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CUTTING EDGE

Cutting Edge: TCR Contacts as Anchors: Effects on Affinity and HLA-DM Stability[1]

Matthew W. Anderson and Jack Gorski[2]

Peptides presented via the class II MHC (MHCII) pathway are selected based on affinity for MHCII and stability in the presence of HLA-DM. Currently, epitope selection is thought to be controlled by the ability of peptide to sequester "anchor" residues into pockets in the MHCII. Residues exhibiting higher levels of solvent accessibility have been shown to contact TCR, but their roles in affinity and complex stability have not been directly studied. Using the HLA-DR1-binding influenza peptide, hemagglutinin (306–318), as a model, we show that side chain substitutions at these positions influence affinity and HLA-DM stability. Multiple substitutions reduce affinity to a greater extent than the loss of the major P1 anchor residue. We propose that these effects may be mediated through the H-bond network. These results demonstrate the importance of solvent-exposed residues in epitope selection and blur the distinctions between anchor and TCR contact residues. The Journal of Immunology,* 2003, 171: 5683–5687.

A critical step in an effective $CD4^+$ T lymphocyte response is the generation and presentation of peptides bound to class II MHC (MHCII)[3] molecules. Crystallographic analyses of peptide/MHCII complexes indicate that peptide affinity for MHCII is mediated by the sequestration of residues in pockets in the MHCII protein and by the formation of a conserved hydrogen bond (H-bond) network between main chain atoms of the peptide and the MHCII (1). Peptide side chains that act as "major" anchors can typically be identified by very low solvent accessibility when bound to MHCII. Other residues can lie on or partially under "shelves" in the MHC structure and have been referred to as "minor" or "auxiliary" anchors (2, 3). Due to the higher degree of solvent exposure at these positions, these side chains are also available to make contacts to TCR. Currently, the outcome of epitope selection is thought to be a direct consequence of the ability of a peptide to form complementary side chain/pocket interactions at the major 1, 4, 6, and 9 pockets.

Once bound to MHCII, a peptide epitope must also undergo a series of peptide exchange reactions catalyzed by HLA-DM (DM) (4). Therefore, DM plays a large role in determining which peptide/MHCII complexes are presented at the surface of an APC (5, 6). Less well understood is how the sequence characteristics of the peptide affect its resistance to DM-mediated peptide exchange (DM stability). Previous studies have identified features that can affect DM stability (7–9). However, more recent data suggest that the relationship between peptide sequence, intrinsic stability, and DM stability is more complex (10). Thus, the relationship between peptide sequence, affinity and DM stability remains poorly understood.

In this study, we show that side chains that exhibit an intermediate degree of solvent accessibility can dramatically influence both the affinity of the peptide for MHCII and the DM stability of the complex. These results extend the range of peptide "anchors" to include more solvent exposed positions in the peptide/MHCII interface, and reveal a previously unappreciated role for TCR contact residues to impact epitope selection events.

Materials and Methods

Peptide synthesis

All peptides were derived from the sequence GPKYVKQNTLKLAT, representing residues 306–318 of the hemagglutinin (HA) protein from influenza A virus (H3 subtype). The N-terminal Gly facilitated labeling. Peptides were synthesized by standard solid-phase methods, purified by HPLC, and confirmed by mass spectrometry. N-terminal labeling with FITC (Molecular Probes, Eugene, OR) or LC-LC biotin (Pierce, Rockford, IL) was performed on the resin before deprotection, then peptides were cleaved and purified as above.

Expression and purification of recombinant soluble HLA-DR1 and HLA-DM

Recombinant soluble DR1 was produced and immunoaffinity purified from a stably transfected *Drosophila* S2 cell line essentially as described (11). Soluble FLAG epitope tagged DM was purified from a stably transfected *Drosophila* S2 cell line as described (12). Purity was confirmed by SDS-PAGE, and both DR1 and DM proteins were buffer exchanged into PBS (pH 7.4) before use.

Competitive peptide binding assay

Relative binding affinities were determined by a competitive binding assay essentially as described (13). DR1 protein (2 nM) was incubated with 2 nM biotinylated HA (306–318) peptide in PBS (0.01% Tween 20, 0.1% BSA, 0.1 mg/ml 4-(2-aminoethyl)-benzene sulfonyl fluoride, 0.1 mM iodoacetamide, 5 mM EDTA, 0.02% NaN_3, pH 7.2) in the presence of varying amounts of inhibitor peptides for three days at 37°C. Bound biotinylated peptide was detected using a solid-phase immunoassay and Eu^{2+} labeled streptavidin. Plates were read using a Wallac VICTOR counter (PerkinElmer Wallac, Gaithersburg, MD). IC_{50} values were obtained from the curve fit of the binding data and converted to K_D values by the equation, $K_D = (IC_{50})/(1+([bHA]/K_{D,bHA}))$, in which

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[3] Abbreviations used in this paper: MHCII, class II MHC; HA, hemagglutinin; NNC, sodium phosphate, sodium citrate buffer.

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$K_{D,bHA}$ was set equal to 17.7 nM based on the results of the direct binding of biotinylated HA peptide to DR1 (data not shown). Each point represents the mean and SD of five independent experiments.

Native PAGE analysis of peptide binding

DR1 protein (100 nM) was incubated with 1 μM FITC-labeled peptide in the presence of 1 mM Pefabloc (Roche, Basel, Switzerland), 1 mM NaN_3, 1 mM EDTA (pH 8.0), and 0.1 mM iodoacetamide overnight at 37°C in 50 mM NaH_2PO_4 and 50 mM sodium citrate buffer (NNC) (pH 5.3). The binding mixtures were resolved on 5/12% native PAGE using a Bio-Rad minigel electrophoresis apparatus. FITC-peptide/DR1 complexes were visualized using a FluorImager 575 (Molecular Dynamics, Sunnyvale, CA), and comigration of DR1 protein on the gel was confirmed by Coomassie blue staining.

Measurement of HLA-DM-mediated peptide dissociation

DR1/peptide complexes were formed by incubating 1 μM DR1 with a 10-fold excess of FITC-labeled peptide as described above. DR1/peptide complexes were then purified from unbound peptide by buffer exchange into PBS (pH 7.4) with a Centricon-30 (Millipore, Bedford, MA) spin filter. Purified DR1/peptide complexes (100 nM) were then incubated with 100-fold excess of unlabeled HA (306–318) peptide in the presence of 1 μM DM. Reactions were performed at 37°C in NNC (pH 5.3). At various timepoints, aliquots of the reaction were quenched with 0.5 M Tris-HCl (pH 8.0) in gel loading buffer, placed immediately on ice before loading on a 5/12% native PAGE gel, and resolved by electrophoresis at 150 V for 30 min. FITC-peptide/DR1 complexes were visualized using a FluorImager. Data is expressed as the percentage of FITC-peptide/DR1 complex remaining relative to the complex at $t = 0$.

Stability of DR1/peptide complexes in the presence of 1-propanol

DR1/peptide complexes (100 nM) and unlabeled HA peptide (10 μM) were incubated in NNC (pH 5.3) with or without 1-propanol (0.5 M final) for 5 h at 37°C. All reactions were quenched with 0.5 M Tris-HCl (pH 8.0) in gel loading buffer and analyzed via native PAGE as described above.

Modeling of HLA-DR1/HA (306–318) interactions and solvent accessibility

The DR1/HA structure (2) was visualized with PyMOL (14). Solvent accessibility was determined using the analysis program "Surface" (15) and provided by Dr. L. Stern (University of Massachusetts Medical School, Worcester, MA).

Results

Crystallographic data (2) was used to calculate the solvent accessibility of each side chain of the HA peptide when bound to DR1 (Fig. 1*A*). We used solvent accessibility to define minor anchor residues in the DR1/HA complex. Minor anchor positions 2, 3, 7, and 10 show an intermediate solvent accessibility ranging from 42% for Val at P2 to 15% for Ala at P10. TCR contact with the side chain of P2 and P3 has been confirmed by crystallographic analysis of the HA1.7 TCR bound to DR1/HA (16) and HLA-DR4/HA (17). The P7 side chain may also be important for TCR contact as shown by the structure of the murine scD10-CA/I-A^k complex (18). In all peptide/MHCII/TCR structures to date, the TCR makes significant main chain contacts in the P7 region. Although the P10 side chain has not been shown to make TCR contacts, other peptide residues outside the MHCII groove have been shown (P-1, P11) to make important TCR contacts for a subset of $CD4^+$ T cells (19, 20), raising the possibility that there may exist TCR with a dependency on a P10 residue. However, in the DR1/HA structure the P7 and P10 side chains are largely buried and thus may be less available for TCR contact. The position of these side chains when bound to DR1 is shown in Fig. 1*B*. Targeted amino acid substitutions at these four positions in the HA peptide included a hydrophobic to polar V→S change at the P2 position, a basic to acidic K→D change at P3, an L→P change at P7 to increase steric constraint at this shallow pocket, and an A→G change to reduce hydrophobicity at the P10 position. The nature of the substitutions together with the length of the peptide made it very unlikely that the binding register was affected. FITC-labeled peptides containing single or multiple substitutions at these positions were examined for DR1 binding in an overnight binding assay under saturating conditions (Fig. 1*C*). We observed that while the P2 and P3 substitutions did not differ greatly from the unsubstituted HA peptide, the P2/3 VK→SD double substitution showed a significant reduction in binding. In contrast, the P7 and P10 single and double substitutions had little effect on binding. Surprisingly, a peptide incorporating all

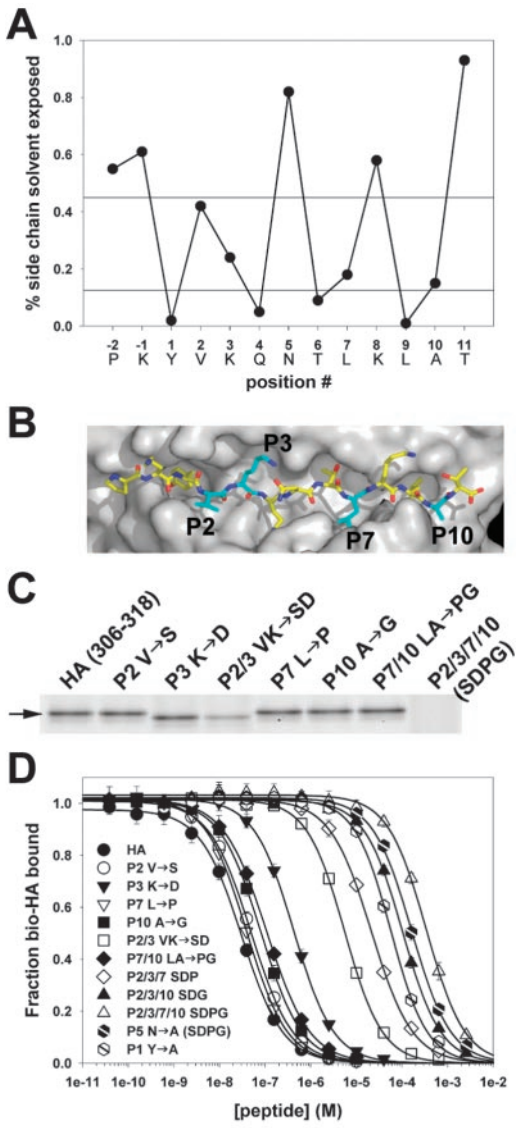

FIGURE 1. Side chain substitution at positions 2, 3, 7, and 10 in the HA peptide affects affinity for DR1. *A*, Solvent accessibility plot of HA peptide bound to DR1. Lines indicate the range of solvent accessibility for minor anchor positions 2, 3, 7, and 10. The sequence and position number of amino acids in the HA peptide is identified on the *x* axis. *B*, Visualization of the DR1/HA structure (2) using PyMOL (14). The view is from the top of the binding groove. The carbon atoms of minor anchor position side chains 2, 3, 7, and 10 are colored blue. *C*, Native PAGE analysis of substituted HA peptide binding to soluble DR1. FITC-labeled peptide/DR1 complexes were visualized using a FluorImager. The arrow indicates the migration of DR1/peptide complexes as detected by Coomassie blue stain. Complexes containing the K→D substitution migrate slightly faster due to the charge difference. *D*, Competitive binding analysis of substituted HA peptides. Data points represent the mean and SD of five independent experiments.

the substitutions at these positions (P2/3/7/10 SDPG) did not bind DR1 at all. These results suggested that despite the presence of the HA major anchor side chains at P1/4/6/9, multiple substitutions at the P2/3/7/10 positions were capable of reducing or abrogating binding.

The effects of these amino acid substitutions on HA affinity for DR1 were then examined by competition binding experiments in which the concentration of unlabeled substituted peptides was varied (Fig. 1*D*). Each substitution showed an effect on relative affinity. The P3 K→D change, resulting in a 14-fold loss in relative affinity, was the largest decrease for a single substitution. The data are tabulated by ranking the various substitutions by K_D (Table I) and are in agreement with previous studies analyzing M13 peptide libraries (21) and single substitutions in the HA peptide for their effect on DR1 binding (22, 23). Multiple substitutions reduced the affinity from 3.5-fold (P7/10 LA→PG) to 11,000-fold (P2/3/7/10 SDPG) over the native HA sequence (Fig. 1*D* and Table I). Interestingly, two of the peptides (P2/3/10 SDG and P2/3/7/10 SDPG) reduced relative affinity more than the reduction observed when the major P1 anchor Tyr is changed to Ala (P1 Y→A). Therefore, substitutions at these positions can have as important an effect as major anchor substitutions. Furthermore, substitution of the highly solvent exposed P5 Asn to Ala (P5 N→A (SDPG)) resulted in an almost 2-fold increase in affinity over the P2/3/7/10 SDPG peptide.

Next we examined whether these substitutions would also affect the DM stability of the peptide/DR1 complex. Using a native PAGE-based DM release assay to measure the off-rate of substituted peptides from DR1, we found that DM increased the dissociation of each substituted peptide to some degree (Fig. 2*A*, Table I), with the P3 K→D change ($t_{1/2}$ = 39 min) showing the fastest dissociation rate for a single substitution. Overall, the P2/3 change showed the largest increase in DM-mediated dissociation ($t_{1/2}$ = 7.5 min). These effects on DM stability were independent of the major anchor residues, which were identical with the parent HA sequence. Plotting DM stability vs peptide affinity (Fig. 2*B*) showed an inverse relationship, indicating that DM stability is related to the overall affinity of the peptide for DR1.

Since the peptides used in the study kept the major hydrophobic contacts constant, we considered the biochemical basis for the observed effects. One possibility was that substitutions containing -OH groups might interfere with the H-bond network (Ser at P2, Asp at P3), or allow increased solvent access to disrupt the H-bond network (Gly at P10). To test this idea, we took advantage of the ability of small molecules containing -OH groups (e.g., 1-propanol) to disrupt H-bonds and/or partially destabilize MHCII (24). In the presence of 1-propanol the P2, 3, and 10 substitutions significantly decreased complex stability (Fig. 3). In contrast, substituted peptide/DR1 complexes showed essentially identical SDS stability under varying pH conditions (data not shown). Taken together, these results sug-

Table I. *Affinity and DM stability of peptides used in this study*

Name	K_D (nM)	$t_{1/2}$ DM (min)
HA 306–318	27.7 ± 1	545 ± 61
P7 L→P	34.9 ± 1	413 ± 68
P2 V→S	44.0 ± 1	100 ± 8
P10 A→G	72.1 ± 1	148 ± 13
P7/10 LA→PG	97.0 ± 1	118 ± 8
P3 K→D	381.9 ± 9	39 ± 3
P2/3 VK→SD	4960 ± 127	7.5 ± 1
P2/3/7 SDP	19138 ± 556	ND
P2/3/10 SDG	92543 ± 2943	ND
P2/3/7/10 SDPG	305481 ± 11303	ND
P5 N→A (SDPG)	145912 ± 4614	ND
P1 Y→A	51483 ± 1236	ND

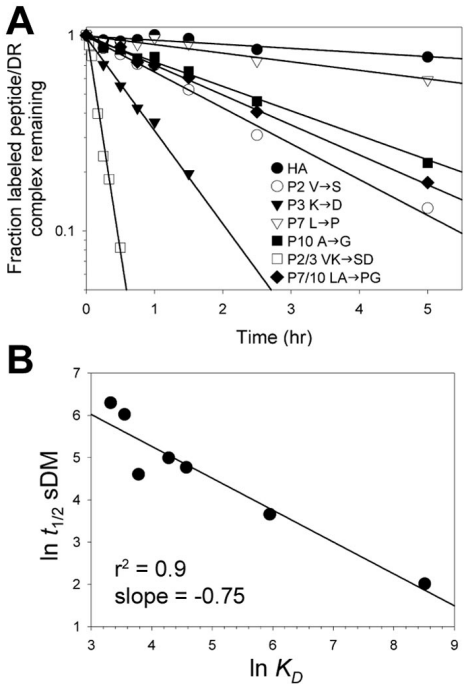

FIGURE 2. Effect of minor anchor substitution on DR1/peptide stability in the presence of DM. *A*, A 100 nM FITC-peptide/DR1 complex was incubated with 10 μM unlabeled HA peptide with 1 μM DM in NNC (pH 5.3) at 37°C. Data is expressed as the amount of FITC-peptide/DR1 complex remaining compared with $t = 0$. The data points represent the mean of three independent experiments. The lines represent the fit of the data to a single exponential function. Values of $t_{1/2}$ are as reported in Table I. *B*, DM stability is inversely related to affinity. The *ln* of $t_{1/2}$ vs *ln* K_D is plotted for each peptide.

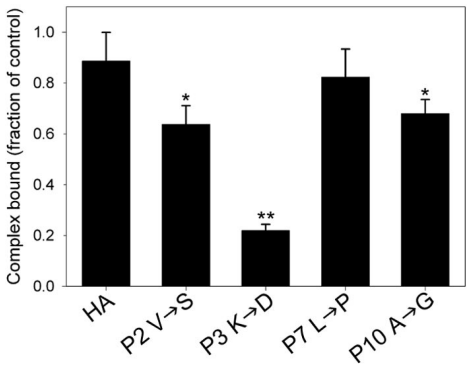

FIGURE 3. Substitutions at the 2, 3, and 10 positions decrease complex stability in the presence of 1-propanol. Peptide/DR1 complexes were incubated for 5 h in NNC (pH 5.3) at 37°C with or without 1-propanol (0.5 M final). Data for each peptide/DR1 complex is plotted relative to the average stability of each complex in the absence of 1-propanol (data not shown), and the data represents the mean and SD of four independent experiments. Single asterisk (*) indicates $p < 0.05$, while (**) indicates $p < 0.005$ relative to the stability of unsubstituted HA/DR1 complex in the presence of 1-propanol.

gested that these substitutions did not increase SDS destabilization of the major anchor P1 Tyr (25), but decreased resistance to H-bond disruption directly or indirectly mediated by 1-propanol.

Discussion

The experiments presented here demonstrate that peptide side chains that reside in more highly solvent-exposed positions in the peptide/MHCII interface can significantly affect the outcome of epitope selection. This is in contrast to most models of epitope selection, which assume that hydrophobic pocket/anchor interactions predominate. Similar effects of solvent exposed residues on peptide affinity for MHC have been observed with two MHC class I binding peptides (26, 27). In both these cases it has been suggested that the increased binding observed could be due to formation of a salt bridge to the MHC. For DR1-binding peptides, an Arg at P2 results in increased affinity (28) because of the formation of an additional H-bond (29). While these changes increase interactions, our 1-propanol data indicates that the negative effects of substitutions at P2, 3 and 10 could be due to disruption of H-bonds or increased solvent access. The details of how changing these side chains results in H-bond disruption will require further investigation.

We show that multiple substitutions at the P2, 3, 7, and 10 positions can dominate peptide binding. More detailed analysis of the data indicates negative cooperativity, i.e., that multiple substitutions show a greater negative impact than expected from single substitutions. This cooperativity is observed with poorly binding peptides and its relationship to MHCII folding and energetics is the subject of a separate study (M. W. Anderson and J. Gorski, manuscript in preparation). However, the multiple substitution data support distributive peptide binding in which positions across the MHCII groove can have an effect.

DM stability is affected by changes at solvent accessible residues (Fig. 2*A*) and overall is related to peptide affinity (Fig. 2*B*). Thus, DM discriminates among peptide sequences based on the total binding energy resulting from distributed interactions across the peptide-binding groove. A practical outcome is that a high affinity peptide should be stable in the presence of DM. This complements recent experiments in the HEL/I-A^k system that show DM preferentially selecting for high affinity peptides (30).

We propose that the current paradigm of assigning peptide side chains a role in either MHC binding or TCR contact must be reevaluated. Each residue should be viewed by a quantitative assessment of contribution to MHCII binding and TCR contact. Although some positions may predominate in one mode or the other, our data shows that under certain conditions, even the most highly solvent exposed residue, P5, can mediate positive contributions to affinity (Fig. 1*D*). It has also been shown that a buried P6 anchor interaction can dramatically affect T cell recognition (31). Thus, epitope selection and TCR stimulation are functions of the entire peptide. Such a view of epitope selection is complex, but by carefully isolating the individual components and defining the mechanisms by which multiple minor effects dominate, a rational predictive model can be obtained. This complements the empirical matrix-based algorithm approach (32) to epitope selection.

The ability of TCR contact substitutions to influence epitope selection leads to a number of important consequences. First, the nature of side chains available for TCR contact might be restricted. Second, it forces a reevaluation of the role of affinity and DM stability in the design of TCR contact substituted peptide antagonists. Finally, it defines an additional mechanism for pathogen evasion of the immune response.

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