How H13 Histocompatibility Peptides Differing by a Single Methyl Group and Lacking Conventional MHC Binding Anchor Motifs Determine Self-Nonself Discrimination


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How H13 Histocompatibility Peptides Differing by a Single Methyl Group and Lacking Conventional MHC Binding Anchor Motifs Determine Self-Nonself Discrimination

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The mouse H13 minor histocompatibility (H) Ag, originally detected as a barrier to allograft transplants, is remarkable in that rejection is a consequence of an extremely subtle interchange, P5Val/Ile, in a nonamer H2-Dβ-bound peptide. Moreover, H13 peptides lack the canonical P5Asn central anchor residue normally considered important for forming a peptide/MHC complex. To understand how these noncanonical peptide pMHC complexes form physiologically active TCR ligands, crystal structures of allelic H13 pDβ complexes and a P5Asn anchored pDβ analog were solved to high resolution. The structures show that the basis of TCRs to distinguish self from nonself H13 peptides is their ability to distinguish a single solvent-exposed methyl group. In addition, the structures demonstrate that there is no need for H13 peptides to derive any stabilization from interactions within the central C-pocket to generate fully functional pMHC complexes. These results provide a structural explanation for a classical non-MHC-encoded H Ag, and they call into question the requirement for contact between anchor residues and the major MHC binding pockets in vaccine design. The Journal of Immunology, 2002, 168: 283–289.

The H13 minor histocompatibility (H) Ag provides one of the most subtle examples of self/nonself discrimination. Snell et al. (1) originally detected H13 as a barrier to both tumor and skin allograft transplants, and using histogenetic techniques, isolated this H Ag via the production of H13 congenic mouse strains. Reciprocally reactive CD8+ CTLs can be readily generated by immunization of mice from H13 congenic partner strains. This alloreactivity is conferred by the conservative Val/Ile polymorphism in the naturally processed H2-Dβ-bound SSV(V/I) GVWYL nonamer peptide present at fewer than 50 copies per target cell (2). Moreover, because reciprocal CTL responses can be generated, the respective self peptides must act as negatively selecting self ligands in the respective hosts. In keeping with this subtle difference, self/nonself discrimination is incomplete in that H13 self-peptides act as partial agonists. Within the narrow window that distinguishes self from nonself, CTLs are readily generated, leading to the graft rejection phenotype. This model offers a unique opportunity to investigate T cell recognition of naturally processed and presented variant peptides that are responsible for substantial biological activity in vivo: T cell selection leading to allograft rejection.

An equally remarkable facet of the H13 system is that the allelic H13 peptides do not conform to the conventional MHC motif paradigm (3–5). MHC allelic variation gives rise to differential peptide binding due to the presence of polymorphic residues, which give distinct chemical and size characteristics to six pockets (A–F; Refs. 6 and 7) within the peptide binding groove. Allele-specific binding motifs were initially revealed by sequencing peptides eluted from MHC molecules (8, 9). For example, peptides eluted from Dα exhibit XXXXAsnXXXMe/Leu, where X is any amino acid. Both crystallographic and biochemical analyses support the importance of the centrally positioned P5 asparagine (P5Asn) side chain in stabilizing the pDβ complex by forming hydrogen bonds (H-bonds) with C-pocket residues Gln70, Gln97, and Tyr156 in the Ag binding domain (4, 10). Strikingly, P5 of H13 peptides is glycine (2), which cannot make the canonical contacts or occupy the C-pocket of the Dβ molecule. To understand how H13 peptides interact and stabilize Dβ in the absence of the P5 anchor residue and how T cells distinguish self from nonself based on such subtle antigenic peptide differences, we describe high resolution crystal structures of allelic H13 peptide/Dβ complexes, as well as compare them with the structure of a pDβ complex formed with a peptide modified to contain the canonical asparagine anchor residue.

Materials and Methods
Preparation of H2-Dβ complexes

Using a method adopted from Young et al. (10), Escherichia coli inclusion bodies of H2-Dβ and β2-microglobulin were separately denatured in 8.0 M urea and 20 mM Tris, pH 8.0, and were separately mixed with the synthetically prepared peptides representing the H13 peptides: H13H, (SSVGVVWYL), H13I, (SSVIGVWYL), and the P5 analog (SSVVNVWYL) in a mass ratio of 3:1:0.5 in 6 M urea and 20 mM Tris, pH 8.0. These
mixtures were refolded by dialysis against 10 mM Tris, pH 8.0, at 4°C in Spectra/Por CE dialysis tubing (MWCO 500) for 48 h. The dialysate was subsequently centrifuged at 15,000 × g and concentrated (30,000 MWCO; Amicon, Beverly, MA). The supernatant was chromatographed in a buffer containing 20 mM Tris and 150 mM NaCl, pH 8.0, on a Superdex75 column (Pharmacia Biotech, Uppsala, Sweden). Fractions containing the highest concentration of pDβ complexes were pooled from multiple runs, dialyzed against water, and concentrated (30,000 MWCO; Centricon, Bedford, MA). The synthetic peptides were produced by the Peptide Synthesis Facility of the Albert Einstein College of Medicine and Research Genetics (Huntsville, AL) using solid-phase F-moc chemistry, purified by HPLC, and confirmed by mass spectrometric analysis.

Crystallographic analysis and MHC binding capacity of H13 peptides

Table I. Crystallographic analysis and MHC binding capacity of H13 peptides

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<th>SVL9</th>
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* Values in parentheses are for the highest resolution shell.

* Rcryst = Σ|hi–|Fi|/Σ|Fi|, where h specifies unique reflection indices, i indicates symmetry equivalent observations of h.

* Rfree = Σ|Fobs – |Fc|/Σ|Fobs| for all reflections, where Fobs and Fcalc are the observed and calculated structure factors, respectively.

* Rfree was calculated against 10% of the reflections removed at random from the refinement.

* Shown are the sequence, relative binding affinity, and IC50 of peptide binding in nanomoles.
Peptide binding assays

The binding of peptides to D\(^\alpha\) molecules was assessed by measuring the amount of peptide required to inhibit by 50% the binding of a known radioligand to soluble purified D\(^\alpha\), as previously described (21).

\( H13 \) minigene expression constructs

To determine the effect of amino acid substitutions in the H13 peptide on T cell recognition, minigene constructs were prepared as described (2, 22). Briefly, the minigene constructs were produced to encode Met-SSVGVWYL, Met-SSVGVXWYL, or Met-SSVGYXIL, in which (X) is a random amino acid substitution of the core SSVGVWYL H13\(^\alpha\) peptide.

Cell lines, T cell activation, and cytotoxic assays

Ag-specific responses of T cell hybrids were determined by the production of \( \beta \)-galactosidase (lacZ) activity (23). The H13\(^\alpha\)-specific hybrid 30NX/ B10Z and the H13\(^\beta\)-specific hybrid B/NXZ have been described (2). In brief, 3–10 \( \times \) 10\(^4\) cells were cocultured overnight in duplicate with 2–5 \( \times \) 10\(^5\) LMK cells cotransfected with H13 minigene constructs, D\(^\alpha\), and B7.2 cDNA. The peptide/MHC-induced T cell response was assayed as lacZ activity using the substrate chlorophenol red \( \beta \)-galactopyranoside. The conversion of this substrate to chlorophenol red was measured at 595 and 655 nm as a reference wavelength with a 96-well microplate reader (Bio-Rad, Richmond, CA). To generate bulk anti-H13\(^\alpha\) CTLs, B10.CE(30NX)-Sn male mice and were restimulated in vitro with cells from female CE(30NX)-H13\(^\beta\)/Sn male and were restimulated in vitro with cells from female CE(30NX)-H13\(^\beta\)/Sn mice. Specificity of the CTLs for naturally presented H13\(^\alpha\) and H13\(^\beta\) minor H Ags, respectively, was confirmed by conventional cell-mediated lympholysis analysis of female B10.CE(30NX)-Sn mice (maintained at The Jackson Laboratory, Bar Harbor, ME) were primed twice with 2 \( \times \) 10\(^5\) spleen cells from H13\(^\alpha\) male C57BL/10Sn (B10) mice, and were then restimulated for 5–6 days with spleen cells from 2000 rad-irradiated female B10 mice in DMEM medium supplemented on day 3 of culture with 10–30 U/ml IL-2 as described (24). To generate bulk anti-H13\(^\beta\) CTLs, female B10 mice were primed similarly with cells from B10.CE(30NX)-H13\(^\beta\)/Sn male and were restimulated in vitro with cells from female CE(30NX)-H13\(^\beta\)/Sn mice. The standard 51 Cr release cell-mediated lympholysis assay as described previously was used for assessment of cytotoxic activity against synthetic peptide pulsed target cells (2). The target cells were T2 cells transfected to express H2-Db, kindly provided by P. Cresswell (Yale University, New Haven, CT). Synthetic peptide-pulsed T2-D\(^\alpha\) target cells were prepared by adding 5 \( \times \) 10\(^4\)Cr-labeled target cells to V-bottom microtiter wells carrying varying peptide concentrations for 1 h. Effector cells were then added at E:T ratio of 10:1 or 20:1 and were incubated for 4 h. The percentage of specific lysis was calculated from the amount of 51 Cr released into the culture supernatant and is the mean of triplicate cultures.

Results

Water molecules fulfill the role of the anchor residue in stabilizing the C pocket of the D\(^\alpha\) clef

To investigate the question of how noncanonical peptides compensate for the lack of a conventional anchor residue, we solved crystal structures of D\(^\alpha\) in complex with both allelic forms of H13: H13\(^\alpha\) (termed SVL9: SSVGVWYL) and H13\(^\beta\) (SIL9: SSVIGVWYL; Table I). In addition, we determined the structure of D\(^\alpha\) complexed to a peptide analog of H13\(^\alpha\) in which we have imposed a P5\(^\text{Asn}\) anchor residue (SVNL9: SSVVNWYL). Omit electron density maps for the regions corresponding to the H13 peptides are shown in Fig. 1.

Two water molecules are observed in the C pocket of H13\(^\alpha\) (SVL9) and H13\(^\beta\) (SIL9) peptide/D\(^\alpha\) complexes (Figs. 1 and 2), which form H-bonds to C pocket side chains (Gln\(^70\), Gln\(^97\), and Tyr\(^115\)). These interactions stabilize the architecture of the groove and enable the anchorless H13 peptides to assume backbone conformations characteristic of conventionally anchored peptides.

The structural similarity between H13 peptides and canonical peptide conformation is shown in Fig. 3A in which the H13\(^\alpha\) peptide, SVL9, is superimposed on a D\(^\alpha\)-bound peptide that contains the P5\(^\text{Asn}\) anchor residue, SEV9 (FAPGYNPAL; Ref. 23). The MHC binding strategy used by the H13 peptides is distinct from that used by a previously described peptide, p1027 (FAPGVFPYM; Ref. 24), which binds D\(^\beta\) despite the lack of a P5\(^\text{Asn}\) anchor by bulging in the central region of the peptide to allow the P6\(^\text{Phe}\) side chain to serve as a new anchor (Fig. 3B). These results demonstrate that noncanonical peptides are capable of adopting multiple conformations to accommodate MHC binding, importantly including the conventional conformation as demonstrated by both allelic forms of H13 (Fig. 3, A and C).

Allelic discrimination of H13 epitopes depends upon the presence/absence of a single methyl group poised for TCR CDR3 recognition

The only difference between the allelic H13 peptides is the presence of either P4\(^\text{Val}\) or P4\(^\text{Ile}\). As shown in Fig. 4, the P4\(^\text{Ile}\) side chain of SIL9 assumes a similar side chain rotamer conformation as P4\(^\text{Val}\) in SVL9, with the additional methyl group (C6\(^\text{Ile}\)) positioned directly between the \( \alpha2 \) and \( \alpha2 \) helices of D\(^\alpha\). P4\(^\text{Ile}\) in the SIL9 peptide is more exposed to solvent than P4\(^\text{Val}\) in SVL9, and is predicted to protrude directly into the central portion of the TCR-binding interface (Fig. 4A; Refs. 27–29). The P4\(^\text{Val}\) ↔ P4\(^\text{Ile}\) substitution results in only minimal alteration in the P4 side chain at a site readily accessible to TCR contact.

Comparison of the SIL9 and SVL9 pD\(^\alpha\) crystal structures revealed two notable differences in addition to those at the P4 side chain. First, although the H13 peptides have similar backbone conformations, the buried P3\(^\text{Val}\) side chains exhibit alternate rotamer
conformations in SIL9 compared with SVL9 (Fig. 3, C and D). Second, a H-bond between Glu163 and the hydroxyl group of P5 Ser is observed in H13b (SIL9/pDb), but not in H13a (SVL9/pDb). This H-bond is also found in SVNL9 (Fig. 4C) and promotes formation of a salt bridge across the Ag binding cleft from Lys66 of the α1 helix to Glu163 of the α2 helix (Fig. 4C). The variability observed at Glu163 in pDb crystal structures (Fig. 4C) suggests conformational plasticity at this site.

Mapping sites critical for T cell recognition of H13

Analysis of solvent accessibility indicates that among the H13 peptide residues, P4 Val/Ile, P6 Val, P7 Trp, and P8 Tyr are most accessible to the TCR (17). The relative contribution of these amino acid side chains to T cell recognition and activation was analyzed. First, LMtk- cells expressing Dα cells were transfected with H13 minigenes encoding H13 peptides that have incorporated random sub-}

(49). H-bonding interactions between water molecules and side chains of C pocket residues Gln70, Gln97, and Tyr156 are depicted in C. D. The P5Asn anchor residue forms interactions with Gln70, Gln97, and Tyr156. C and D. The orientation is such that C termini of the peptides are behind the plane of the page, and the N termini of the peptides are in front of the plane of the page. C and D were made with SETOR.

Structural and functional effects of an imposed central anchor

The identification of antigenic T cell epitopes and the design of agonist and antagonist ligands have in large part relied on the elaboration of allele-specific binding motifs and the definition of anchor residues (30–34). Immune responses to tumor Ag peptides lacking anchor residues have been reported to be enhanced by modifications that provide a surrogate anchor (35, 36). However, the extent to which peptides with imposed anchors improve antigenicity remains uncertain. The H13 model provides a sensitive and robust system to investigate this issue. pMHC complexes rendered stable by the imposition of anchor residues would be expected to enhance antigenicity, presumably by increasing cognate pMHC density on the plasma membrane. Alternatively, peptides modified so that they have improved anchors could alter the conformation of the peptide, and in doing so, disrupt TCR binding. To test these possibilities, the effects of adding a central anchor residue to H13 peptides on MHC binding and T cell recognition were analyzed. Analysis of the crystal structures of the anchorless and anchored pDb complexes then allowed for the correlation of functional parameters with structural alterations.

Consistent with a role for the conventional P5 anchor in pMHC stabilization, replacement of the native P5 Ser of SVL9 with a P5 Asn increased its affinity for Dα ~10-fold (Table I). This increased affinity is consistent with the crystal structure of SVNL9, showing that the P5Asn side chain participates in prototypic H-bonds with amino acid side chains of C pocket residues Gln70, Gln97, and Tyr156 (Fig. 2D).

To determine the consequence of P5Asn substitutions on CTL recognition, CTLs generated after reciprocal H13 congenic strain immunization were analyzed. As previously shown (2), allelic discrimination by normal CTLs occurs within a remarkably narrow peptide concentration range: ~1–100 pM for anti-H13a CTLs and ~1–1000 pM for anti-H13b CTLs (Fig. 5, C and D). H13 peptides substituted with P5Asn anchors were no more active than the naturally occurring SIL9 and SVL9 peptides in this assay (Fig. 5, C and D).

The P5Asn-substituted peptides actually inhibited recognition by anti-H13a CTL (Fig. 4C), suggesting that the improved anchors induced structural changes that modified the complementarity of the anti-H13a TCR with the pMHC interface to outside the biologically optimal range. Overall, the imposed P5Asn anchor pulls the central portion of the SVNL9 peptide backbone more toward the α2 helix compared with SVL9 and SIL9 (0.6 Å at the CA atoms of P4). In addition, the imposed anchor induced a change in the rotamer conformation of P4 Val (Fig. 4B), the key TCR contact residue in the H13 response.

It is not clear how TCR binding affects the conformation of the anchor-imposed SVNL9 peptide. Because structures of TCR complexed to pMHC have shown that TCR binding can alter the peptide conformation (37–40), TCR binding may induce a conformational change in SVL9. One explanation that may account for the inhibited T cell response to SVNL9 is that the main chain of SVNL9 is restricted from undergoing a conformational change induced by TCR binding. Despite observed and proposed structural changes, it is important to note that allelic discrimination, i.e. the ability of the CTLs to discriminate allo-P4Hec from self-P4 Val substitutions, was preserved in the context of the P5Asn-modified H13 peptides. This is supported by the ranges of concentrations required for inducing the CTLs, which demonstrate that self and allo agonist activity was minimally affected by the P5Asn substitution (data not shown). Therefore, side chains of P4, P7, and P8, but not P6 are essential components in the binding of both anti-H13a and anti-H13b TCRs.
In the case of anti-H13 CTLs, the P5 Asn modification did not abrogate allelic discrimination, but rather acted additively to increase the concentration required to achieve equivalent levels of cytolysis. These results suggest that structural changes induced by the P5Asn substitution are independent of those involved in allelic discrimination.

**Discussion**

The structural basis of the H13 transplantation Ag

Minor H Ags are allelically variant self peptides that pose a serious clinical concern in organ and bone marrow transplants, even under conditions of an MHC match (41). An increasing number of minor H Ags are being identified at the molecular level, but none have been examined at the structural level (42). This characterization of Snell’s classical H13 Ag provides a model for understanding how the most subtle molecular changes are sensed by T cells, ultimately resulting in transplant rejection. The topologies of the H13 pMHC complexes are remarkably similar, with the most notable difference being the extension of the P4 side chain by a single methyl group in SIL9 compared with SVL9. Modeling of the 2C TCR juxtaposed onto H13 peptides suggests that while a large surface area engages the TCR CDR loops, CDR3/H9251 is positioned for direct contact with the terminal end of the P4 side chain (40). Allelic discrimination was preserved by anti-H13 CTLs, even in the context of modest reorganization of the peptide conformation imposed by the substitution of a P5Asn anchor (Fig. 5, C and D), suggesting...
that CDR3α has sufficient flexibility to bind the P4Val side chain repositioned by the P5Asn substitution, and in doing so, trigger the TCR. X-ray crystallographic studies of agonist and superagonist pKb ligands for the 2C11 TCR suggested that the ability to form H-bonds between CDR3 loops and a critical peptide side chain is necessary to induce optimal TCR triggering (40). The presence/absence of a single methyl group may be an even more subtle trigger in which a small number of van der Waals contacts between the TCR CDR3α and the P4 side chain forms the basis of H13 allelic discrimination.

However, it is also possible that TCRs specific for H13b recognize SVL9 in a way that alters the peptide conformation, which can occur with valine and other side chains (Fig. 4A), but not isoleucine. Structures of TCR complexed to pMHC have shown that TCR binding can distort the peptide conformation (37–40). The crystal structures of H13 specific TCRs bound to respective ligands may assist in distinguishing these possibilities.

**Anchor imposition decreases antigenicity by causing conformational changes in a key TCR contact residue**

Allele-specific MHC motifs have been a lynchpin of antigen epitope definition and peptide-based vaccine design. Previous studies have all been consistent with obligate contacts between central anchor residues and MHC, either by direct interaction or by water-mediated H-bonds. Water molecules are frequently observed to fill cavities at molecular interfaces such as the TCR/pMHC interface (43) and the peptide/MHC interface (25). Because the loss of a single H-bond can decrease affinity by several orders of magnitude (44), direct or water-mediated H-bonds between anchor residues and MHC have been previously thought to be necessary for display of the characteristic peptide backbone configurations for each specific MHC allele (6, 7). However, some naturally occurring peptides, including the non-MHC encoded H Ags H13 (2) and H47 (45), are immunogenic despite the lack of a canonical central anchor residue. A clearer understanding of how such nonmotif peptides accomplish this task is a matter of considerable importance. In this report, we show two unprecedented features of the naturally occurring nonmotif H13 peptides (containing P5Val instead of P5Asn): neither direct nor water-mediated H-bond interactions bridge anchor residues and MHC; and they assume the prototypic backbone structure for Dα motif-bearing peptides. Thus, our studies provide evidence that interactions between central anchor residues and MHC side chains are not necessary for Ag presentation to T cells. In addition, the ability of water to stabilize the C pocket without the assistance of a peptide side chain has implications on the initial formation of pMHC complexes in the endoplasmic reticulum as well as intra- and extracellular peptide exchange.

Innovative insights into unresolved problems associated with modification of T cell epitopes are provided by the structure and function of an anchor-modified minor H Ag (Figs. 1D, 2B, 3B and 4D, 4B, and 5B). The extent to which peptides can be improved as immunogens by modification of anchor residues is a significant unresolved issue. Although vaccination of melanoma patients with an anchor-imposed peptide derived from the gp100 melanoma-associated Ag, gp100209–212 M, was reported to be significantly more efficient in generating clinical responses to melanoma in clinical trials (46), a study assessing the effect of modified anchors on melanoma-reactive CTL reported that only 2 of 47 modified peptides actually increased binding, immunogenicity, and recognition by established CTL lines (47). X-ray crystallographic analysis of the H13 SVNL9 pDβ complex indicates that the P5Asn side chain is engaged in prototypic H-bond contacts with the Dβ C pocket, which can account for the increased stability of the SVNL9 pDβ complex compared with that of the SVL9 pDβ (Table I). However, despite substantially increased binding, structural changes induced by the P5Asn anchor not only failed to increase agonist activity, but for SVL9 pDβ, the P5Asn substitution went so far as to reduce agonist activity of the SVL9 pDβ complex (Fig. 4C). Our results provide the first direct structural evidence that anchor imposition decreases antigenicity by causing subtle conformational changes in critical TCR contact residues of the peptide. Careful consideration of the consequences of anchor residues and water in peptide-MHC interactions should assist in epitope identification and vaccine design.

**FIGURE 5.** TCR sensitivity to substitutions in the polymorphic position P4 and the P5 anchor. Functional analysis of anti-H13 TCR recognition of naturally occurring and unnatural P4 and P5Asn anchor substitutions. A and B, Individual minigene constructs encoding random amino acid substitutions (X) at position 4 of the H13 antigenic peptide SSV(X)GVWYL were cotransfected into LMTk- cells with Dα MHC class I and B7-2 eDNA constructs as described (H47 REF). Specific recognition of the transfected LMTk cells by H13a-specific T cell hybrid 30NX/B10Z.A and the H13β-specific T cell hybrid B/NXZ.B was then measured by the lacZ assay. The minigene constructs were sequenced to determine the identity of the “X” residue, shown by single letter code above the bars. Duplicate transfectants were screened with the H13a-specific hybrid 30NX/B10Z.A and the H13β-specific T cell hybrid B/NXZ.B was then measured by the lacZ assay. The minigene constructs were sequenced to determine the identity of the “X” residue, shown by single letter code above the bars. Duplicate transfectants were screened with the H13a-specific hybrid 30NX/B10Z.A and the H13β-specific T cell hybrid B/NXZ.B was then measured by the lacZ assay.
Acknowledgments
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References