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_J Immunol_ 2001; 166:3994-3997; doi: 10.4049/jimmunol.166.6.3994
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A Structural Difference Limited to One Residue of the Antigenic Peptide Can Profoundly Alter the Biological Outcome of the TCR-Peptide/MHC Class I Interaction

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The vesicular stomatitis virus (VSV) octapeptide RGYVYQGL binds to H-2Kb and triggers a cytotoxic T cell response in mice. A variant peptide, RGYVYEGL (E6) with a glutamic acid for glutamine replacement at position 6 of the VSV peptide, elicits a T cell response with features that are quite different from those elicited by the wild-type VSV peptide. The differences found in the nature of the T cells responding to the E6 peptide include changes in both the Vβ elements and the sequences of the complementarity-determining region 3 loops of their TCRs. Further experiments found that the E6 peptide can act as an antagonist for VSV-specific T cell hybridomas. To determine whether these differences in Vβ usage, complementarity-determining region 3 sequences, and the switch from agonism to antagonism are caused by a conformational change on the MHC, the peptide, or both, we determined the crystal structure of the variant E6 peptide bound to H-2Kb. This structure shows that the only significant structural difference between H-2Kb/E6 and the previously determined H-2Kb/VSV is limited to the side chain of position 6 of the peptide, with no differences in the MHC molecule. Thus, a minor conformational change in the peptide can profoundly alter the biological outcome of the TCR-peptide/MHC interaction.

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The Journal of Immunology, 2001, 166: 3994–3997.

In an attempt to explore the structural basis of Ag recognition by cytotoxic T cells, recent studies have focused on an increasingly detailed examination of the interactions between TCR and peptide/MHC class I (pMHC) complex, including those comprised of altered peptide ligands (1–3). Using the H-2Kb/vesicular stomatitis virus (VSV) complex as a model system, we have explored the biological effect of amino acid substitutions of both peptide and MHC residues that are likely to interface with the TCR. We have shown that the Q→E substitution at position 6 (E6) of the naturally occurring VSV peptide, can profoundly alter, both in vitro and in vivo, T cell response. Analysis of the TCR-pMHC binding patterns of a set of T cell clones specific for the VSV peptide showed that the Q→E substitution alters the interface of TCR binding to the MHC compared with the wild-type VSV peptide (4). Further, using mice with a transgenic TCR α-chain, Wang et al. (5) have shown that immunization with E6 elicits T cells with TCRs containing different complementarity-determining region (CDR)-3β motifs than the TCRs on T cells arising after immunization with VSV peptide. In addition, when the Vβ element usage of these peptide-specific T cell populations was analyzed, it was found that E6 peptide immunization induced a different TCR Vβ usage than that found in VSV peptide-immunized mice (6). Thus, the Q→E substitution at position 6 of the peptide results in significant differences in the nature of the TCRs of the responding T cell populations. In addition to these findings, we have recently observed that the E6 peptide is able to antagonize an H-2Kb/VSV-specific TCR (N30.7), indicating that this alteration in the VSV peptide sequence is enough to inhibit the recognition of the wild-type peptide by this particular TCR (7).

Given the significant biological effects of the Q→E substitution on the TCR structure and function, we decided to investigate whether these functional alterations in the T cell responses were due to structural changes on the heavy chain of the H-2Kb molecule induced by this peptide variant. With this aim, we have determined the crystal structure of the H-2Kb/E6 complex and compared it with the H-2Kb/VSV structure. We found that the Q→E substitution at position 6 of VSV peptide resulted in structural changes limited to the peptide and that did not involve the heavy chain of the MHC molecule. Thus, subtle conformational changes in the ligand can cause profound alterations in the type of T cell response triggered by the TCR-pMHC interaction.

Materials and Methods

Synthetic peptide production

E6 (RGYVYEGL) was synthesized by the Peptide Synthesis Facility of the Albert Einstein College of Medicine. The peptide was purified to >98% homogeneity by reversed-phase HPLC and the identity of the peptide was confirmed by mass spectrometry.

Production, folding, and crystallization of H-2Kb/E6 complex

As previously described by Zhang et al. (8), transformed BL21 (DE3)pLysS cells were grown in Luria-Bertani medium, lysed with a French press, and then processed by several cycles of centrifugation and washing, resulting in >95% pure inclusion bodies. The H-2Kb and β2-microglobulin (β2-m) inclusion bodies were solubilized in a buffered urea

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0022-1767/01/$02.00

Received for publication November 6, 2000. Accepted for publication January 2, 2001.

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1 This work was supported by National Institutes of Health Grants RO1 AI07289-32, ST52CA09173-23, RO1 AR42533-5, RO1 AI41240, and The Welch Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 Abbreviations used in this paper: pMHC, peptide/MHC class I; VSV, vesicular stomatitis virus; CDR, complementarity-determining region; β2-m, β2-microglobulin.
solution. Peptide, H-2K\(^b\), and \(\beta_m\) were mixed in a molar ratio of 10:1:1 at an overall concentration of 0.3 mg/ml; the mixture was dialyzed against aqueous buffer in 500 MWCO dialysis. After dialysis, the material was concentrated and then purified on an ion exchange column. The homogeneity and molecular mass of the complex was assayed by gel exclusion chromatography (8, 9). Conditions for crystallization were similar to those previously established by Zhang et al. (8): using the hanging drop vapor diffusion method with a reservoir buffer of 0.1 M calcium acetate/0.1 M cacodylate buffer (pH 6.5) 15–20% and polyethylene glycol 8000.

**Data collection, molecular replacement, and refinement**

X-ray data was collected at the X-9B beamline at Brookhaven National Laboratories Synchrotron Light Source on a Mar345 image plate system using a single crystal at \(-165^\circ\text{C}\) (Brookhaven Instruments, Holtsville, NY). Data were processed with Denzo and Scalepack. The crystal belongs to the space group \(P_{2_1}2_1\) (unit cell \(a = 89.3 \text{ Å}, b = 82.3 \text{ Å}, c = 66.4 \text{ Å} \) and \(\beta = 111.0^\circ\)) containing two pMHC complexes per asymmetric unit. See Table I for dataset statistics.

Although previous H-2K\(^b\) structures with a \(P_{2_1}\) spacegroup and very similar unit cell parameters have been reported, this crystal form has substantially different packing, necessitating molecular replacement. The search model used was the previously published H-2K\(^b/\beta_m\) with the peptide coordinates removed (10). Using data to 4 Å, AmoRe (11) located unambiguous rotation and translation solutions with a correlation coefficient of 31.8. This result was accepted and a second H-2K\(^b/\beta_m\) complex was introduced. Rotation and translation solutions were acquired, bringing the correlation coefficient to 51.8 and the \(R\) factor to 44.8%. AmoRe rigid body refinement reduced the \(R\) factor to 36.7%.

X-Plor version 3.851 (12) was used to refine the structure through multiple cycles of model building. A random set of 5% of reflections were sequestered from further refinement and used for calculation of \(R_{free}\) (1735 reflections for data to 2.3 Å). Bulk solvent corrections were applied to the Fo throughout the refinement (13). Data were gradually extended to 2.3 Å with a \(F_o/F_c\) cutoff of 2.0, and individual atomic B factors were used at the end of the refinement process. Areas with poor geometry were fit using simulated annealing omit maps. Although clear electron density for peptide could be seen from the initial stages of model building, peptide residues were not included until the final stages of refinement.

Because the \(F_o\) was noted to be somewhat anisotropic, an overall anisotropic B factor correction (\(B_1, B = 21,094, B_{33} = 9,853, B_{33} = 21,044\)) was applied, resulting in a reduction of the \(R_{free}\) by 1.5% and a significant improvement in electron density maps. The crystal form has two pMHC complexes per asymmetric unit; these complexes are quite similar, with Cα carbon RMS deviation for the Ag presentation domains of 0.61 Å. RMS deviation between the two peptides is 0.377 Å. There was no use of non-crystallographic restraints during any part of the refinement. One complex contains a peptide with a higher average B factor than the other (71 Å\(^2\) vs 46 Å\(^2\)). Average B factors for the two heavy chains are more similar (53 Å\(^2\) vs 59 Å\(^2\)). Differences in average peptide B factor may be due to crystal packing, but omit electron density maps (calculated from randomized coordinates to reduce model bias) of both peptides are of high quality.

**Results and Discussion**

Residue 6 of the peptide is positioned differently in H-2K\(^b\)/VSV and in H-2K\(^b\)/E6

To evaluate the structural changes that are responsible for the different biological outcomes that result from the Q to E substitution at position 6 of the VSV peptide, H-2K\(^b/\beta_m/E6\) was folded, crystallized, and its structure was determined to 2.3 Å by x-ray crystallography. This crystal structure was compared with the previously reported H-2K\(^b/\beta_m\)/VSV structure with the same space group (10), demonstrating that the glutamic acid at position 6 of the E6 peptide (Fig. 1, A and B) is positioned differently in the cleft than the glutamine of position 6 in the VSV wild type. The glutamic acid side chain of the E6 peptide is \(-\sim\) 1.5 Å closer to the \(\alpha\) helix than the position 6 glutamine of the wild-type VSV peptide; this orientation is conserved in both complexes of the asymmetric unit (see Fig. 1, C and D). Although shifted, position 6 of E6 remains solvent exposed and a likely TCR contact residue. This structural difference is attributable to the hydrogen bond observed between the NE2 atom of the Q6 residue and the glutamic acid at position 152 on the \(\alpha\) helix of the H-2K\(^b\) molecule. The negatively charged side chain at position 6 of the E6 peptide no longer hydrogen bonds to the E152 residue of H-2K\(^b\) (Fig. 1, C and D). The position and conformation of other nearby residues in the peptide and the Ag-binding groove are not significantly altered by the Q\(\rightarrow\)E replacement, including the position of E152. In both the H-2K\(^b/E6\) and H-2K\(^b/VSV\) complexes, the carboxylate of E152...
forms a salt bridge with the positively charged side chain of R155, a nearby amino acid on the α2 helix; this may account for the relative immobility of residue 152 compared with the position 6 of the peptide. Thus, the only significant conformational difference between the two structures attributable to the Q→E substitution is limited to position 6 of the peptide. No other structural differences attributable to the substituted amino acids are noted. The Q→E replacement does alter the electrostatic surface of the molecule, and this electrostatic change is likely the only other significant difference between the two structures.

In TCR-α transgenic mice immunized with the VSV peptide, position 98 of the CDR3β loop of TCRs expressed on VSV-specific T cells contained either a valine or threonine residue (5). In contrast, immunization with the E6 peptide induced the expansion of T cells containing positively charged amino acids at position 98 of the TCR CDR3β loop. This compensatory charge at position 98 in the CDR3β sequence appears to be required for specific recognition of the of the E6 peptide, adding further support to the notion that position 6 of the VSV peptide is a critical contact for TCR recognition.

To further evaluate the role of the antigenic peptide structure in controlling the nature of the responding TCR repertoire, the Vβ family usage in CD8⁺ T cell populations obtained from TCR-α transgenic mice immunized with VSV or E6 peptide was analyzed (6). Thus, in VSV peptide-immunized mice, the predominant TCR Vβ gene element in CD8⁺ CTL populations was Vβ13 (80%) (Fig. 2A). This Vβ element represented <5% of CD8⁺ T cells obtained from naive TCR-α transgenic mice (p < 0.0005). No significant expansion of any other Vβ element was observed (6). However, when TCR-α transgenic mice were immunized with the E6 peptide variant, the predominant Vβ gene element expressed by CD8⁺ T cells was Vβ7 (80%) (Fig. 2A). This Vβ family was significantly expanded compared both with naive and VSV-immunized mice (p < 0.0005). The alteration in the TCR Vβ usage caused by the Q to E substitution at position 6 of VSV could be the result of a direct interaction between that position of the peptide and the germline-encoded CDR loops of the TCRs from the T cell clones expanded after peptide immunization. This is noteworthy, because Ag recognition has been perceived as a CDR3-mediated event, whereas the germline-encoded CDR1 and CDR2 loops have been considered as being important for the MHC restriction aspect of recognition (14). However, the crystal structure of TCR-pMHC complexes have shown that both CDR1α and CDR1β are likely to contact residues on the N- and C-terminal half of the peptide, respectively, indicating that CDR1 loops could be involved in the recognition of specific peptides (15–17). Because our data indicate that the Q→E substitution affects only the peptide bound to the H-2Kb groove, it seems unlikely that the heavy chain of the H-2Kb molecule could play a role in the Vβ usage changes induced by the Q to E substitution at position 6 of VSV. This observation supports the notion that the Vβ usage change caused by the Q→E replacement would be necessitated by an interaction between the position 6 side chain of the E6 peptide and the TCR CDR1β loop of the responding CD8⁺ T cell (6).

To examine whether the E6 peptide had other effects than those already described, we tested whether the E6 peptide was able to specifically antagonize H-2Kb/VSV-specific N30.7 TCR (7). Ag prepulse antagonism assays have demonstrated that the E6 peptide specifically antagonizes recognition of VSV wild-type peptide by N30.7 TCR. This inhibition was not due to competition for binding to H-2Kb molecules, because a Sendai virus-derived peptide that binds to H-2Kb did not antagonize recognition of VSV by N30.7 TCR (Fig. 2B) (7). Furthermore, the E6 peptide did not antagonize another H-2Kb/VSV-specific TCR, N15 (Fig. 2B). This observation further supports the E6 antagonism of N30.7 as a specific process and not simply due to competition between the VSV and E6 peptides for binding to the H-2Kb-binding groove.

Our observation that the E6 peptide antagonizes the H-2Kb/VSV-specific N30.7 TCR indicates that structural changes limited to a single position of the antigenic peptide are sufficient to cause TCR antagonism. Two previous studies in which the structures of MHC molecules complexed with antagonist peptides carrying single amino acid replacements were compared with the wild-type agonist peptide showed minor structural changes in MHC residues (18–21). In agreement with our findings, those studies indicated that alteration of TCR contact residues results in only a small conformational change of the TCR-binding surface of the pMHC complex. In addition to providing evidence that TCR antagonism can
be caused by limited changes in conformation and charge of peptide residues that contact the TCR, our study indicates that these minor conformational changes could also alter the TCR repertoire of the entire CD8^+ T cell population in response to peptide immunization. Moreover, the limited conformational changes on the TCR-binding surface of the pMHC complex caused by the Q->E substitution of position 6 of the VSV peptide also support the notion that alterations in the recognition contacts of T cell clones (4, 22) caused by single amino acid replacements in the antigenic peptide are unlikely to be due to a major structural rearrangements of the pMHC complex.

Considered together, the biological and structural observations presented here indicate that subtle structural changes in the pMHC complex can lead to profound changes in the biological response of T cells. The molecular understanding of the interactions controlling the T cell response is important for the design of strategies aimed to manipulating immune responses.

Acknowledgments
We thank Drs. Steve Almo, Anne Davidson, Betty Diamond, and Matthew Scharff, as well as Matthew Roden for critical reading of the manuscript. We also thank Edith Palmieri for folding and purification of H-2Kb/E6 and Marie Muranelli for secretarial assistance.

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