predicted small increase in peptide association rate could also play a role in influencing immunogenicity, the ~7-fold greater half-life of the complex with the native peptide indicates that in the time for 50% of the modified peptide to dissociate from HLA-A2, only ~0.8% of the native peptide would remain bound. The greater numbers of gp100_{209–2M}/HLA-A2 complexes on the surfaces of APCs could thus yield a more stable immunological synapse with Ag-specific T cells, leading to more productive signaling.

**Comparison with other studies of the effects of the T2M-substitution in gp100_{209}**

Our results contradict those of Clay et al. and Denkberg et al. (44, 45), who concluded that significant structural changes result from the T2M substitution in the gp100_{209} peptide. Clay et al. identified T cell “clouds” that were capable of recognizing the T2M-modified but not the native gp100_{209} peptide presented by HLA-A2. In light of our data, the findings of Clay et al. may be best attributable to TCR affinity or avidity: T cells expressing TCRs with weak affinity for the native gp100_{209} peptide presented by HLA-A2 may not respond well to the native peptide as the fast peptide dissociation rate results in too few TCR-peptide/MHC interactions to support the enhanced immunogenicity of the T2M-modified peptide.

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**Figure 7.** Eyring plots for the peptide dissociation data indicate the thermodynamic basis for the enhanced stability of the gp100_{209–2M}/HLA-A2 complex. Slopes of these lines are proportional to the enthalpic barriers for dissociation, whereas intercepts are proportional to entropic barriers. The steeper slope for the native peptide indicates a more unfavorable enthalpic barrier for dissociation, whereas the greater intercept indicates a more favorable entropic barrier. As the entropic component is larger, the native peptide dissociates faster.

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**Table III. Kinetic data for the dissociation of fluorescein-labeled peptides from HLA-A2 at 37°C**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Percent amplitude</th>
<th>$k_1$ ($s^{-1}$)</th>
<th>$t_{1/2}$ (hours)</th>
<th>Fold Rate Decrease for T2M Substitution</th>
<th>Percent amplitude</th>
<th>$k_2$ ($s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein at position 5</td>
<td>Native</td>
<td>90 ± 1</td>
<td>5.1 ± 0.2 × 10^{-5}</td>
<td>3.8 ± 0.2</td>
<td>6.4 ± 0.9</td>
<td>10 ± 2</td>
</tr>
<tr>
<td></td>
<td>T2M-mod</td>
<td>87 ± 3</td>
<td>7.9 ± 1.0 × 10^{-6}</td>
<td>24.5 ± 3.1</td>
<td>ND</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Fluorescein at position 4</td>
<td>Native</td>
<td>89 ± 1</td>
<td>7.0 ± 0.2 × 10^{-5}</td>
<td>2.7 ± 0.1</td>
<td>ND</td>
<td>11 ± 6</td>
</tr>
<tr>
<td></td>
<td>T2M-mod</td>
<td>69 ± 7</td>
<td>8.7 ± 1.2 × 10^{-6}</td>
<td>22.1 ± 3.0</td>
<td>ND</td>
<td>31 ± 7</td>
</tr>
<tr>
<td>Fluorescein at position 7</td>
<td>Native</td>
<td>82 ± 2</td>
<td>1.7 ± 0.1 × 10^{-4}</td>
<td>1.2 ± 0.1</td>
<td>ND</td>
<td>18 ± 2</td>
</tr>
<tr>
<td></td>
<td>T2M-mod</td>
<td>100</td>
<td>2.2 ± 0.1 × 10^{-5}</td>
<td>8.8 ± 0.4</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

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**Table IV. Activation energies for dissociation of the native gp100_{209} peptide and the T2M-modified variant from HLA-A2**

<table>
<thead>
<tr>
<th></th>
<th>$\Delta H^\ddagger$</th>
<th>$\Delta S^\ddagger$</th>
<th>$\Delta H^\ddagger$</th>
<th>$\Delta S^\ddagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>25.4 ± 2.4</td>
<td>3.3 ± 0.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>T2M-mod</td>
<td>19.3 ± 2.5</td>
<td>−19.4 ± 5.8</td>
<td>−6.2 ± 3.5^b</td>
<td>−22.7 ± 5.8^b</td>
</tr>
</tbody>
</table>

^a^ Enthalpies in kilocalories per mole; entropies in calories per mole per K.

^b^ Values for the native peptide subtracted from the values for the T2M-modified variant.
the formation of a stable immunological synapse. Conversely, with the T2M-modified peptide, the increased density of peptide/MHC complexes on the APC cell surface increases the likelihood that sufficient TCR-peptide/MHC interactions will occur, despite weak TCR affinity. This interpretation is supported by the experiments of Yu et al. (10), where CTL recognition of APC pulsed with the native gp100209 peptide was substantially reduced when a significant time delay was introduced into the experiment, whereas the those pulsed with the T2M-modified peptide were unaffected.

Denkberg et al. based their conclusions of structural alterations resulting from the T2M modification on an Ab (1A9) that could recognize the T2M-modified but not the native gp100209 peptide bound to HLA-A2 (45). These experiments, which were unlikely to be influenced by the more rapid dissociation of the native peptide, are clearly at odds with our findings. As indicated above, there are no physical differences in the surfaces of the gp100209 and gp100209–2M/HLA-A2 molecules, and electrostatic calculations of surface potentials do not show any effects stemming from removal of the hydrogen bond made from Thr2 to Glu63 (calculations not shown). The temperature factors in the two structures do not suggest different levels of motion between the two peptides. It is conceptually possible that there are differences not visible in the two structures even at the relatively high resolution of 1.8 Å, but if so, this would imply a level of discrimination not previously observed in Ab-Ag recognition. Although it is thus impossible to tell from the structures what, if any, differences the 1A9 Ab may be discerning, we do note that other studies have shown clear differences between Ab and TCR recognition of peptide/MHC complexes (46). Finally, we note the possibility that, as with any x-ray structure, the ensemble-averaged conformation present in vivo may differ from what is observed crystallographically. However, from the data presented in this study, there is no reason to expect that any conformational differences between in vivo and in vitro structures should differ between the complexes of gp100209 and gp100209–2M with HLA-A2.

**Implications for vaccine design**

The structure with the native gp100209 peptide indicates how the polar threonine side chain is accommodated in the HLA-A2 P2 pocket, forming a hydrogen bond with Glu63 of the HLA-A2 α1 helix. Given the polar environment around the rim of the pocket (Fig. 4), the hydrogen bond made to Glu63 is likely to be strongly stabilizing, as there should be little desolvation penalty for formation of this hydrogen bond. This interpretation is supported by the thermodynamic measurements summarized in Table IV: the native peptide has a much higher enthalpic barrier for dissociation than the T2M-modified peptide, consistent with the need to break a strong hydrogen bond between Thr2 and Glu63, which is not present in the structure with the T2M-modified peptide. Dissociation of the T2M-modified peptide is unfavorable entropically, consistent with the greater hydrophobicity of methionine over threonine (47). Thus, hydrophobicity is the likely reason for the increased stability (and thus immunogenicity) of the gp100209–2M/HLA-A2 complex (although side chain conformational entropy could play a role, its contribution is expected to be small relative to hydrophobicity; Refs. 48 and 49). However, the increase in hydrophobicity is offset considerably by the loss of the Thr2–Glu63 hydrogen bond, accounting for the relatively small ~9-fold enhancement in binding affinity for the T2M-modified peptide (5) (one caveat to this thermodynamic argument is we have measured activation energies for dissociation, rather than complete binding thermodynamics).

When the native and T2M-modified structures are superimposed, there is no overlap of the β-hydroxyl group of threonine at position 2 of the native peptide with any of the atoms of the methionine in the modified peptide (Fig. 2C). This suggests that it should be possible to achieve further gains in peptide/MHC stability through addition of a β-hydroxyl moiety to methionine—essentially crossing threonine and methionine, producing a β-hydroxy–methionine amino acid capable of hydrogen bonding to Glu63. As there are no structural differences induced by replacement of threonine with methionine, it is reasonable to hypothesize that the structure of gp100209 with β-hydroxy-methionine at position 2 should likewise be unchanged. Thus, our results suggest an approach for engineering the gp100209 peptide to achieve further gains in peptide/MHC stability and, potentially, immunogenicity. Such an approach may also be applicable to other TAA presented by HLA-A2, particularly those for which anchor-fixing using standard amino acids has failed to achieve immunologically significant gains.

In conclusion, we have shown that the T2M substitution in the gp100209 TAA does not alter the structure of the gp100209/HLA-A2 complex. Consistent with this finding, we were unable to identify higher frequencies of T cells specific for either the native or modified peptide in PBMC from a large number of human subjects. These data strongly support the conclusion that the greater immunogenicity of the gp100209–2M peptide is due to the enhanced stability of the peptide/MHC complex, validating the anchor-fixing approach for generating therapeutic vaccine candidates. Finally, the structural and thermodynamic data suggest a route for generating improved APL vaccine candidates using nonstandard amino acids.

**Acknowledgments**

We thank Drs. Yue Chen and Eva Skrzypczak-Jankun, John Clemens, Tommy Schad, the staff of the Argonne Structural Biology Center, and Joe Miller of Shamrock Structures for assistance.

**Disclosures**

B. M. Baker has a patent pending for the use of β-hydroxy amino acids at the first primary anchor for increasing peptide/MHC-I stability.

**References**


8. Baker, B. M., S. J. Gagnon, W. E. Biddison, and D. C. Wiley. 2000. Conversion of hydroxyl amino acids at position 2 of the native peptide with any of the atoms of the methionine in the modified peptide (Fig. 2C). This suggests that it should be possible to achieve further gains in peptide/MHC stability through addition of a β-hydroxyl moiety to methionine—essentially crossing threonine and methionine, producing a β-hydroxy–methionine amino acid capable of hydrogen bonding to Glu63. As there are no structural differences induced by replacement of threonine with methionine, it is reasonable to hypothesize that the structure of gp100209 with β-hydroxy-methionine at position 2 should likewise be unchanged. Thus, our results suggest an approach for engineering the gp100209 peptide to achieve further gains in peptide/MHC stability and, potentially, immunogenicity. Such an approach may also be applicable to other TAA presented by HLA-A2, particularly those for which anchor-fixing using standard amino acids has failed to achieve immunologically significant gains.

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