T Cell Receptor (TCR) Recognition of MHC Class I Variants: Intermolecular Second-site Reversion Provides Evidence for Peptide/MHC Conformational Variation

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Brief Definitive Report

Summary

We investigated mechanistic differences in antigen presentation between murine MHC class I variants H-2Kb and H-2Kbm8. H-2Kbm8 differs from H-2Kb by four residues at the floor of the peptide-binding site, affecting its B pocket which interacts with the second (P2) residue of the peptide. The rest of the molecule, including the T cell receptor (TCR)-contacting residues, is identical to H-2Kb. Due to this variation, CTLs that recognize the ovalbumin257_264 and HSV gB498_505 peptides on H-2Kb cannot recognize them on H-2Kbm8. This could be due to impaired peptide binding or an altered peptide:H-2Kbm8 conformation. Peptide binding studies ruled out the first explanation. Molecular modeling indicated that the most obvious consequence of amino acid variation between peptide/H-2Kb and peptide/H-2Kbm8 complexes would be a loss of the conserved hydrogen bond network in the B pocket of the latter. This could cause conformational variation of bound peptides. Intermolecular second-site reversion was used to test this hypothesis: P2-substituted OVA and HSV peptides, engineered to restore the hydrogen bond network of the B pocket, were the only ones which restored CTL recognition. These results provide a molecular understanding of peptide/MHC conformational variation.

MHC class I molecules are the most polymorphic molecules in mammals (1). This polymorphism can influence T cell recognition by: (a) changing the class I surface that directly interacts with the TCR; (b) changing a set of peptides that can be presented to the TCR; and (c) binding the same peptide(s) in a different conformation. While the first two mechanisms are well understood (2-4), the third one is still obscure. In theory, conformational variation could work as follows: MHC alleles X and Y differ from each other only at peptide-binding residues, and both can bind peptide A. As a consequence of differential interactions in the peptide-binding site, A/X and A/Y would structurally differ from each other. Some of this variation may be accessible to the TCR, and TCRs that recognize A/X may not be able to recognize A/Y. Studies using MHC or peptide variants invoked conformational variation as a cause for altered T cell recognition whenever peptide: MHC binding appeared to be intact (reviewed in reference 5). One study implicated conformational variation as a major cause for alloreactivity (6): the same HPLC fraction eluted from H-2Kbm8 and H-2Kb molecules, presumably containing a shared self-peptide(s), was differentially recognized by CTLs when bound to H-2Kb versus H-2Kbm8. However, neither of the above studies employed methods that could reveal the structural mechanism of variation. To address this issue, we used chemically, and, in some cases, crystallographically, defined peptides and natural MHC class I variants, and the technique of intermolecular second-site reversion.

Materials and Methods

Mice, Immunization and 51Cr-release Assay. C57BL/6 (B6, H-2b) and B6.C-H-2bm8 (bm8, H-2bm8) mice were obtained from the NCI (Frederick, MD) and Dr. J. Sprent (The Scripps Institute, La Jolla, CA), respectively. The latter were bred at the MSKCC vivarium. Peptide and virus immunization, restimulation, and 51Cr-release assay were performed exactly as described (7).

Peptides and the Peptide:MHC Binding Assay. Peptide-MHC binding was quantified by determining relative expression of sti-
Results and Discussion

**T Cell Recognition of HSV and OVA Peptides Complexed to K^b and K^{bms}.** When immunized with intracellularly loaded ovalbumin or live virus, or with OVA or HSV glycoprotein B_{p88-505} peptides, B6 mice mount CTL responses directed against naturally processed epitopes SIINFEKL (OVA) and SSIEFARL (HSV), presented by H-2K^b (7, Table 1). These two peptides share the same principal anchor residues, in positions five and eight (P5F and P8L), and a medium-size residue at P2 (I and S, respectively). P5F and a medium-size residue at P2 (in contrast to tyrosine [P5Y] and a small P2 residue in H-2K^b-binding peptides VSV [RGYVYQGL] and SEV [FAPGNYPAL]) characterize the “OVA” submotif of H-2K^b-binding peptides (11). Peptides with this submotif contact the central H-2K^b pocket C by P5F, but, unlike the VSV and SEV peptides, also interact with pocket B via P2 (11-13). This interaction has the potential to affect T cell recognition (14, 15).

B6 anti-OVA CTLs cannot recognize OVA when presented by H-2K^{bms} (5, 16, 17, Table 1). HSV, another peptide with the “OVA” submotif, also failed to be recognized when presented by H-2K^{bms} (Table 1). H-2K^{bms} differs from H-2K^b by three β-strand substitutions, Y22→F, M23→I, and E24→S, and a loop substitution A30→N (18), all located on the floor of the peptide binding site (11, 19). Crystal structure analysis (11, 20) and site-directed mutagenesis (21, 22) have shown that the mutations Y22→F and E24→S occur in the pocket B of the peptide binding groove, where they may directly affect peptide binding, but have no solvent accessibility and thus are predicted not to interact directly with the TCR. Residues 23 and 30 point away from the peptide binding groove and are functionally irrelevant for peptide binding and TCR recognition (12, 20, 21).

**Impaired Peptide Binding Does Not Account for the Lack of OVA/K^{bms} and HSV/K^{bms} Recognition.** The inability of H-2K^{bms} to present OVA and HSV could be explained by: (a) significant H-2K^{bms}-induced, peptide-independent, structural changes that alter TCR contact; (b) impaired peptide binding; or (c) subtle, peptide-dependent local conformational variation of the peptide/MHC complex. H-2K^b and H-2K^{bms} are indistinguishable as assayed by a panel of conformation-sensitive mAb, directed against the α-helical regions of the α1 and α2 domains (5, 16). Furthermore, H-2K^{bms} can present other peptides (e.g., the VSV and SEV peptide) to B6 CTLs (10, R. Dyall, unpublished data). These findings argue strongly against the first possibility.

Binding of OVA and HSV peptides to H-2K^b and H-2K^{bms} was tested using a class I-stabilization assay (8, Fig. 1). Identical results were obtained using a modified stabilization assay (not shown), that took into account the peptide off-rates (by thoroughly washing the peptide away before a 4-h incubation at 37°C). The results were, therefore, representative of the binding capacity of peptides tested. HSV bound equally well to H-2K^b and H-2K^{bms} (Fig. 1, a and b). OVA did not bind to H-2K^{bms} as well as to H-2K^b (Fig. 1, c and d). However, impaired binding alone could not explain the lack of OVA/K^{bms} recognition, because even at the OVA concentration which produced substantial H-2K^{bms} stabilization (10^{-6} M, Fig. 1 d), there was still no recognition of this complex (Table 1). These results, therefore, rule out the second explanation.

**Could a local structural variation explain the phenotype of H-2K^{bms}?** Crystal structures of H-2K^b complexed to

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**Table 1. Second-site Reversion of the H-2K^{bms} Presentation Defect by Substituted Peptides**

| Strain | Immunization | Targets | % 51Cr-release (−log M peptide concentration) |
|--------|--------------|---------|---------------------------------------------|
|        |              |         | 6  | 8  | 10 | 12  |
| B6     | OVA          | K^b/OVA | 77 | 70 | 79 | 55  |
|        |              | K^b/O2E | 6  | 4  | 7  |      |
|        |              | K^b/O2A | 71 | 75 | 50 | 40  |
|        |              | K^b/O2D | 4  | 3  | 1  | 0   |
|        |              | K^{bms}/OVA | 0 | 0  | 0  |      |
|        |              | K^{bms}/O2E | 42 | 28 | 3  | 0   |
|        |              | K^{bms}/O2A | 11 | 0  | 0  |      |
|        |              | K^{bms}/O2D | 7  | 2  | 0  |      |
| B6     | HSV          | K^bHSV  | 71 | 70 | 46 | 16  |
|        |              | K^b/H2E | 8  | 5  | 2  | 1   |
|        |              | K^{bms}/HSV | 1 | 0  | 1  | 0   |
|        |              | K^{bms}/H2E | 42 | 40 | 17 | 2   |
OVA (Fig. 2 a, reference 11), VSV and SEV (12) show that all three complexes share a conserved hydrogen bond network in the B pocket, and the HSV/Kb complex is likely to preserve it (Fig. 2 b). The substitutions Y22→F and E24→S in H-2Kbm8 would be predicted to alter the conserved hydrogen bonding network (see a naive model of Kbm8-HSV, Fig. 2 c). We conclude that OVA/H-2Kbm8 and HSV/H-2Kbm8 complexes are unlikely to be isostructural with their H-2Kb counterparts. By methods employed here, it is not possible to determine which peptide and/or MHC residues would move. However, likely candidates for this predicted structural variation include the TCR-accessible backbone atoms of peptide residues P1-P4, as well as TCR-accessible MHC residues K66 and N70 which sit atop the B pocket.

**Intermolecular Second-site Reversion Restores CTL Recognition and Provides Evidence for Conformational Variation.** A specific prediction of this model is that the reconstitution of the hydrogen bond network in the B pocket may restore TCR recognition of peptide/Kbm8. To test this prediction, we adapted a genetic technique of second-site reversion (23, 24), whereby protein (usually enzyme) point-mutants are functionally rescued by spontaneous mutations at a distant (second) site, providing information on functionally important intramolecular interactions. We used substituted peptide as an intermolecular second site. If we were correct, re-introduction of a peptide side chain of medium size, which could participate in the B pocket hydrogen bonding network, might restore the recognition of peptide/Kbm8 complexes. Of several substituted OVA and HSV peptides, those bearing glutamic acid at P2 (O2E and H2E) were recognized by B6 CTLs (Table 1, Fig. 3, and not shown). The second-site effect depended on the position, the size and the nature of peptide substitution. Other P2 variants, with smaller acidic (O2D) or medium (OVA wt, H2I) or small (O2A) hydrophobic side chains, or a small hydrophilic side chain which could not form hydrogen bonds with MHC side chains in the B pocket (O2S) could not rescue recognition (Table 1; not shown). It could be argued that the second-site reversion may not strictly depend on the B pocket hydrogen bond network, and that it could be achieved by other substitutions in the peptide as well. We did not test all possible peptide variants, and thus cannot formally exclude this possibility. However, we consider it highly unlikely, for two reasons. First, neither of several TCR-exposed or buried substitutions tested (O4R, O2E4E, O7E, O7Q, O4E, O5Y, H5Y, and H7K) could restore recognition (not shown). Second, OVA peptides substituted at all buried or all exposed residues with amino acids from the VSV peptide (SGYNYEKL and RIIVFQGL, respectively) also failed to restore recognition (not shown). These results strengthen the conclusion that the original Kbm8 mutation and the second-site peptide substitution have to be spatially close and likely interact directly.

The rescue of recognition was obtained with mono- (Fig. 3 A), oligo- (Fig. 3 B) or polyclonal (Table 1) CTLs, indicating that reversion occurs for the majority of αOVA and αHSV CTLs, and not just for a few clones. This was reminiscent of the effect of H-2Kbm8 on OVA presentation and positive selection, which affect the entire CTL repertoire (16, 17). The rescue of recognition for long-term CTLs (Fig. 3) was more efficient than for “primary” CTLs (Table 1), where E/Kbm8 was recognized only at high peptide concentrations (10-8, as opposed to 10-12 for wt/Kb), and resulted in lower lysis levels. We explain this discrepancy by differential affinity of primary versus long-term
Conformational Variation Best Explains the Effects of H-2Kbm8 Mutation and of Second-site Revertant Peptides on T Cell Recognition. We next tested whether the gain in recognition might be explained by improved binding of P2 variant peptides to H-2Kbm8. Both wild-type peptides, as well as the conservative substitution O2A, bound strongly to H-2Kb, while O2E, H2E, and O2D bound poorly, probably owing to unfavorable steric and electrostatic interactions of their acidic P2 residues with MHC24E in the B pocket. O2E stabilized H-2Kbm8 similarly or slightly worse than OVA and O2A (Fig. 1 d), but was nevertheless recognized by OVA/H-2Kb-specific CTLs, while OVA and O2A were
not (Table 1, Fig. 3 A). The lack of recognition of OVA/H-2Kb8 therefore, may be explained by peptide:MHC structural variation. Likewise, since HSV and H2E stabilized H-2Kb8 equivalently (Fig. 1 b), conformational variation most plausibly explains why only the H2E/H-2Kb8 complex was recognized (Table 1 and Fig. 3 B).

The side chain of P2 is buried in the groove, is inaccessible to the TCR, and therefore is unlikely to influence TCR recognition directly (12, 25). Recognition of the 2E/H-2Kb8 complexes by H-2Kb-restricted CTLs implies that these complexes could assume conformations similar to that of the wt peptide/H-2Kb complexes. Indeed, computer modeling predicts that a single amino acid change of P2 to E in HSV and OVA has the potential of creating H-2Kb8 complexes which are nearly isostructural to that of HSV/H-2Kb and OVA/H-2Kb, owing to the restoration of an extended hydrogen bonding network in the B pocket (Fig. 2 d). Thus both the inability of H-2Kb8 to efficiently present OVA and HSV peptides to H-2Kb restricted CTLs, and the rescue of this presentation by second-site 2E revertants, can be explained by altered physicochemical interactions in pocket B, which in turn affect the TCR-interacting surface of the peptide:MHC complex. A similar situation was found for the T4 phage lysozyme (23), where intramolecular second-site revertants compensated for the loss of hydrogen bonds via new hydrogen-bonding interactions. By contrast to OVA and HSV, VSV and SEV peptides are presented by H-2Kb8 (17, and R. Dyall, unpublished observation), since the side chains of these peptides do not interact with the B pocket, they may be less affected by hydrogen bond network changes. Thus, the loss of a B pocket hydrogen bond network would influence TCR recognition in a peptide-specific manner.

Recent findings have indicated that the MHC class I:peptide interaction is not static. MHC side chains have been observed to adopt different rotamers when associated with different peptides (11, 12, 26, 27). Peptide-associated changes in the class I:peptide complex could also be detected by monoclonal antibodies directed at different parts of the α1 and α2 domains (28–30), and some may have been induced by indirect, conformational effects (30). Consistent with the above reports, and the one on the role of conformational variation in alloreactivity (6), our results make a strong case for the influence of MHC:peptide conformational variation on TCR recognition of H-2Kb8 and the role of the B pocket hydrogen bonding network in it.

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