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Recognition of an MHC Class I-Restricted Antigenic Peptide Can Be Modulated by para-Substitution of Its Buried Tyrosine Residues in a TCR-Specific Manner

Naoyuki G. Saito,* Hsiu-Ching Chang, † and Yvonne Paterson‡

Conformational dependence of TCR contact residues of the H-2Kb molecule on the two buried tyrosine side chains of the vesicular stomatitis virus (VSV)-8 peptide was investigated by systematic substitutions of the tyrosines with phenylalanine, p-fluorophenylalanine (pFF), or p-bromophenylalanine (pBrF). The results of peptide competition CTL assays revealed that all of the peptide variants, except for the pBrF analogues, had near-native binding to the H-2Kb molecule. Epitope-mapped anti-H-2Kb mAbs detected conformational differences among H-2Kb molecules stabilized with these VSV-8 variants on RMA-S cells. Selective recognition of the VSV-8 analogues was displayed by a panel of three H-2K b-restricted, anti-VSV-8 TCRs. Thus, these substitutions result in an antigenically significant conformational change of the MHC molecular surface structure at both C and D pockets, and the effect of this change on cognate T cell recognition is dependent on the TCR structure. Our results confirm that the structure of buried peptide side chains can determine the surface conformation of the MHC molecule and demonstrate that even a very subtle structural nuance of the buried side chain can be incorporated into the surface conformation of the MHC molecule.

The ability of buried residues to modulate this molecular surface augments the number of residues on the MHC-peptide complex that can be recognized as “foreign” by the CD8 T cell repertoire and allows for a higher level of antigenic discrimination. This may be an important mechanism to expand the total number of TCR specificities that can respond to a single peptide determinant. The Journal of Immunology, 1999, 162: 5998–6008.

C

rystallographic analysis of the MHC class I molecule revealed that antigenic peptides bind in a groove created by a pair of antiparallel α-helices located above a β sheet in the α1 and α2 domains of the heavy chain (1, 2). This binding groove consists of six pockets, named A through F, where the amino terminus and some of the side chains of antigenic peptides can be found buried (3). Bulk sequencing analysis of peptides eluted from various MHC class I molecules gave additional insight into how a unique MHC class I molecule binds peptides of seemingly diverse sequence by selecting those with appropriate side chains that fit in allele-specific pockets (4). From these and other observations, amino acid residues whose side chains seem to occupy some of these binding pockets became known as “anchor residues,” while those solvent-exposed residues that seem to orient themselves outward from the binding groove became known as “TCR contact residues.” A number of studies, however, challenged the anchor/TCR contact residue dichotomy model. One of the first pieces of evidence came from a study in which some anchor residues of the antigenic peptide failed to target CTL lysis, despite the fact that these variants were perfectly capable of binding to the MHC molecule (5). More convincing evidence came from studies where mutations of MHC residues lining the binding pockets, or amino acid substitution of buried residues within the peptide, resulted in a serologically observable topological change in the MHC-peptide complex (6–13), as well as the findings that variations in anchor residue can influence T cell recognition (14–17).

Despite these findings, the role of buried peptide side chains on MHC molecular conformation has yet remained elusive. The most obvious reason is that all of the studies so far have employed nonconservative amino acid substitutions within the peptide sequence, mostly alanine scanning, to determine conformational effects (6–17). It is quite possible that such a drastic structural perturbation is very likely to affect the thermodynamic and kinetic parameters of the MHC-peptide interaction. Furthermore, many of these observations have been made by multiple amino acid substitutions within the peptide sequence. In this case, serologically observed differences may have been derived from structural changes within the peptide itself.

In this study, we have examined the conformational dependence of H-2Kb, a murine MHC class I molecule, on the buried side chain structure of vesicular stomatitis virus (VSV)3-8, an antigenic octapeptide derived from VSV nucleoprotein, using a panel of VSV-8-specific H-2Kb-restricted T cell clones and H-2Kb-specific mAbs. In the native peptide sequence, amino acids at the third and the fifth positions (P3 and P5) are both tyrosines. In this study, we replaced these residues with phenylalanine, p-fluorophenylalanine (pFF), or p-bromophenylalanine (pBrF). In effect, we substituted the p-hydroxyl group on tyrosines with hydrogen, fluorine, or

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3 Abbreviations used in this paper: VSV, vesicular stomatitis virus; MFI, mean fluorescence intensity; pFF, p-bromophenylalanine; pPF, p-fluorophenylalanine.
Materials and Methods

Peptide synthesis, purification, and characterization

VSV-8 peptide (RGYYYQGL), its P3 and P5 side chain variants, as well as OVA-8 peptide (SIINFEKL) were manually synthesized in our laboratory utilizing r-butyloxy carbonyl chemistry on polystyrene support resins suspended in methylene chloride inside glass reaction vessels (18), except for phenylalanine and pBrF analogues of VSV-8, which were purchased from Research Genetics (Huntsville, AL) as unpurified cleavage products. VSV-8 is the immunodominant H-2Kb-restricted CTL epitope derived from VSV (19), OVA-8 is the immunodominant H-2Kb-restricted OVA CTL epitope (20).

All peptides were purified by reverse-phase HPLC on a Vydac C18 Column (Separations Group, Hesperia, CA) using a 30-min 0–50% acetonitrile gradient in 0.1% trifluoroacetic acid. HPLC purification of the crude synthetic products yielded isolated singlet absorbance peaks, which were collected and analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Obtained mass spectra were consistent with the expected structures of the peptides. HPLC-purified peptides were stored dry at 4°C within a vacuum desiccator.

The nomenclature system for the P3 and P5 side chain analogues of VSV-8 is as follows. Designation of point substitution is in the context of the native VSV-8 sequence. The first number, either 3 or 5, identifies the position of the tyrosine whose side chain is modified. The atomic symbol (H, F, or Br) after the hyphen following the residue number identifies the nature of the substitution at the para position on the phenyl ring. For example, 3-F VSV-8 contains a pFF in place of P3 tyrosine, in the context of the native VSV-8 peptide sequence. The structures of the side chain analogues of VSV-8 are illustrated in Fig. 1.

Mice

Six- to eight-wk-old female C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in the University of Pennsylvania School of Medicine Laboratory Animal Facility (Philadelphia, PA). They were maintained and sacrificed in full accordance with the university policy on animal research.

Cell culture media

All mammalian cells were cultured in a 37°C incubator under 10% CO2 and 95% humidity. One of four growth media, DMEM (Mediatech, Hertford, VA), IMDM Mediatech, and RPMI-1641 (Mediatech, Manassas, VA), HybriCare Medium (American Type Culture Collection (ATCC), Manassas, VA), and RPMI-1640 (Mediatech, Gaithersburg, MD) were utilized for the cell culture experiments. All mammalian cells were cultured in a 37°C incubator under 10% CO2 and 95% humidity.

APC

EL4 is a dimethylbenzanthracene-induced thymoma cell line derived from a C57BL/6 mouse (21). N1 is a cell line established by stable transfection of EL4 with VSV nucleoprotein gene (22). EL4 transfectants were grown under selection with 400 μg/ml geneticin (Life Technologies, Gaithersburg, MD). EL4 and N1 were kindly provided by Dr. James M. Sheil (West Virginia University, Morgantown, WV). E.G7 was a cell line established by stable transfection of EL4 with the OVA gene (23). Both of these EL4 transfectants were grown under selection with 400 μg/ml geneticin (Life Technologies, Gaithersburg, MD). EL4 and N1 were kindly provided by Dr. James M. Sheil (West Virginia University, Morgantown, WV). E.G7 was kindly provided by Dr. Stephen C. Jameson (University of Minnesota, Minneapolis, MN). P815 is a mastocytoma cell line derived from a methylcholanthrene-induced tumor from a DBA/2 (H-2d haplotype) mouse (24). RMA is a cell line derived from RBL-5, a Rauscher virus-induced T cell line (30). All of the above cell lines were maintained in the RPMI medium, except for R8, which was maintained in the DMEM medium.

MHC class I-restricted T cell clones

The H-2Kb-restricted VSV-8-specific CD8+ CTL Clone 33 (31) was kindly provided by Dr. James M. Sheil. The H-2Kb-restricted OVA-8-specific CD8+ CTL Clone B3 (32) was kindly provided by Dr. Stephen C. Jameson. Both CTL clones were maintained with the IMDM medium within 24-well plates (2 ml/well). These CTL clones (1.0 × 105 cells/ml) were restimulated and expanded weekly with irradiated (20,000 rads) N1 cells (1.0 × 105 cells/ml) for Clone 33 and E.G7 cells (1.0 × 105 cells/ml) for Clone B3, in addition to irradiated (2000 rads) C57BL/6 whole splenocytes (5 × 105 cells/ml) and 20 μl of murine IL-2.

The H-2Kb-restricted N15 and N26 TCRs are derived from VSV-8-specific CD8+ CTL clones of the same name (33), which were originally isolated from the spleen of C57BL/6 mice inoculated with VSV (34). 58αβ, a TCR-negative, CD3-positive cell line derived from the DO11.10 T cell hybridoma subline (35) was transfected with TCR α and β-chain cDNA, as described earlier (36, 37). To allow for an effective activation of the N15 and N26 TCR transfectants by the VSV-8 peptide in complex with the H-2Kb molecule, cDNAs of the murine α-chain, CD8α, and CD8β-chains were also transfected into the recipient hybridoma line. Results of flow cytometric, quantitative immunofluorescence analyses confirmed that both N15 and N26 transfectants express comparable levels of CD8α, CD8β, and their respective TCRs (36). They were grown in the RPMI medium with 100 μg/ml of genetinidin and 200 μg/ml of hygromycin B (Boehringer Mannheim, Indianapolis, IN).

Hybridomas and mAbs

These H-2Kb-specific hybridomas were obtained from the following sources. 34-4, 205 (38) and 26-13-35 (39) were purchased from ATCC. Y-3 and Y-25 (40) were kindly provided by Dr. Charles A. Janeway, Jr. (Yale University, New Haven, CT). 5F1 (41) was kindly provided by Dr. Linda A. Sherman (Scripps Research Institute, La Jolla, CA). EH-144 (42)
was kindly provided by Dr. Stanley G. Nathenson (Albert Einstein College of Medicine, Bronx, NY). S198 (43) and K9-178 (44) were kindly provided by Dr. Ulrich Hämmerling (Memorial Sloan-Kettering Cancer Center, New York, NY). All hybridomas were grown with HyberCare-10 medium, and cell culture supernatant was used for cell surface staining. Affinity purified R-PE-conjugated goat anti-mouse IgG or IgM were also purchased from Caltag (South San Francisco, CA).

**CTL assays**

Modification of a standard \(^{51}\)Cr-release assay, as described earlier, was performed (45). Briefly, 1.0 \(\times\) \(10^6\) target cells were resuspended in 50 \(\mu\)l of aqueous 2 M\(\text{Ci/ml} \text{Na}_2\text{CrO}_4\) (NEN Life Science Products, Boston, MA) and incubated for 1 h at 37°C, under 10% CO\(_2\) and 95% humidity. Target cells were then washed twice and added to each well of a 96-well round-bottom plate at 1.0 \(\times\) \(10^4\) cells/well. Peptide was then added at the desired concentration to each well. In the case of peptide competition assays, two different peptides were added, one at a constant concentration and another at decreasing concentrations via serial dilution. Finally, an appropriate number of effector cells were added to each well to achieve the desired E:T ratio. The total volume in each well was kept constant at 200 \(\mu\)l. After 4 h of incubation, 100 \(\mu\)l of supernatant was removed from each well, and the level of \(^{51}\)Cr in each supernatant was measured with a Gamma 4000 gamma counter (Beckman Coulter, Fullerton, CA) or MicroBeta TriLux liquid scintillation and luminescence counter (EG&G Wallac, Gaithersburg, MD). Percent specific lysis was calculated using the following formula: \(\text{specific cpm} = \text{total cpm} - \text{spontaneous cpm}\).

Spontaneous cpm was determined from the supernatant of wells containing only 1.0 \(\times\) \(10^4\) labeled target cells. Total cpm was determined by lysing 1.0 \(\times\) \(10^6\) labeled target cells in aqueous 1% Triton X-100 (Sigma, St. Louis, MO). Each experiment was performed in triplicate and repeated three times on different occasions with consistent trends. One set of representative data is shown.

**RMA-S H-2K\(^b\) stabilization assays**

Slight modifications were made to an assay described earlier (46, 47). Briefly, RMA-S cells were incubated for 20 h at 27°C to induce H-2K\(^b\) expression. A total of 1 mM of each peptide was added to respective wells of a 24-well plate containing 2.0 \(\times\) \(10^3\) RMA-S cells in 2 ml final volume of the RPMI medium. After an additional 1-h incubation at 27°C, the cells were moved to a 37°C incubator. After 4 h of incubation at 37°C, the cells were washed, subdivided, and stained with an excess of the anti-H-2K\(^b\) mAb of interest. After further washing, fluorochrome-conjugated secondary Ab was applied. R-PE-conjugated goat anti-mouse IgG secondary Ab was used for all primary mAbs, except for 28,13,3, in which case anti mouse IgM was used. Cells were again washed and fixed in 0.5% paraformaldehyde. Fluorescence of stained cells was determined on a FACScan flow cytometer and analyzed with CellQuest software (Becton Dickinson Immunochemistry Systems, Mountain View, CA). Each sample contained \(5 \times 10^4\) target cells, and 30,000 events were recorded on the flow cytometer. Data were collected on a logarithmic scale, and linear mean fluorescence intensity (MFI) of each sample was obtained for further analysis. The experiments were repeated three times on different occasions with consistent trends, although actual levels of MFI differed on different days of assay. The arithmetic mean of the three sets of data are reported, and statistical analysis of MFI values were performed using the general linear model procedure available from SAS (Cary, NC) software version 6.12. Initially, group \(p\) values signifying the statistical significance of differences of MFI values among the four peptides for specified individual mAb data sets were obtained by an ANOVA appropriate for a two-factorial design (peptide and replicate) under the assumption of no second order (peptide by replicate) interaction. In the cases of mAb data sets with the statistically significant peptide group \(p\) values of \(<0.05\), pairwise comparisons between the native VSV-8 peptide and its analogues were further evaluated by Tukey’s least significant difference multiple comparison test to determine which differences contributed significantly to the overall test of the null hypothesis of no differences among peptides. Strictly for graphical purposes, individual MFI values were also represented in terms of percent relative MFI using the following formula: \(\text{MFI of VSV-8 analog} / \text{MFI of low temperature}\) - 100.

**IL-2-release assays**

Assays to determine the level of IL-2 release by N15 and N26 TCR transfectants were done using a modified version of a protocol described earlier (36, 37) using CTL2, an IL-2-responsive cell line (48–50). CTL2 was maintained in the RPMI medium supplemented with 1 U/ml of murine rIL-2 (Boehringer Mannheim).

Irradiated (3000 rads) 1.0 \(\times\) \(10^5\) R8 cells in 100 \(\mu\)l of the RPMI medium were added to individual wells in a 96-well plate with various concentrations of VSV-8 or its side chain analogues. One hour later, 1.0 \(\times\) \(10^5\) TCR transfectants in 100 \(\mu\)l of the RPMI medium with 10 ng/ml of PMA (NEN Life Science Products) were added. After 24 h, culture supernatant was removed, freeze-thawed, and serial dilutions added to 1.0 \(\times\) \(10^5\) CTL2 for 24 h. The cells were then pulsed with 0.5 \(\mu\)Ci per well of \[^{3}H\]thymidine (NEN Life Science Products), harvested, and counted. Experiments were performed in triplicate, and raw data from appropriate dilution series was used for analysis. One set of representative data is shown.

**Computational molecular modeling**

All molecular simulations were performed on an Indy Workstation (Silicon Graphics, Mountain View, CA) with 100-MHz IP 22 processor and 24-bit graphics. Using Insight II software (BIOSYM/Molecular Simulations, San Diego, California) and its Builder module, the structure of VSV-8 in complex with H-2K\(^b\) (51) was rendered based on its Brookhaven Protein Data Bank (52, 53) coordinates (filename: 2vaa). First-order models of the P3 and/or P5 analogues of the VSV-8 peptide were created by simply replacing the corresponding para-hydroxy groups with appropriate atoms. All models were constructed in the context of consistent-valence forcefield, a generalized valence forcefield (54). In the Bump module of the Insight II program, a van der Waals overlap is defined to exist when the distance between any two atoms is sufficiently less than the sum of the predefined van der Waals radii of the atoms. The extent of van der Waals overlaps were calculated as percentages of the interatomic distance. In some cases, the extent of atomic overlaps were also calculated in angstroms.

**Results**

Clone 33 recognizes 5-F VSV-8, but not 3-F VSV-8 and 3,5-F VSV-8

To define the minimum threshold for the perturbation of the H-2K\(^b\) conformation, we began our studies by examining the binding and recognition of pFF analogues of VSV-8, since pFF resembles tyrosine more than any other p-substituted phenylalanine. To study the ability of pFF substituted VSV-8 analogues to mimic the native peptide, a CTL assay was performed using EL4 cells as H-2K\(^b\)-bearing targets and Clone 33 as effector cells (Fig. 2). 5-F VSV-8, the P5 substituted analogue, was capable of targeting the EL4 cells to be lysed by Clone 33 at approximately the potency and efficacy of the native peptide. In contrast, 3-F VSV-8 and 3,5-F VSV-8, the two analogues with P3 substitution, were unable to elicit detectable levels of CTL-mediated lysis, even at very high concentrations.
Substitution of VSV-8 P3 and P5 tyrosine residues with pFF does not affect its ability to bind the H-2Kb molecule at the cell surface

To investigate the mechanism of the failure of Clone 33 to recognize P3-substituted analogues, a peptide competition assay using Clone B3, an H-2Kb-restricted OVA-8-reactive CTL clone, was performed (Fig. 3). Though there are several methods to measure the relative stability of peptides on the MHC molecule, this method is known to be one of the most sensitive available (55). VSV-8 and all three of the pFF-substituted analogues were able to compete with OVA-8 for occupancy of the H-2Kb peptide binding site at similar concentrations, suggesting a similar capacity of the analogues to bind to the MHC molecule.

Abs sensitive to changes in Pockets D and C detect pFF substitutions

To determine whether the failure of recognition of the 3-F and 3,5-F VSV-8 analogues by Clone 33 is due to a conformational perturbation of the H-2Kb molecule, we obtained a panel of mAbs specific for the H-2Kb molecule that had previously been determined to have varying degrees of tolerance to structural modifications of the MHC class I molecule induced by peptide binding. The binding sites of 5F1 and K9-178 are thought to include H-2Kb residues surrounding pocket D (the P3 side chain binding site), in addition to some spreading to pocket A (37, 44, 56). 28-13-3S is an Ab that binds residues on both α1 and α2 helices near pockets A and in close proximity to pocket D (37). Y-25 is mapped to residues adjacent to pockets C and F (44). The ability of these four mAbs to distinguish the structural differences among the VSV-8 and its pFF analogues bound to Kb was determined by RMA-S stabilization assays. All of these mAbs recognize the differences among the four peptide structures (Fig. 4); although some of the differences are small, they are significant according to a rigorous statistical analysis described in Materials and Methods (the group p values and pairwise p values are given in the figure legend). 5F1 and K9-178 showed diminished levels of recognition of P3 analogues, while recognizing the 5-F analogue at near native levels; in
contrast, 28-13-3S showed a higher than native level of recognition of P5-substituted peptides. Finally, Y-25 displayed a decreased recognition of the 3-F analogues.

Abs that bind to regions not involving pockets D and C did not detect pFF substitutions

34-4-20S and EH-144 are sensitive to specific mutations within the α1 and/or α2 domains of the H-2Kb molecule, as well as to the sequence of peptides that bind to the MHC molecule and bind to regions at opposite ends of the H-2Kb peptide binding groove (37, 57). Therefore, we used these Abs to study the extent of pFF-driven structural perturbations within the region of the H-2Kb molecule away from the peptide binding groove. Neither of these Abs detected the pFF substitutions (Fig. 5, top panels).

One possibility for the serological differences among the H-2Kb molecules stabilized with the pFF analogues of VSV-8 is that the different peptides sustain unequal levels of H-2Kb expression on the RMA-S cells. Y-3 and S19.8 were used for their generally nonselective reactivities in respect to mutations in H-2Kb or variations in peptide sequence to determine whether pFF substitution of the VSV-8 peptide results in analogues that sustain higher or lower levels of H-2Kb expression on RMA-S cells. The binding site of Y-3 has recently been mapped to an area between pockets D and F (37, 56) that is not involved in peptide binding, and it is equally reactive to H-2Kb in complex with a wide variety of peptides (47). S19.8, which is raised against the b2-microglobulin (β2m b), is reactive to all intact H-2Kb molecules (58). Binding of mAbs Y-3 and S19.8 to the pFF-substituted peptides was not significantly different to that of VSV-8 (Fig. 5, lower panels).

The effect of pFF substitution is TCR-specific

So far, we have shown that there is a >5-fold reduction in the level of recognition by Clone 33 of 3-F VSV-8 and 3,5-F VSV-8 peptides in complex with the H-2Kb molecule compared with VSV-8 (Fig. 2). In addition, we showed that pFF substitutions at both P3 and P5 result in MHC-peptide complexes with altered surface conformations recognized by a panel of Abs (Fig. 5). However, it was not clear if the serologically observed changes, especially those near pocket C induced by P5 substitution, were significant enough to influence the recognition of the MHC-peptide complex by other CTLs, and if so, whether these effects are TCR-specific.

To answer these questions, we tested these pFF analogues on TCR-transfected hybridomas that release detectable amounts of IL-2 upon antigenic stimulation. Structural information about these TCR, especially in terms of their contacts with the MHC-peptide complex, have been investigated in detail (33, 36, 37, 59, 60). In one of these studies, the effects of alanine scanning of the VSV-8 peptide on the recognition by the CTL clones that express these TCRs have been compared (59). In that study, lysis by Clone N15 was mildly (20%, <60%) affected by P3 substitution and severely (<20%) affected by P5 substitution, while lysis by Clone N26 was severely (<20%) affected by either P3 or P5 substitution.

The results of our IL-2-release assays show that the N15 transfectants responded to all pFF variants of VSV-8 in complex with the H-2Kb molecule (Fig. 6, top). Quite interestingly, 5-F VSV-8 and 3,5-F VSV-8, P5 analogues of VSV-8 and 3-F VSV-8, respectively, elicited significantly enhanced levels of IL-2. In contrast, all pFF variants of VSV-8 failed to elicit IL-2 release from N26 transfectants, while VSV-8 at concentrations 10 nM and higher elicited
The effect of para-substitution is dependent on both size and the chemistry of the substituent atom

To further analyze the mechanism by which para-substitution of P3 and P5 residues within the VSV-8 sequence could affect immunorecognition by T cells, we designed four more analogues of VSV-8, substituting P3 or P5 tyrosine with either phenylalanine (3-H and 5-H VSV-8) or pBrF (3-Br and 5-Br VSV-8).

Results of the peptide competition assay using Clone B3 indicate that the phenylalanine analogues of the VSV-8 peptide were capable of binding to the H-2K\(^b\) molecule at native levels, while the capacity of the P3 and P5 pBrF analogues to bind to the class I molecule seemed to be of order of magnitude less than that of the native peptide (Fig. 7).

Finally, we assessed the ability of these analogues to activate N15 and N26 TCR transfectants. With N15 transfectants, we saw a notably decreased level of IL-2 release elicited by the 3-H analogue, while we saw significantly enhanced levels of IL-2 release elicited by the 3-Br and 5-Br VSV-8, as described in Materials and Methods. E:T ratio = 5. Error bars indicate SEM (n = 3).

FIGURE 7. Peptide competition \(^{31}\)Cr-release CTL assay. \(^{31}\)Cr-labeled EL4 cells were incubated for 4 h with Clone B3, in presence of 50 pM OVA-8 and increasing concentrations of VSV-8, 3-H VSV-8, 5-H VSV-8, 3-Br VSV-8, or 5-Br VSV-8, as described in Materials and Methods. Error bars indicate SEM (n = 3).

In summary, we found that para-substitution of P5 tyrosine within the VSV-8 peptide with hydrogen, fluorine, or bromine (5-H, 5-F, and 5-Br VSV-8) results in N15 TCR superagonists, while the same manipulation at P3 results in subtly suboptimal agonists for the same TCR. In the case of N26 TCR, we found that para-substitution of P5 or P3 position with hydrogen, fluorine, or bromine results in a null peptide, except for 5-Br VSV-8, which elicited near native levels of IL-2 release.

Computer models of the VSV-8 analogues show negative steric effects of pBrF substitution of the P3 tyrosine

To explore the structural effects of para-substitutions on the binding mode of the VSV-8 peptide to the H-2K\(^b\) molecule, we constructed first-order models of these P3 and/or P5 analogues based on the crystallographic structure of the VSV-8 peptide with the H-2K\(^b\) molecule. First, we examined the van der Waals overlaps between the side chain atoms and the residues lining their respective pockets. At pocket C, where P5 is located, we observed no substantial (>10% atomic overlap) van der Waals interactions between the peptide and the MHC molecule, regardless of the nature of the para-substituent. On the other hand, examination of the pocket D revealed a minor van der Waals overlap between the acidic hydrogen on glutamate 152 and either hydroxyl oxygen of the native VSV-8 peptide or the p-fluorine of the pFF analogues. The bromine atom on the 3-Br analogue participates in a set of substantial overlaps with atoms from glutamate 152 and arginine 155. No substantial atomic overlap was found between the phenylalanine side chain of the 3-H VSV-8 analogue and the D pocket (Table I).

Discussion

The influence of buried peptide residues on immunorecognition

It has been known for some time that most immune receptors (both TCR and Igα) that interact with MHC class I molecules actually recognize the antigenic surface derived from the combination of the MHC molecule and the antigenic peptide (11). Also, several
studies have shown that the overall topology of the binding groove and the specificities of the individual pockets do partially determine the peptide sequence by only allowing peptides containing defined "anchor residues" located at appropriate positions along the sequence to bind to the groove (5, 34, 61–64).

There have been numerous reports showing the role of buried peptide residues with respect to overall MHC structure and Ag presentation. Crystallographic structures of H-2Kb in complex with two different viral peptides have revealed that glutamine 152 and arginine 155, residues forming the D pocket, adopt alternate conformations in response to the nature of the bound peptide (51). Other studies showed that a change in these buried side chains of the peptide could affect the surface topology of the MHC molecule (6, 7, 9, 10, 65). Furthermore, recent reports of structural and biochemical analysis of soluble, peptide free MHC class I molecule (66), as well as the measurement of kinetic and thermodynamic constants of interaction with an antigenic peptide (67), suggest that this process of conformational change can be analyzed quantitatively. In addition, this anchor side chain-induced effect is implicated in the process of peptide-induced CTL antagonism (68–71).

In this study, we show that the conformation of TCR contact surface within the H-2Kb molecule is exquisitely dependent on the structure of buried peptide side chain structure and results in immunologically significant modulation of cognate T cell responses. Implicit in our discussion of the differences in recognition of the para-substituted analogues is that the subtle changes in peptide structure invoked by changing the atomic radius of the aromatic substitutions at P3 and P5 do not radically change the peptide backbone nor the residues that occupy the peptide binding pockets. In other words, we assume here that the P3 side chains are all bound to pocket D and the P5 side chains remain bound to pocket C, regardless of the para-substitutions.

The comparison of tyrosine with p-substituted phenylalanines

To interpret our results, we must consider the structural differences between tyrosine, the P3 and P5 residue within the native VSV-8 peptide, and the various p-substituted analogues of phenylalanine of which pFF most closely resembles tyrosine. When covalently bonded to a phenyl ring, the fluoro group has an atomic radius of ~1.73 Å, which is very close to that of the hydroxyl group, which is ~1.61 Å, according to the consistent-valence forcefield scheme (54). Thus, pFF is a structural analogue of tyrosine that should have minimal steric perturbation. In general, the hydroxyl group on a tyrosine phenyl ring is highly electron-donating due to its resonance effect, while the fluorine on the pFF phenyl ring is

Table I. Van der Waals interactions of the P3 side chains from VSV-8 and its para-substituted analogues with the H-2Kb residues forming the D pocket

<table>
<thead>
<tr>
<th>Peptide</th>
<th>P3 Atom</th>
<th>H-2Kb Atom</th>
<th>Overlap (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV-8</td>
<td>p-Oη</td>
<td>Glu152: Hε2</td>
<td>0.33 (13.5%)</td>
</tr>
<tr>
<td>3-H VSV-8</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-F VSV-8</td>
<td>p-F</td>
<td>Glu152: Hε2</td>
<td>0.28 (11.7%)</td>
</tr>
<tr>
<td>3-Br VSV-8</td>
<td>p-Br</td>
<td>Glu152: Oε2</td>
<td>0.90 (27.3%)</td>
</tr>
<tr>
<td></td>
<td>p-Br</td>
<td>Arg155: Nε</td>
<td>0.69 (20.6%)</td>
</tr>
<tr>
<td></td>
<td>p-Br</td>
<td>Arg155: Cζ</td>
<td>0.83 (23.7%)</td>
</tr>
<tr>
<td></td>
<td>p-Br</td>
<td>Arg155: Nγ2</td>
<td>0.58 (17.3%)</td>
</tr>
</tbody>
</table>

*These contacts and the overlap values were calculated using Insight II software, as described in Materials and Methods.
weakly electron-withdrawing due to its inductive effect. Due to this effect, hydrophobicity of the phenyl ring on pFF is roughly similar to that of unsubstituted phenylalanine. Therefore, pFF can be considered as a hydrophobic analogue of tyrosine. In some model systems, this relative hydrophobicity effect allows the pFF side chain to bind more tightly to some hydrophobic ligands (72, 73). Like all other halogens, bromine is also a weakly electron-withdrawing phenyl substituent. However, bromine is a large atom, with an atomic radius of 2.13 Å. Therefore, pBrF side chain can be considered to be a phenylalanine-like hydrophobic phenyl ring with a bulky p-substituent slightly larger than that of a methyl group (1.95 Å).

The ability of a panel of TCRs to detect pFF at P3 and/or P5

Our first set of experiments showed that Clone 33, an H-2Kb-restricted CTL clone originally raised to recognize the VSV-8 epitope, could detect the presence of pFF at P3 but not at P5. To determine the effect of pFF substitution on the recognition by other TCRs, we took advantage of the better characterized N15 and N26 TCR transfectomas (36, 37). In the case of recognition of VSV-8 by N15, which is mildly and severely impaired by alanine substitution at P3 and P5 positions, respectively, the pFF substitution at P3 did not result in any observable change. However, the presence of pFF at P5 made the native peptide and the pFF analogue more effective agonists for N15, especially at suboptimal peptide concentrations. Thus, we can conclude that pFF substitution results in more limited structural perturbation than that which occurs with alanine scanning, allowing N15, respectively, to recognize these analogues. On the other hand, pFF substitution at P3 and P5 rendered the MHC-peptide complex unrecognizable by the N26 TCR at concentrations up to 1 μM. Thus, we can conclude that the conformation of the TCR contact surface of the H-2Kb molecule is, in fact, influenced by both buried peptide residues. However, there is clearly a marked difference among the TCRs in respect to their tolerance to the conformational variation of the MHC class I molecule. In addition, different MHC class I haplotypes may vary in their susceptibility to structural perturbations driven by buried peptide side chains. For example, the “anchor residues” of an H-2Dk-restricted antigenic peptide can be interchanged to increase MHC-peptide affinity without losing its ability to activate the polyclonal population of cognate CTLs (74).

The detection of buried residue-dependent variations of H-2Kb surface residues by mAbs

To further investigate the extent of MHC molecular conformational variation due to buried tyrosine side chains, H-2Kb stabilization assays on RMA-S cells were performed using a panel of mAbs that recognize the MHC molecule. We observed a consistent decrease in the level of recognition of the MHC-peptide complex by mAbs that are sensitive to changes in pocket D (SF1 and K9-178), when the H-2Kb molecules were stabilized with P3-substituted VSV-8, while we saw a pattern of slightly increased recognition by these mAbs when P5 was substituted with pFF. We also observed a decrease in recognition of 3-F VSV-8 by Y-25, a mAb that binds residues adjacent to pocket C. A serological pattern similar to the pocket D-sensitive Abs was seen in 28-13-3S, whose epitope includes part of pocket A directly adjacent to pocket D. This suggests that the pFF-induced conformational alteration could also involve those residues slightly removed from those forming the pockets. Our results obtained from mAbs mapped to areas distant from the D and C pockets showed that pFF-induced structural rearrangements are generally limited to those residues in proximity to the buried side chains.

Table II. H-2Kb residues that form pockets C and D

<table>
<thead>
<tr>
<th>Pocket</th>
<th>Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Val9, Tyr22, Asn70, Ser71, Phe74, Val97, Ser99, Gln114, Tyr116</td>
</tr>
<tr>
<td>D</td>
<td>Asn39, Ser99, Gln114, Glu132, Arg153, Tyr159</td>
</tr>
</tbody>
</table>

Compilation of H-2Kb residues that contribute to formation of pockets C and D

A hydrophobicity-based structural hypothesis

To address the mechanism by which the change in hydrophobicity of the P3 and P5 tyrosine phenyl ring could result in the effects described here, we created phenylalanine and pBrF analogues of the VSV-8 peptide. We chose these analogues to test the mechanistic model that the difference between pFF and tyrosine is mainly that of phenyl ring hydrophobicity. Although both pockets C and D of the H-2Kb molecule are formed by both polar and nonpolar residues (Table II) (51, 75), the exposed groups that line these pockets are strictly nonpolar, and there exists no hydrogen-bonding interactions between the hydrophilic groups of the peptide side chains and the MHC molecule (76, 77). Thus, it is very possible that the increased hydrophobic nature of the pFF side chain will allow these nonpolar groups to position closer to the altered phenyl ring.

If our hydrophobicity hypothesis is correct, we should see a similar, if not enhanced, effect with phenylalanine-substituted analogues, since the nonsubstituted phenyl ring is more hydrophobic than the tyrosine side chain, and the lack of p-substituent will allow for better “collapse” of the pocket residues. In fact, we observed this effect with 3-H and 5-H VSV-8. On the other hand, pBrF is substituted with a halogen atom much larger than the hydroxyl group. Thus, even though the phenyl ring on pBrF is much more hydrophobic than that on tyrosine, it is very possible that the bulky substituent will inhibit optimal binding to and/or collapse of the C and D pockets. Our observations with 5-Br VSV-8 are consistent with this idea. However, in the case of 3-Br VSV-8, an analysis of our first-order model of the peptide analogue in complex with the H-2Kb molecule suggests that the bromine atom at P3 participates in an extensive steric interaction with the residues lining the C pocket that could overcompensate the hydrophobic “collapse” effect of the phenyl ring (Table I). We propose that the apparent decrease of the binding of 3-Br VSV-8 to the H-2Kb molecule, as well as its lack of recognition by N26 is due to a sterically driven rearrangement of the residues forming the C pocket.

The function of “anchor” residues

It seems that the traditional analogy in classifying the residues within an antigenic peptide into either “anchor” or “TCR contact” residues has been overinterpreted to support an untested assumption that buried “anchor” residues are “invisible” to TCR. The first piece of evidence against this assumption came from the fact that “anchor” residues are not the only component of the peptide that is buried. The single peptide-loaded structures of the H-2Kb molecules have revealed that a sizable portion (varying from 75% to 83%) of the peptide molecular surface becomes solvent-inaccessible upon binding to the class I molecule (51, 78). It is highly unlikely that only a minor (17–25%) portion of the antigenic peptide is “visible” to the TCR, since this will result in near total degeneracy of the antigenic information encoded by the peptide. In
addition, there has been an increasing number of reports suggesting that the sequence of antigenic peptides is a source of conformational variation of surface residues from the MHC-peptide complex (10, 11, 14–17, 47, 79).

The analyses of crystallographic structures of TCRs in complex with their respective MHC class I molecules loaded with cognate peptide Ags (A6 and B7 complexed to HLA-A2/HIV tax peptide) reinforced the importance of MHC residues in immunorecognition (80, 81). Despite the fact that both A6 and B7 recognize the same MHC-peptide complex, only 1 of the 17 TCR residues contacting the antigenic complex is conserved. However, the crystallographic structures revealed that this pair of TCRs bind to the MHC-peptide complex in a similar diagonal conformation. Only two of the peptide residues, P5 and P8 tyrosines, make extensive contacts with the TCRs. Furthermore, it was found that many of the same residues on the HLA-A2 molecule were contacted by these two different TCR molecular surfaces. These observations imply that peptide-induced conformational variation of MHC surface residues could modulate the process of TCR-mediated immunorecognition.

The role of buried peptide residues in immunorecognition can also be argued in terms of the process of molecular evolution. The mammalian MHC class I-restricted T cell response has evolved to recognize the invasion of foreign entities by the display of antigenic peptides within the MHC class I molecules, while maintaining the ability to distinguish self from nonself. In light of this function, it is highly unlikely that the pockets within the peptide binding grooves of the MHC molecules have evolved to partially hide the identity of the peptide Ag, since that would be counterproductive to the task of the molecule. The results of our study with the P3 and/or P5 analogues of the VSV-8 peptide showed that the structure of the buried anchor residues are crucial to the formation of the molecular surface, created by the combination of the peptide and the MHC molecule, that is recognized by the TCR and that the antigenicity of the MHC-peptide complex is exquisitely dependent on the atomic-level structure of the buried side chain. In fact, substitution of the P5 tyrosine hydroxyl group of VSV-8 creates a set of VSV-8 superagonist analogues with higher than native level of recognition by the N15 TCR. Though numerous reports describing the activities of T cell partial agonist and antagonist peptides; Dr. Kim A. Sharp for use of his computational resources; and Dr. Andrew J. Cucchiara for his expert assistance in the statistical analysis of some of our experimental data.

**References**


main-chain atoms of the amino terminus.


antibodies and H-2 mutant mice.


protein into the class I pathway of antigen processing and presentation.


and viral antigen processing.


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