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C-Terminal Anchoring of a Peptide to Class II MHC via the P10 Residue Is Compatible with a Peptide Bulge

Maryam Yassai, Amin Afsari, Jason Garlie, and Jack Gorski

The binding of antigenic peptide to class II MHC is mediated by hydrogen bonds between the MHC and the peptide, by salt bridges, and by hydrophobic interactions. The latter are confined to a number of deeper pockets within the peptide binding groove, and peptide side chains that interact with these pockets are referred to as anchor residues. T cell recognition involves solvent-accessible peptide residues along with minor changes in MHC helical pitch induced by the anchor residues. In class I MHC there is an added level of epitope complexity that results from binding of longer peptides that bulge out into the solvent-accessible, T cell contact area. Unlike class I MHC, class II MHC does not bind peptides of discrete length, and the possibility of peptide bulging has not been clearly addressed. A peptide derived from position 24–37 of integrin β₂ can either bind or not bind to the class II MHC molecule HLA DRB3*0101 based on a polymorphism at the P9 anchor. We show that the loss of binding can be compensated by changes at the P10 position. We propose that this could be an example of a class II peptide bulge. Although not as efficient as P9 anchoring, the use of P10 as an anchor adds another possible mechanism by which T cell epitopes can be generated in the class II presentation system. *The Journal of Immunology*, 2002, 168: 1281–1285.

The recognition of MHC-bound peptide by the TCR lies at the center of the adaptive immune response. There are two series of interactions that are required; the first is the binding of the peptide to the MHC and the second is the recognition of novel epitopes revealed by this binding. The nature of the peptide binding is restrained by the structure of the MHC. Thus, the structure of the class I MHC allows for the binding of short peptides whose N-terminal and C-terminal ends are anchored into pockets located at each end of the peptide binding groove (see Ref. 1 for a recent review). Structural studies have indicated that the portion of the peptide recognized by the T cell corresponds to the central, solvent-exposed portion of the peptide (2). Although the binding of the peptide may change the structure of the MHC to a small extent, the major source of non-self epitopes to be recognized by self-tolerant TCR is provided by the peptide side chains that point into the solvent. An additional level of epitope complexity arises from the binding of peptides of different lengths, with many of the longer peptides being accommodated by a bulging of the central portion of the peptide (3–8).

Peptide binding to class II MHC differs from the binding to class I in that the peptides are not constrained in size. There are major binding pockets at each end of the peptide binding groove; however, the position of the helices allows the peptide to exit at either end (reviewed in Ref. 9). The central region of the binding groove consists of three or four shallow pockets that are involved in peptide binding interactions. A large portion of allelic specificity in peptide binding is a function of the nature of these shallow pockets as the polymorphism in the terminal pockets is relatively restricted.

The interactions in the central region can be sufficient to stabilize those peptides that do not show extensive anchoring in the terminal regions (10, 11). Thus, the current view of class II MHC peptide interactions is one of a peptide lying in a relatively shallow groove with multiple contacts across the entire extent of the peptide binding groove. This view has not included the possibility of the peptide bulging out from the groove as has been reported for class I.

The most important peptide anchor residue is referred to as P1 (or i) and the final anchor occurs at P9 (i + 8). The identification of peptide anchors past P9 could be indicative of a peptide bulge. There have been reports that peptides binding to HLA-DR1 can use P10 as an anchor (12, 13). The human class II MHC molecule HLA-DRw52a (DRB3*0101) has a peptide binding motif that includes a primary hydrophobic anchor at P1 and another hydrophobic anchor at P9 (14). There is an allele-specific requirement for an acidic group at P4. Using an HLA-DRw52a-binding peptide derived from integrin β₃, we explore the alternate use of a hydrophobic anchor at P10 in lieu of P9. The results are interpreted in the context of a peptide bulge between the anchors at P6/P7 and P10.

Materials and Methods

Expression of recombinant soluble HLA-DRB3*0101 proteins

Soluble human class II HLA-DRB3*0101 proteins were generated in Sf9 insect cells using a baculovirus expression system as described (15). Human cells grown in serum-free medium Excell 405 (JRH Biosciences, Lenexa, KS) supplemented with l-glutamine were infected with recombinant DRasol and DRB3*0101sol viruses at a multiplicity of infection of 10 for each virus. At 72 h postinfection, cells were removed and the culture medium was concentrated 10-fold and used for immunofluorescence purification using immobilized anti-HLA-DR mAbs, L243. HLA-DR molecules were eluted as described (16) and concentrated into PBS using Centricon-30 (Amicon, Beverly, MA). The concentrations of purified HLA-DR proteins were determined by UV absorbance at 280 nm using an extinction coefficient of 77,000 M⁻¹ cm⁻¹ (16).

Peptides

Peptides derived from amino acid residues 24–37 of integrin β₂ are described in Table I. These peptides were synthesized by standard solid-phase methods, purified by HPLC, and confirmed by mass spectrometry. The peptides were labeled with FITC at the N-terminal end and purified by reversed-phase HPLC.
Table I. Integrin $\beta_2$ (residues 24–37) peptides used in this study

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLG</td>
<td>AWCSDEALPLGSPR</td>
</tr>
<tr>
<td>PPG</td>
<td>AWCSDEALPGSPR</td>
</tr>
<tr>
<td>PPL</td>
<td>AWCSDEALPPGSPR</td>
</tr>
<tr>
<td>PGG</td>
<td>AWCSDEALPPGSPR</td>
</tr>
<tr>
<td>PGL</td>
<td>AWCSDEALPGSPR</td>
</tr>
<tr>
<td>AGG</td>
<td>AWCSDEALPGSPR</td>
</tr>
<tr>
<td>AGL</td>
<td>AWCSDEALPGSPR</td>
</tr>
<tr>
<td>K26Q29PPL</td>
<td>AKYSDQALPPGSPR</td>
</tr>
<tr>
<td>K26Q29PLL</td>
<td>KMRATPLLQGMA</td>
</tr>
</tbody>
</table>

* The peptides are named after the amino acids in positions 32–34, respectively. The CLIP peptide used for generating the structural models is included. Anchor residues are underlined and positions are numbered.

Peptide binding assays

A total of 10 nM purified soluble HLA-DRB3*0101 proteins were used in 50 $\mu$L of binding mixtures with indicated amounts of labeled and/or unlabeled peptides. The binding conditions were 37°C in PBS with 1 mM EDTA, 1 mM PMSE, 0.1 mM iodoacetamide, and 3 mM NaN$_3$, at pH 5.5 for 18 h. The binding mixtures were resolved on native PAGE using Bio-Rad (Hercules, CA) minigel electrophoresis apparatus for the fast separation of bound from unbound peptides. The binding of FITC-labeled peptides was detected and quantitated by ImageQuant software of FluorImager.

Visualization/modeling

To view possible peptide structures the DR3:class II-associated invariant chain peptide (CLIP)$_3$ crystal structure (17) was used as a basis for further modeling. HLA-DR and peptide residues were modeled to generate a DRw52a sequence with the $\beta_3$ (residues 24–37) peptide in the binding groove using Sybyl (Tripos, St. Louis, MO). The occurrence of a proline residue at P9 might play a role in the ability of the P10 residue to function as an anchor. It is possible that a proline can introduce a kink in the peptide structure that might favor a P10 interaction with the peptide binding groove.

Results

**Loss of peptide binding due to changes at the P9 anchor can be partially offset by P10 modification**

The peptide derived from region 24–37 of integrin $\beta_3$, AWCSDEALPGSPR, implicated in the response to the platelet alloantigen $\alpha_\text{IIb}$, has been shown to bind to DRw52a (DRB3*0101). As part of this previous analysis (14) a motif for DRw52a was defined as an aromatic/hydrophobic P1 residue, Trp$_{25}$, an acidic P4 residue, Asp$_{28}$, and a hydrophobic P9 residue. As residue P9 was changed to Leu, a competition binding assay was performed (Fig. 3). The ability of unlabeled PPL to compete with the binding of labeled PLG was compared with the ability of unlabeled PPL to compete with the labeled peptide. The results indicate that PPL is a good competitor of the binding interaction. These data are in relatively good agreement with the direct binding assay and indicate a similar mode of binding for the two peptides.

**L34 facilitates peptide binding in the absence of Pro at position 33**

The occurrence of a proline residue at P9 might play a role in the ability of the P10 residue to function as an anchor. It is possible that a proline can introduce a kink in the peptide structure that might favor a P10 interaction with the peptide binding groove. The role of proline at position 33 was investigated by modifying this position. After testing a number of residues with smaller side
chains, it was observed that a peptide with glycine at the P9 position, PGG, showed minimal binding to DRw52a. To determine whether the substitution of leucine at the P10 will also anchor a peptide with Gly at position 33, the binding of the PGG peptide was compared with that of the PGL peptide. The results (Fig. 4) show that there is an 8-fold increase in the binding of the PGL peptide over the PGG peptide. However, it should be noted that the levels of binding achieved are lower than those with Pro at position 33. Using equivalent amounts of DRw52a, the PPL peptide binds over 4-fold better than does the PGL peptide (Fig. 4D); thus, there appears to be an effect of the proline in allowing for P10 anchoring.

The effect of Leu at P10 is not due to use of a different peptide binding register

One trivial explanation of the binding of the Pro<sup>33</sup>/Leu<sup>34</sup> peptide is the use of an alternate peptide register. The only possible alternate register would be AWCSDEALPPL, with Cys and Glu acting as the P1 and P4 anchors. To show that the Leu at position 34 was acting in the original register, we eliminated the two side chains in question, substituting the Cys with Lys and the Glu with Gln, and assayed the ability of the resulting peptide to bind to DRw52a (Fig. 5). This peptide, K26Q29PPL, which does not have the required hydrophobic and acidic residues at P1 and P4 in an alternate register, also bound to DRw52a (Fig. 5, lane 1). As expected, the binding was stronger with the K26Q29PLL peptide (Fig. 5, lane 2). It should be pointed out that these peptides also have Y substituted for W at the P1 anchor (Table I) with no adverse effect.

L34 facilitates peptide binding in the absence of Pro at position 32

To further understand the effect of other peptide side chains on the ability of the P10 residue to recapture binding, we determined whether there is a requirement for a Pro at position 32. As described earlier for position 33, proline may play a unique role in the ability of P10 to anchor a peptide. Therefore, peptides with

![Image](http://www.jimmunol.org/)

**FIGURE 2.** Plot of peptide binding data. The data from Fig. 1 are shown plotted as relative fluorescence units (rfu) for each concentration. The relative fluorescence data in the peaks were transferred from the FluorImager to an Excel spreadsheet and plotted directly without fitting. Sources of data points are identified in the legend.

**FIGURE 3.** Plot of peptide competition results. The binding of labeled PLG peptide was performed in the presence of increasing concentrations of the peptide identified in the legend.

**FIGURE 4.** Binding of peptides with glycine at position P9. A, Native PAGE of binding experiments for the PGG peptide. B, Native PAGE of PGL binding. C, Plot of binding curves for PGG and PGL. D, Plot of binding curves of PLG and PPL peptides performed at the same time. The PPL peptide is better at binding than PGL but, as expected, binds at ~20% of the PLG peptide.

![Image](http://www.jimmunol.org/)

**FIGURE 5.** Native PAGE of peptide binding using peptides modified to eliminate any possibility of alternate registers. Lane 1, K26Q29PPL peptide; lane 2, K26Q29PLL peptide; lane 3, PLG peptide. The two K26Q29 peptides also have a Y for W substitution at 25 (P1). Peptides concentration was 1 μM in the binding assays.
as equivalent concentrations of the APL peptide and the PPL peptide bound with the same efficiency (Fig. 6).

Discussion

The experiments described in this report indicate that a hydrophobic side chain at P10 can partially reconstitute the loss of binding of a peptide when the P9 anchor residue is substituted by Pro or Gly. The pocket generated at the C-terminal end of the binding groove is, in general, the second most profound pocket and thus would be expected to contribute to the overall stability of a bound peptide. There are two possible interpretations of this observation that differ in the presumed geometry of the bound peptide. In the first of these, the side chain at P10 acts at an independent site on the edge of the peptide binding groove to compensate for the loss of the P9 anchor. In the second, the residue at P10 interacts with the pocket that is normally occupied by P9, resulting in a partially bulged peptide.

To help visualize these possibilities and to insure that structural integrity was maintained, we modeled these possibilities. The x-ray crystal structure of DR3 was used to generate the DRw52a structure by replacement. Simultaneously, the peptide residues were changed from those of the CLIP peptide, originally bound to the DR3, to those of the β1 integrin with Leu at 33. Energy minimization without electrostatics was performed to eliminate incompatible geometries. The Trp in the β1 sequence was replaced by Tyr in the primary anchor position. A peptide with a Tyr replacement works as well as the Trp peptide and shows the same P10 effect (Fig. 5 and data not shown). The orientation of the peptide is shown in Fig. 7B.

In the first model, the peptide backbone is left in its original position and the side chains at P9 and P10 are simply exchanged for Pro and Leu. In this manner, the aliphatic P10 residue would provide an additional interaction with the class II MHC, anchoring the peptide close to the outside of the binding groove. The resulting peptide geometry (Fig. 7A) is compared with the model for the original β1 peptide (Fig. 7B), in which Leu at 33 is an anchor extending into the nonpolar pocket (Fig. 7B, second arrow pointing up).

There are two examples of class II structures in which this side chain to slip out of the pocket to some extent. The two prolines fill the groove, and the side chain at 32 bulges up slightly. The three peptide structures are overlapped to show the putative “bulge” structure that would result with the second possible geometry (Fig. 7D). This level of modeling provides an approximation of the possible actual structures, eliminating those disfavored on basic steric grounds.

It is possible that the third structure would provide a greater chance of stabilizing peptide binding. In this model, the position of Leu at P7, which extends into a side pocket under the ridge of the β-chain α helix, is maintained. A large part of the hydrogen bonding between the peptide and the MHC would also be maintained. Those disruptions that take place occur in the C-terminal portion of the peptide, which may be less influential than if they took place in the N-terminal portion (21). The relatively conserved α69Asn H bonding to P8 is affected as the amino group of P9 is not available for bonding. The distance between the α69Asn amide nitrogen and the carboxyl group of P7 has increased due to the kink and bulge. The α67Arg and β61Trp H bonds are maintained. It should be pointed out that the DR2-myelin basic protein structure showed that peptide binding could be maintained without hydrogen bonding in the C-terminal half of the peptide (11). The Val polymorphism at β57 in DRw52a precludes that side chain from participating in hydrogen bonding or forming the β57-α76 salt bridge. This polymorphism might result in a larger P9 pocket, as has been observed for I-Aβ7 (19, 22). A certain degree of flexibility in the use of the pocket that normally binds the P9 side chain has been

**FIGURE 6.** Role of proline at position 32 on P10-mediated binding. Native PAGE analysis of peptides with substitutions at position 32 (P8). Side chains at each position are identified above the lanes. Peptide concentrations were at 1 μM in the binding reactions. The results of two independent binding experiments are shown for the PPL and APL peptides. A PLG peptide-binding experiment was performed as a positive control.

**FIGURE 7.** Model of peptides bound to HLA-DRw52a. A, P33L34 in extended form. B, L33G34 in extended form. C, P33L34 with L34 extending into the P9 pocket. D, Overlay of all three structures. The peptide positions are identified. P7 is pointing out of the plane of the paper. P8, P9, and P10 show the largest displacement for form C (blue). Arrows pointing up show positions of the P1 and P9 pockets.
shown in the case of I-A\textsuperscript{g7}-bound GAD221–235 (Ref. 39 cited in 22). In this case Gly is the P9 residue but the P9 pocket is occupied by a side chain from an adjacent molecule in the crystal lattice (22). It will be of great interest to determine the actual structure of a peptide-MHC complex in which the P9 residue is implicated in stabilizing binding.

The P10 residue, also referred to as i + 9, has been implicated in providing peptide-anchoring function in HLA-DR1 (12, 13) and HLA-DR2a (DRB5*0101) (11). In the case of DR1 it was shown that a polyalanine helix with hydrophobic anchors at P1 and P10 were sufficient to result in high-affinity binding (12). In the case of the DR2a, moving the P10 anchor side chain to P9 increased the binding of the peptide. The authors also modeled a possible kink in the peptide backbone (23). However, the role of P10 in antigenic peptides is not yet fully appreciated. We demonstrate in this study that DR\textsubscript{w52a} also shows this characteristic, indicating that P10 anchoring may be a general phenomenon for many if not all class II alleles. It will be interesting to determine whether certain class II alleles or isotypes can anchor peptides sufficiently at both C and N termini to allow the much larger bulges that have been reported for class I.

We provide direct data that the elimination of peptide binding by changing the side chain of an anchor residue can be compensated for by an additional change in a neighboring peptide side chain. We interpret these results to indicate that by flexing the peptide, the neighboring side chain can interact with the MHC pocket with sufficient efficiency for maintaining the binding of the peptide. This would result in a local perturbation of the peptide, in part resembling the bulges described for class I peptide binding. It can be envisioned that certain peptides will have the ability to use both the P9 and P10 residues for anchoring. In such cases, there exists the possibility of generating two conformational epitopes from the same peptide-MHC complex. It would be likely that T cells would distinguish the difference between the two conformations, thus increasing the immunogenicity of such a peptide.

Acknowledgments

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References