Strategic Mutations in the Class I Major Histocompatibility Complex HLA-A2 Independently Affect Both Peptide Binding and T Cell Receptor Recognition*

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Received for publication, March 26, 2004, and in revised form, April 30, 2004

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This paper is available online at http://www.jbc.org

Mutational studies of T cell receptor (TCR) contact residues on the surface of the human class I major histocompatibility complex (MHC) molecule HLA-A2 have identified a “functional hot spot” that comprises Arg65 and Lys66 and is involved in recognition by most peptide-specific HLA-A2-restricted TCRs. Although there is a significant amount of functional data on the effects of mutations at these positions, there is comparatively little biochemical information that could illuminate their mode of action. Here, we have used a combination of fluorescence anisotropy, functional assays, and Biacore binding experiments to examine the effects of mutations at these positions on the peptide-MHC interaction and TCR recognition. The results indicate that mutations at both position 65 and position 66 influence peptide binding by HLA-A2 to various extents. In particular, mutations at position 66 result in significantly increased peptide dissociation rates. However, these effects are independent of their effects on TCR recognition, and the Arg65-Lys66 region thus represents a true “hot spot” for TCR recognition. We also made the observation that in vitro T cell reactivity does not scale with the half-life of the peptide-MHC complex, as is often assumed. Finally, position 66 is implicated in the “dual recognition” of both peptide and TCR, emphasizing the multiple roles of the class I MHC peptide-binding domain.

Recognition of a peptide bound to a major histocompatibility complex protein (peptide-MHC) by the αβ T cell receptor (TCR) is necessary for the initiation and propagation of a cellular immune response, as well as the development and maintenance of the T cell repertoire. TRCs bind peptide-MHC in a diagonal-to-orthogonal fashion, interacting with elements of both the peptide and the MHC (1–3). Numerous studies have probed the interfaces between TCRs and their ligands through the use of altered peptides or site-directed mutagenesis of the MHC (e.g. see Refs. 4–8). These studies have been useful in identifying hot spots within individual interfaces, as well as predicting the biophysical mechanisms by which T cell receptors bind their ligand (9).

In our studies of TCR recognition of the human class I MHC HLA-A*0201 (referred to as HLA-A2) presenting the Tax peptide (sequence LLFGYPYVV; see Ref. 10), we identified a functional hot spot consisting of arginine 65 and lysine 66 on the HLA-A2 α1 helix (4). In a mutagenesis experiment involving 15 HLA-A2 amino acids contacted by the Tax-HLA-A2-specific αβ T cell receptor A6, only two mutations, Arg65 → Ala (R65A) and Lys66 → Ala (K66A), resulted in significantly reduced T cell effector functions when the mutants were used to present the Tax peptide to T cells expressing the A6 receptor. Similar results were found with T cells expressing a different Tax-HLA-A2-specific TCR, B7. Lys66 was also identified as a critical position influencing T cell reactivity in at least one other mutagenesis study of HLA-A2 (8).

The general nature of the Arg65-Lys66 hot spot in the recognition of Tax-HLA-A2 was confirmed by functional assays with 201 additional T cell lines, bringing the total number of T cell lines studied to 203 (4). The R65A mutation significantly reduced activity for 67% of these lines. The K66A mutation was even more detrimental, negatively affecting 98% of this large panel. Extension of this study to T cell lines specific for four additional peptides presented by HLA-A2 (influenza M1, MART-1, pp65, and gp100) indicated that the K66A mutation was detrimental for the majority of T cell lines tested, independent of peptide specificity (11). Similar results were also observed for panels of CTL lines specific for the hapten dinitrophenyl conjugated to Tax and M1 peptides presented by HLA-A2 (12). These findings raise the possibility that Lys66 is a critical position for recognition by HLA-A2-restricted T cell receptors, perhaps contributing to the phenomenon of MHC restriction or to the dependence of T cell receptors on a single MHC subtype regardless of peptide specificity.

These interpretations, however, are predicated on the assumption that the mutations do not drastically alter the peptide-MHC interaction. As the strength of a T cell response is related to how well the peptide is bound by the MHC (13), a reduction of T cell effector functions in a functional assay could be attributable to weaker peptide binding. We previously used circular dichroism to monitor the thermal unfolding of the wild-type and the R65A and K66A mutant peptide-MHC molecules (4). Because of the linkage between peptide binding and protein stability, thermal unfolding is frequently used as a probe of peptide binding affinity (14). As neither the R65A nor the K66A mutation resulted in a significant change in the
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In this report, we used a combination of fluorescence anisotropy, T cell functional assays, and Biacore binding studies to demonstrate that the Arg65-Lys66 functional hot spot represents a true hot spot for TCR binding. We find that although the alanine mutations that defined this hot spot do influence the peptide-MHC interaction, this is independent from their effects on T cell recognition. Lysine 66 in particular is implicated in the dual recognition of both peptide and TCR, emphasizing the multiple roles of the class I MHC peptide-binding domain. We also make the observation that an increase in thermal stability is insufficient to result in a loss of peptide dissociation from the MHC molecule, which is insufficient to result in a loss of T cell recognition, potentially due to the anisotropy of peptide dissociation. We used anisotropy and fluorescence anisotropy to study this phenomenon, and we observed that the peptide-MHC half-lives do not necessarily result in improved potency.

EXPERIMENTAL PROCEDURES

Proteins and Peptides—Fully assembled soluble HLA-A2 peptide-MHC was refolded from bacterially expressed inclusion bodies as described previously (21). Mutations were introduced in the HLA-A2 gene using standard molecular biology techniques. Excess MHC was refolded from bacterially expressed inclusion bodies as described previously (16). Briefly, fully assembled heterotrimer was unfolded, fractionation, and renaturation of refolded protein as described previously (22–24).

Peptide-free K66A and K66R heterodimers were purified by denaturation, fractionation, and renaturation of refolded protein as described previously (16). Briefly, fully assembled heterotrimer was unfolded in 6 M guanidine HCl, pH 10. Heavy chain, βm, and peptide were then separated chromatographically under denaturing conditions. Fractions containing heavy chain and βm were combined and dialyzed against buffer to allow assembly of peptide-free heavy chain/βm heterodimer. After dialysis, samples were filtered and concentrated to 100 μg/ml (250 μl of peptide stock concentration). Protein was frozen in aliquots at −80 °C immediately after preparation.

Peptides were synthesized commercially (Sigma Genosys). For measuring binding and dissociation, a derivative of the Tax peptide (LLF-GYPVV) was used; the peptide was substituted with Lys to increase solubility, and Tyr was substituted with fluorescein-derivatized lysine (peptide referred to as Tax-3K5Flc). Dissociation measurements were performed in the presence of an excess of unlabeled peptide with the sequence LLKGPYVV (Tax-3K). A polyglycine peptide, labeled at position five with a fluorescein-derivatized lysine (GGGGK[Flc]GGGG), was used as a negative control for binding measurements. Extinction coefficients at 280 nm (units of μ M−1 cm−1) were 12,060 for Tax-3K, 105,600 for HLA-A2-Tax-3KFlc, 1210 for Tax-3K, 10,850 for the negative control peptide, 93,410 for heavy chain/βm heterodimer, and 19,180 for βm.

Fluorescence Anisotropy—Fluorescence anisotropy was measured with a Beacon 2000 polarization instrument (PanVera, Madison, WI) as described previously (16). Data were analyzed using the program Origin (OriginLab, Northampton, MA). Error analysis was performed using standard error propagation techniques (25). All measurements were performed in 10 mM Hepes, 150 mM NaCl, pH 7.4.

Peptide Dissociation Kinetics—Dissociation kinetics for each of the HLA-A2 mutants were measured with fluorescence anisotropy as described previously (16), with 7.5 nM peptide-MHC loaded with Tax-3KFlc in the presence of 7.5 μM Tax-3K. Data were fit to single or biphasic functions of the form

\[ y(t) = y_0 + \sum \left( A_i \exp\left(-k_it\right) \right) \quad (1) \]

in which \( y_0 \) is the baseline offset, the summation is over the number of phases \( i \) (1 or 2), \( A_i \) is the amplitude for phase \( i \), and \( k_i \) is the rate constant for phase \( i \) and \( t \) is time.

Peptide-MHC Equilibrium Binding—Equilibrium binding of Tax-3KFlc to peptide-free K66A and K66R HLA-A2 heterodimer, also measured using fluorescence anisotropy as described recently (16), was performed at 25 °C with 120 nM of peptide-free heterodimer in the presence of ~600-fold excess βm and varying amounts of Tax-3KFlc. Samples were incubated for 3–6 h prior to analysis. Note that because we used anisotropy, data from these experiments differ from that seen in a typical binding isotherm. At high peptide concentration the response is low because of the anisotropy of excess peptide, whereas at low peptide concentration the response is high because of the anisotropy of the peptide-MHC complex.

To fit the binding data, we took advantage of the fact that anisotropy measures concentrations and provides direct information on the fraction bound and free (26). Anisotropy was converted into the fraction bound as a function of free peptide and fit to a multiple equal and independent site model varying the number of sites and the equilibrium binding constant (16). In this case, the fitted number of sites is equivalent to the activity of the heavy chain/βm heterodimer. 

TCR Surface—The CD8+ CTL clone RS56 (A6) (27), 10B7 (expressing the B7 TCR) (27), and 1E7 (not previously described) were transfected with HLA-A2 wild-type and Lys66 mutant-transfected Hmy2.C1R cells (28). The gene usage and CDR loop sequences for the TCRs expressed by T cell clones RS56 (A6), 10B7 (B7), and 1E7 are shown in Table I (1E7 expresses two productively rearranged TCR surface).

Wild-type and Lys66 mutant transfectants displayed very similar levels of cell surface HLA-A2 molecules as quantitated by cell surface staining with the 2B7 antibody (4). Cytotoxicity was quantified by a time-resolved fluorometric assay as described previously (29).

Biacore Biosensor Measurements—Steady-state equilibrium binding of wild-type and mutant Tax-HLA-A2 to the A6 TCR was monitored at 25 °C using a Biacore 3000 surface plasmon resonance biosensor. The TCR was coupled to a CM5 sensor chip using amine coupling to ~250–500 maximum response units (RU)max. Separate chips were used for each HLA-A2 molecule studied. 25 μl of freshly prepared peptide-MHC at various concentrations was injected at a flow rate of 5 μl/min (300-s contact time). The flow was directed over a mock surface to which no protein was bound, followed by the TCR surface. Responses from the TCR surface were corrected for the response from the mock surface and

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| α chains | CD1 | CD2 | CD3 |
|---------|-----|-----|-----|
| 1E7a: AV1581, Jα29 | SSSTYLYWYQK | YIFSNMDMK | SITQASGNTPLVF |
| 1E7b: AV2221, Jα21, Jα32 | ATQYPSLFWYQV | KATRADDKG | LSPDGGATKMLF |
| 10B7 (B7): ADV21SIA12, Jα24 | TDAFQFPWYQGF | HIPRVSIEK | AMEGAQKLYVF |
| RS56 (A6): AV2S1A2, Jα24 | DRGSQFFWYRFQ | SIYSNDEKE | TTDWGGKLFQ |

* Clone 1E7 expresses two productively rearranged α chains.

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Gene usage and CDR loop sequences for the TCRs expressed by T cell clones RS56 (A6), 10B7 (B7), and 1E7 are shown in Table I (1E7 expresses two productively rearranged TCR surface).
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RESULTS

Peptide Dissociation Kinetics for the R65A and K66A Mutants Indicate Clear Effects on Peptide Off Rates—Fig. 1 shows dissociation of Tax-3K5Flc from R65A and K66A at 25 °C. Dissociation data from wild-type HLA-A2 are shown for comparison (Fig. 1, dotted line; see Ref. 16). As with peptide dissociation from the wild-type molecule (16, 18), these data did not fit to a single exponential decay but were adequately fit by a biexponential decay function. The second, faster phase has a half-life of only —14 min at 25 °C. Thus, at least for the Tax derivative studied here, lysine at position 66 is optimal for ensuring a long peptide-MHC half-life. The observation that the replacement of lysine with arginine still results in fast dissociation indicates there are stringent orientational requirements for the positive charge at position 66.

Clues to the cause of the dramatic increase in peptide dissociation seen with the K66A mutation can be found in the structure of the Tax-HLA-A2 complex (32, 33). The charged nitrogen of the Lys66 side chain is very close to the carbonyl oxygen of peptide position 2 (P2), suggesting that loss of a hydrogen bond between Lys66 and the P2 oxygen is responsible for the faster peptide off rate. However, as diagrammed in Fig. 2, Lys66 also forms an ion pair with the side chain of Glu65. To ascertain whether the increase in the peptide dissociation rate can be solely attributed to the loss of the Lys66-P2 hydrogen bond, we investigated the effects of changing Glu65 to alamine (63A). As indicated in Table III, the E63A mutation also results in a large increase in the rate of peptide dissociation. Thus we cannot credit the increase in peptide off rate seen with the K66A mutation solely to the loss of the Lys66-P2 hydrogen bond, as loss of the Glu65-Lys66 ion pair also affects the rate.

Functional Effects of Position 66 Mutants: T Cell Activity Does Not Correlate with Peptide-MHC Half-life—The effects of the position 66 mutations on TCR recognition were assessed by lysis of Tax peptide-pulsed transfectants by three different Tax peptide-HLA-A2-specific CTL clones, each expressing a different T cell receptor (Fig. 3). The results demonstrate that the K66A, K66L, K66N, and K66Q mutations completely abolish recognition by the TCR clone RS56 (Fig. 3A) and greatly diminished recognition by the TCR clone 10B7 (Fig. 3B). The K66R mutation had no discernable effect with either clone. The observation that clones RS56 and 10B7 can still lyse K66R targets indicates that although lysine at position 66 is required for slow peptide dissociation, neither the lysine nor a slow peptide dissociation rate is requisite for T cell recognition. It also indicates that the receptors expressed by RS56 and 10B7 are dependent on a positive charge at position 66.

In contrast to RS56 and 10B7, the 1E7 clone (Fig. 3C) was not affected by the K66A mutation but was negatively affected by all of the other Lys66 mutations. These findings demonstrate that the K66A mutation does not diminish the ability of all TCRs to recognize Tax-HLA-A2, despite the increased peptide dissociation rate.

Activation Thermodynamics for Peptide Dissociation from the Lys66 Mutants Indicate That Faster Dissociation Is due to Entropic Effects—To further probe the effects of the position 66 mutations on peptide dissociation, the dissociation data as a function of temperature were analyzed via Eyring analysis (34). From transition state theory, Eyring analyses permit extraction of the thermodynamics (ΔH‡, ΔS‡) for moving from the bound state to the peptide-binding transition state. Although interpretation of these values is complicated by the inexact nature of the protein-peptide transition state (35), when compared with the values for the wild-type molecule, the thermodynamic parameters can give insight into the molecular effects of the mutations.

This analysis is shown in Fig. 4 and summarized in Table IV. Every mutation studied has a less favorable enthalpic barrier yet a more favorable entropic barrier (we limit the discussion to relative effects only). Thus for these mutations (K66A, K66R, K66L, K66N, and K66Q), the increase in peptide off rate relative to wild type is entirely due to a greater gain in entropy upon moving from the bound to the transition states, compensated to various extents by a loss in enthalpy (compare ΔH‡ to TΔS‡ in Table IV).
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**TABLE II**

| HLA-A2 molecule | Phase 1: peptide dissociation from fully assembled heterotrimer | Phase 2: peptide dissociation from heavy chain/peptide heterodimer |
|-----------------|-------------------------------------------------------------|---------------------------------------------------------------|
|                 | Amplitude | $k$ | $t_{1/2}$ | Amplitude | $k$ | $t_{1/2}$ |
| **25 °C**       |           |     |          |           |     |          |
| Wild type$^c$   | 90.3 ± 0.4 | 2.55 ± 0.03 × 10⁻⁵ | 7.55 ± 0.10 | 9.7 ± 0.6 | 1.36 ± 0.10 × 10⁻⁴ | 1.42 ± 0.10 h |
| R65A            | 97.6 ± 0.2 | 2.89 ± 0.03 × 10⁻⁵ | 6.66 ± 0.07 | 2.4 ± 0.4 | 2.05 ± 0.48 × 10⁻⁴ | 0.94 ± 0.22 h |
| K66A + β₂m$^d$ | 100       | 2.68 ± 0.01 × 10⁻⁵ | 7.18 ± 0.03 | not detected |                                     |                |
| K66A            | 91.7 ± 0.5 | 1.17 ± 0.01 × 10⁻⁴ | 1.65 ± 0.01 | 8.3 ± 0.4 | 1.57 ± 0.16 × 10⁻³ | 440 s         |
| K66A + β₂m$^d$ | 95.7 ± 0.7 | 1.19 ± 0.01 × 10⁻⁴ | 1.62 ± 0.01 | 4.3 ± 0.5 | 1.55 ± 0.39 × 10⁻³ | 450 s         |
| **37 °C**       |           |     |          |           |     |          |
| Wild type$^c$   | 85.1 ± 0.7 | 1.77 ± 0.04 × 10⁻⁴ | 1.09 ± 0.03 | 14.9 ± 1.0 | 1.4 ± 0.1 × 10⁻³ | 500 s         |
| R65A            | 76.5 ± 0.2 | 2.63 ± 0.02 × 10⁻⁴ | 0.73 ± 0.01 | 23.5 ± 0.3 | 1.25 ± 0.02 × 10⁻³ | 550 s         |
| K66A            | 83.6 ± 0.6 | 1.11 ± 0.01 × 10⁻⁴ | 0.17 ± 0.01 | 16.4 ± 1.3 | 1.51 ± 0.25 × 10⁻² | 46 s          |

$^a$ Data were fit to biexponential decay functions as described under “Experimental Procedures.” Half-lives are calculated as 0.693/k.

$^b$ Units of h or s as indicated.

$^c$ Wild-type data from Ref. 16.

$^d$ Includes ~8000-fold excess β₂m.

**TABLE III**

| HLA-A2 molecule | 25 °C | 37 °C |
|-----------------|-------|-------|
|                 | $k$ | $t_{1/2}$ | $k$ | $t_{1/2}$ |
| Wild type$^b$   | 2.55 ± 0.03 × 10⁻⁵ | 7.55 ± 0.10 | 1.77 ± 0.04 × 10⁻⁴ | 1.09 ± 0.03 |
| K66A            | 1.17 ± 0.01 × 10⁻⁴ | 1.65 ± 0.01 | 1.11 ± 0.01 × 10⁻³ | 0.17 ± 0.01 |
| K66R            | 1.04 ± 0.03 × 10⁻⁴ | 1.85 ± 0.06 | 1.02 ± 0.03 × 10⁻³ | 0.19 ± 0.01 |
| K66N            | 1.38 ± 0.02 × 10⁻⁴ | 1.39 ± 0.02 | 1.54 ± 0.02 × 10⁻³ | 0.13 ± 0.01 |
| K66Q            | 1.03 ± 0.03 × 10⁻⁴ | 1.88 ± 0.06 | 1.93 ± 0.02 × 10⁻³ | 0.10 ± 0.01 |
| K66L            | 8.28 ± 3.93 × 10⁻⁴ | 0.23 ± 0.11 | 1.68 ± 0.58 × 10⁻³ | 0.12 ± 0.04 |
| E63A            | 4.84 ± 0.12 × 10⁻⁴ | 0.40 ± 0.01 | 2.75 ± 0.58 × 10⁻⁴ | 0.70 ± 0.15 |

$^a$ Reports peptide dissociation from fully assembled heterotrimer only.

$^b$ Wild-type values from Ref. 16.

Equilibrium Binding of Peptide to the K66A and K66R Mutants: Both Mutants Retain High Binding Affinity—The effects of the K66A and K66R mutants on peptide binding by HLA-A2 were further examined in an equilibrium binding experiment. These mutants were chosen for these experiments as they have rapid peptide dissociation rates yet can still be efficiently recognized by T cells (albeit by only a very small subset for K66A). Binding was assayed using a recently developed fluorescence assay, in which the affinity of the peptide for the peptide-free HLA-A2 heavy chain/β₂m heterodimer is directly measured (16), as opposed to competition, stabilization, or antibody binding experiments that are commonly performed.

Fig. 5 shows experiments for Tax-3K5Flc binding peptide-free K66A (Fig. 5A) and K66R (Fig. 5B) HLA-A2 heavy chain/β₂m heterodimers. The insets show the raw data, in which the fully saturated protein has a minimum anisotropy due to the presence of excess peptide (Fig. 5). Transforming this data directly into the fraction bound versus the concentration of free peptide permits fitting to an independent site binding model (16, 26). The resulting $K_D$ from analysis of six separate experiments is $51 ± 20$ nM for K66A and $1.7 ± 0.4$ nM for K66R, compared with the value of $18 ± 1$ nM observed for Tax-3K5Flc binding to wild-type HLA-A2 (16). No appreciable binding to either mutant was seen with a fluorescence-labeled polyglycine control peptide.

There was considerable variability (as much as 2.5-fold) in the affinity determined with different preparations of the K66A mutant, accounting for the large error in the measurement. Poor reproducibility with this mutant may stem from the loss of the Lys⁶⁶-Glu³⁴ ion pair (Fig. 2). Furthermore, saturation of the binding curve could not be achieved with K66A because of limitations of the assay. Statistically then, the value of $51 ± 20$ nM thus should be considered an upper limit. Nevertheless, this experiment demonstrates that the K66A mutant retains the capacity to bind peptides with relatively high affinity.

The observation that the K66R mutant binds peptide more tightly than wild type despite a dramatic increase in peptide off rate indicates that the mutation results in either faster peptide association and/or a shift in the conformational equilibrium of the peptide-free molecule toward a more peptide-accessible state (16, 18).

A Positive Charge at Position 66 Is Required for TCR Binding—The effects of the R65A, K66A, and K66R mutations on TCR binding were next examined in a direct binding study using Biacore biosensor technology. A recombinant soluble form of the TCR expressed by the T cell clone RRS6 (Fig. 3A) was used. This Tax-HLA-A2-specific TCR, termed A6, has been used in a number of studies of TCR-peptide-MHC interactions (e.g. see Refs. 6, 23, 24, and 36) and is well characterized structurally and molecularly. Previous work has shown that A6...
type Tax-HLA-A2 was measured as these experiments are shown in Fig. 6. The affinity for wild-type Tax-HLA-A2 binds wild-type Tax-HLA-A2 with an affinity near 1 μM at 25 °C (24).

The A6 TCR was immobilized on a Biacore sensor surface, and the binding of the various HLA-A2 molecules was measured under steady-state equilibrium conditions. The results of these experiments are shown in Fig. 6. The affinity for wild-type Tax-HLA-A2 was measured as ~2 μM, in good agreement with the previously determined value. Little or no binding was seen to the R65A or K66A mutants. In contrast, binding to the K66R mutant was readily detectable, allowing determination of an affinity of ~36 μM. Thus, a positive charge at position 66 is required for binding of the Tax-HLA-A2-specific TCR A6, in accordance with the functional studies.

The lack of detectable binding to the R65A or K66A mutants is in contrast to previous measurements with these mutants, which showed detectable but much weaker binding to A6 (4). However, the steady-state experiments here, with 50–60 μM injected protein, are more rigorous than the previous study, which relied more on interpretation of kinetic association and dissociation phases. Close inspection of the K66A data (Fig. 6B) may indicate some very weak binding, but even if this is the case, the TCR affinity is clearly much weaker for K66A than it is for K66R.

It is interesting that the affinity of A6 for K66R is weaker than wild type, yet T cells expressing this receptor kill K66R targets as efficiently as wild type (Fig. 3A). This adds to a growing list of TCR ligands whose affinity does not scale with activity, a phenomenon that has generated considerable discussion in the recent literature (e.g. see Refs. 20 and 37–39) but for which a clear explanation is still elusive.

**DISCUSSION**

Our previous studies with HLA-A2 mutants identified Arg<sup>65</sup> and Lys<sup>66</sup> on the HLA-A2 α1 helix as amino acids that form important interactions with HLA-A2-specific T cell receptors. This TCR hot spot may be involved in the phenomenon of MHC restriction or in the dependence of T cell receptors on a single MHC subtype independent of peptide specificity. This interpretation, however, is predicated on the assumption that the R65A and K66A mutations do not drastically alter the interaction of the peptide with the MHC molecule. This assumption is particularly important for Lys<sup>66</sup>, which in the A6-Tax-HLA-A2 structure contacts both the peptide and the TCR (23). Here we have shown that although the mutations do influence peptide binding, the loss in T cell activity associated with these mutations cannot be attributed to alterations in peptide binding. Thus the effects on TCR recognition are independent from the effects on peptide dissociation, and Arg<sup>65</sup> and Lys<sup>66</sup> represent true hot spots, directly or indirectly affecting peptide binding.

Our experiments also revealed that immunological potency measured in vitro need not correlate with the half-life of the peptide-MHC complex, as is often assumed. Finally, position 66 is implicated in the dual recognition of both the peptide and the TCR, emphasizing the multiple roles of the class I MHC peptide-binding domain. The various mutations studied and how they relate to these findings are discussed below.

**Arginine 65: A Linkage between Peptide Specificity and TCR Hot Spots**—The R65A mutation has little effect on peptide dissociation at 25 °C but results in a 1.5-fold increase at physiological temperature. The reason for this slight increase in the off rate is difficult to ascertain, as in the Tax-HLA-A2 structure the Arg<sup>65</sup> side chain does not directly contact the peptide or other amino acids of the MHC molecule (16, 18). However, it is ~7 Å away from the carbonyl oxygen of glycine 4 of the peptide, near enough for a favorable long range electrostatic interaction.

The R65A mutation results in a loss of TCR activity with 67% of all Tax-HLA-A2-specific T cells that have been tested (4) but does not impact T cells expressing receptors specific for the influenza M1, MART-1, pp65, and gp100 peptides (11). Considering that 1) the much more dramatic changes in the peptide dissociation rate seen with K66A and K66R are not enough to result in a loss of T cell activity (see below) and that 2) the R65A mutation does not alter the thermal stability of the molecule (4), the effects of this mutation on the activity of Tax-HLA-A2-specific T cells can best be attributed to direct or indirect effects on T cell receptor binding.

In the structures of the A6 and B7 TCRs bound to Tax-HLA-A2, Arg<sup>65</sup> forms an ion pair with a glutamate and an aspartic acid, respectively (32, 33). It appears that the loss of T cell activity stems from reduced TCR binding resulting from the loss of this ion pair, an interpretation supported by Biacore binding studies with the A6 receptor. The fact that T cell receptors specific for other peptides are not affected by this mutation indicates that this critical interaction is specific for a subset of Tax peptide-restricted receptors, highlighting a linkage between TCR hot spots on the MHC molecule, TCR peptide specificity, and individual TCR sequences.

**Lysine 66: A More General Hot Spot Influencing Both the Peptide and the Receptor Interaction**—The K66A mutation has a significant effect in T cell functional assays; of 203 Tax-HLA-A2-specific T cell lines assayed, 98% were negatively impacted...
by this mutation (4). Unlike R65A, however, similar results were found with T cell lines specific for four other peptides presented by HLA-A2 (11) as well as cell lines specific for the hapten dinitrophenyl conjugated to the Tax and M1 peptides presented by HLA-A2 (12). Here, we have demonstrated that the mutation results in a 5–6-fold increase in the peptide dissociation rate compared with wild type. The requirement for lysine at position 66 is quite specific, as neither charged (Arg) nor polar (Gln, Asn) nor hydrophobic (Leu) substitutions at position 66 restored the peptide dissociation rate to wild-type levels. The observation that replacement of lysine with arginine still results in fast dissociation indicates that there are stringent orientational requirements for the positive charge at position 66. Position 66 is a polymorphic position for human class I MHC molecules; however, the extent of polymorphism is small, as the position is lysine in 91% of known HLA-A2 subtypes (40).

As shown in Fig. 2, the K66A mutation removes a hydrogen bond to the bound peptide. It also, however, removes an ion pair with Glu63 just "underneath" Lys66. Changing Glu63 to alanine also has a substantial effect on peptide dissociation. Thus, the increase in peptide dissociation seen with the K66A mutation cannot be directly attributed to removal of the lysine-peptide hydrogen bond, as the Lys66-Glu63 interaction also seems to play a role in ensuring a long peptide-MHC half-life.

Although we might expect the loss of the Lys66-peptide hydrogen bond and/or the loss of the Lys66-Glu63 ion pair to introduce mobility into the bound peptide, the transition state thermodynamics do not support this. All of the position 66 mutants result in a less favorable activation enthalpy and a more favorable activation entropy for peptide dissociation. More mobility in the bound state would be expected to increase the entropic barrier for dissociation (\(\Delta S^\ddagger > 0\)), as there would be less entropy to gain upon moving to the transition state. The effects we have observed (\(\Delta S^\ddagger > 0\)) must result from more dynamic motion in the transition state, a more rigid bound state, or both. In the 1.8-Å crystal structure of Tax-HLA-A2 (32), there is no indication (as evidenced by B-factors) for high mobility of the peptide or MHC in the vicinity of Lys66, and it is difficult to envision a mechanism by which the Lys66 mutations introduce more rigidity to the structure. Alternatively, there is good evidence for dynamic motion in the peptide-free MHC molecule (16, 18, 41), and this may carry over to the

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**Table IV**

| HLA-A2   | \(\Delta H^\ddagger\) | \(\Delta S^\ddagger\) | \(\Delta H^\ddagger\) | \(\Delta S^\ddagger\) |
|----------|----------------------|----------------------|----------------------|----------------------|
|          | cal/mol              | cal/mol              | kcal/mol             | kcal/mol             |
| Wild type | 21.8 ± 1.8           | -6.1 ± 1.0           | 8.3 ± 0.5            | 9.1 ± 1.6            |
| K66A     | 29.2 ± 0.3           | 21.7 ± 1.4           | 7.4 ± 1.8            | 8.3 ± 0.5            |
| K66R     | 30.2 ± 1.1           | 24.3 ± 5.1           | 8.4 ± 2.1            | 9.1 ± 1.6            |
| K66L     | 24.5 ± 0.3           | 10.3 ± 0.8           | 2.7 ± 1.8            | 4.9 ± 0.4            |
| K66N     | 30.5 ± 0.9           | 25.9 ± 4.5           | 8.7 ± 2.0            | 9.5 ± 1.4            |
| K66Q     | 33.6 ± 1.3           | 37.2 ± 7.9           | 11.8 ± 2.2           | 12.9 ± 2.4           |

* a Value for mutant minus value for wild type.
* b At 25 °C.
* c Wild-type values from Ref. 16.

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**Fig. 4.** Eyring analyses for peptide dissociation from the peptide/MHC heterotrimer for each of the position 66 mutants studied. Peptide dissociation was measured over the temperature range of 4–37 °C. The slopes and intercepts of these plots are related to the activation enthalpy and entropy for dissociation, respectively. Dashed lines represent the data for wild-type HLA-A2 (16).
binding transition state. Thus, the molecular effects of the loss of the Lys66-P2 hydrogen bond and the Glu63-Lys66 ion pair may be to increase dynamics in the peptide-free molecule. The higher activation enthalpies, on the other hand, imply a loss of favorable interactions; this could result from weaker protein or peptide interactions with solvent in a more mobile binding transition state. A structure of the K66A mutant complex is required to further substantiate these arguments, and work in this area is underway. Note, however, that the observation that the CTL clone 1E7 efficiently kills K66A targets argues against any dramatic structural perturbations resulting from the mutation.

The faster peptide dissociation from the K66A mutant was surprising given earlier CD melting experiments that showed no change in Tm, thus suggesting little or no change in peptide binding affinity (4). To investigate this, we directly measured peptide binding affinity for the K66A mutant. The resulting KD was 51 ± 20 nM at 25 °C, compared with the wild-type value of 18 ± 1 nM (16). Although statistically this should be considered an upper limit on affinity, the observation that the mutation results in little change in the thermal stability of the molecule (4) supports this relatively high affinity. If the mutation only affected peptide dissociation, we would predict an affinity of ~90 nM (16), slightly weaker than the measured value, even accounting for experimental error. The K66A mutation therefore may have additional effects other than increasing the peptide dissociation rate. The results with K66R are less ambiguous; despite the very fast off rate, the peptide affinity is ~10-fold stronger than wild type. Therefore, the K66R mutation (and perhaps K66A as well) results in a net increase in the rate of peptide binding to the MHC molecule.

An important aspect of peptide-MHC interactions is that peptide binding proceeds via a conformational transition in the heavy chain from a "peptide-inaccessible" state to a "peptide-accessible" state (16, 18, 42). There are thus three ways in which the overall rate of peptide association can be increased: an increase in the association rate constant, a shift in the conformational equilibrium of the peptide-free molecule toward a more peptide-accessible state, or both. In addition to hydrogen bonding with the peptide, Lys66 helps to bury the P2 side chain, suggesting a mechanism by which position 66 alterations could result in a faster net rate of peptide binding.

Although the K66A mutation results in much faster peptide dissociation, functional assays with the RS56, 10B7, and 1E7 Tax-specific CTL clones indicate that faster peptide dissociation cannot be responsible for the loss of T cell activity seen.
with this mutant. The CTL clone 1E7 efficiently kills targets with the K66A mutation, and clones RS56 and 10B7 efficiently kill targets with the K66R mutation, which has a peptide dissociation rate similar to K66A at both 25 and 37 °C. Therefore, the fast peptide dissociation rate resulting from the K66A and K66R mutations is still above some “minimum threshold” for efficient antigen presentation in an in vitro assay of cytotoxicity. Although data that correlate the peptide dissociation rate with in vivo immunogenicity exist (43), our findings indicate that this correlation does not necessarily hold for in vitro experiments. An increased half-life of the peptide-MHC complex has recently been used to explain the enhanced potency of the SIYR peptide in the murine 2C-H2-Kb system (20), but our data indicate that this does not have to be the case. Note, however, that although the peptide dissociation rates for the K66A and K66R mutants are much faster than wild-type HLA-A2, both mutants bind peptide with relatively high affinity, and a correlation between peptide affinity and activity (13, 19) remains intact.

There is a well supported expectation that the steady-state level of a peptide-MHC complex on the surface of an antigen presenting cell will be critical in influencing the immunological potency of an antigenic peptide, whether in vitro or in vivo. However, our data highlight an important difference between in vitro measurements of cytotoxicity, where exogenous peptide is present and can bind at the cell surface, and in vivo situations, where there is little or no exogenous peptide and binding occurs in the tightly regulated environment of the endoplasmic

![Diagram](https://example.com/diagram.png)
Strategic Mutations in HLA-A2 Affect TCR and Peptide Binding

For the loss of the in vitro measured T cell activity with mutations at position 66? The answer must lie with direct or indirect effects on T cell receptor recognition and differs for different T cell clones. For RS66 and 10B7, which express the A6 and B7 T cell receptors, respectively (23, 27, 30), maintaining the positive charge is required; mutations to hydrophobic or polar amino acids do not restore activity, although the mutation of Lys66 to arginine does. This requirement for a positive charge at position 66 was further demonstrated in the Biacore experiments, where K66A was poorly recognized by the A6 TCR (if at all), whereas K66R was efficiently bound.

The crystallographic structures of the A6 and B7 T cell receptors expressed by RS66 and 10B7 both show the nitrogen of the Lys66 side chain partially buried in the TCR-peptide-MHC interface (23, 30). Although it does not form any ion pairs or hydrogen bonds in either structure, in A6, the side chains of Asp66 and Glu30 of CDR1α and Asp68 of CDR3α are all within 9 Å of the Lys66 nitrogen. In B7, the side chain of Asp30 of CDR1α is within 5 Å. Loss of favorable long range electrostatic interactions with the TCR is thus one possible reason for the loss of binding and activity when the charge at position 66 is removed. Electrostatic repulsion resulting from exposure of Glu63 underneath Lys66 could also be a factor, as could any subtle structural perturbations in the peptide-MHC molecule itself.

Unlike RS66 and 10B7, no structural information is available for the TCRs expressed by the "K66A-resistant" CTL clone 1E7 (this clone expresses two productively rearranged α chains). Comparison of the sequences of the TCRs expressed by 1E7 with those of the A6 and B7 TCRs (Table I) reveals numerous differences, particularly in the α chains. Unfortunately, the sequence information provides little information as to why 1E7 is "resistant" to the K66A mutation. It is intriguing that 1E7 can tolerate the loss of the charge at position 66 when lysine is substituted with alanine, yet not with leucine, valine, or asparagine. Perhaps one or both of the receptors on 1E7 accommodate an alanine substitution through structural plasticity, similar to how the A6 TCR accommodates a valine → arginine substitution at position 7 of the Tax peptide (24).

From the observation that 1E7 is resistant to the K66A mutation, which abolishes reactivity for nearly all other HLA-A2-restricted receptors, one might conclude that the elements encoding MHC restriction can vary with the TCR CDR loop sequence. Further experiments to clarify the origin of K66A resistance for the 1E7 clone and to determine whether it is related to the existence of two productively rearranged α chains are currently underway.

Finally, as shown in Fig. 2, the Lys66-peptide hydrogen bond and Lys66-Glu63 ion pair are conserved in all known peptide-HLA-A2 structures (32, 33, 45–56), and it is reasonable to expect that Lys66 will influence to some extent the interaction of most peptides with HLA-A2. Lysine 66 is thus involved in the dual recognition of both peptides and T cell receptors. It is tempting to speculate that this provides a link between peptide selection by the MHC and subsequent TCR selection by the peptide-MHC complex. Non-anchor peptide positions that simultaneously influence both peptide and TCR binding have been found in other class I systems (19, 55), and solvent-exposed potential TCR contact positions have been shown to influence peptide binding in a class II system (44). However, whether this dual recognition is physiologically relevant in terms of simultaneous peptide and T cell receptor selection by the MHC molecule is unknown. Further studies of the relation-
Strategic Mutations in HLA-A2 Affect TCR and Peptide Binding

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Strategic Mutations in the Class I Major Histocompatibility Complex HLA-A2 Independently Affect Both Peptide Binding and T Cell Receptor Recognition

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J. Biol. Chem. 2004, 279:29175-29184.
doi: 10.1074/jbc.M403372200 originally published online May 6, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M403372200

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