Predominant occupation of the class I MHC molecule H-2K\textsuperscript{wm7} with a single self-peptide suggests a mechanism for its diabetes-protective effect

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Transmitting editor: W. Strober

Received 7 July 2009, accepted 23 December 2009

Abstract

Type 1 diabetes (T1D) is an autoimmune disease characterized by T cell-mediated destruction of insulin-producing pancreatic \(\beta\) cells. In both humans and the non-obese diabetic (NOD) mouse model of T1D, class II MHC alleles are the primary determinant of disease susceptibility. However, class I MHC genes also influence risk. These findings are consistent with the requirement for both CD4\(^+\) and CD8\(^+\) T cells in the pathogenesis of T1D. Although a large body of work has permitted the identification of multiple mechanisms to explain the diabetes-protective effect of particular class II MHC alleles, studies examining the protective influence of class I alleles are lacking. Here, we explored this question by performing biochemical and structural analyses of the murine class I MHC molecule H-2K\textsuperscript{wm7}, which exerts a diabetes-protective effect in NOD mice. We have found that H-2K\textsuperscript{wm7} molecules are predominantly occupied by the single self-peptide VNDIFERI, derived from the ubiquitous protein histone H2B. This unexpected finding suggests that the inability of H-2K\textsuperscript{wm7} to support T1D development could be due, at least in part, to the failure of peptides from critical \(\beta\)-cell antigens to adequately compete for binding and be presented to T cells. Predominant presentation of a single peptide would also be expected to influence T-cell selection, potentially leading to a reduced ability to select a diabetogenic CD8\(^+\) T-cell repertoire. The report that one of the predominant peptides bound by T1D-protective HLA-A*31 is histone derived suggests the potential translation of our findings to human diabetes-protective class I MHC molecules.

Keywords: autoimmunity, diabetes, MHC

Introduction

Type 1 diabetes (T1D) is a polygenic T cell-mediated disease characterized by autoimmune destruction of pancreatic islet \(\beta\) cells (1). The strongest predisposing genetic determinant for the disease is the presence of particular class II MHC alleles, with the highest risk genotype being DQB1*0302-DR4/DQB1*0201-DR3 (2). Numerous association studies have suggested that certain class I MHC alleles are also predisposing for T1D (3–10). Recently, Nejentsev
et al. (11) applied sophisticated statistical analyses to several large data sets, which enabled them to localize T1D susceptibility not only to the class II MHC genes HLA-DOB1 and HLA-DRB1 but also to the class I genes HLA-A and HLA-B. Thus, prior associations reported between certain class I MHC alleles and T1D development do not merely reflect linkage disequilibrium with the class II MHC genes. Although HLA-B*39 and HLA-B*18 were found to confer susceptibility to T1D in this study (11), certain other class I HLA alleles (e.g. HLA-A*31 and HLA-B*27) were found to be protective. Earlier association studies also suggested protective effects for particular class I alleles (7, 10), as did observations in the non-obese diabetic (NOD) mouse model of the disease (12–16).

Although it is difficult to evaluate the mechanism responsible for the T1D-protective effect of an MHC molecule in humans, here, we explored this question by performing biochemical and structural analyses of the murine class I MHC molecule H-2K<sup>wm7</sup>, which protects against T1D in the NOD mouse model of the disease (12, 13). The T1D-protective effect of H-2K<sup>wm7</sup> was first described by Hattori et al. (13), who crossed NOD (K<sup>A</sup>, A<sup>q7</sup>, D<sup>b</sup>) to B10.A(R209) mice (K<sup>wm7</sup>, A<sup>k</sup>, E<sup>k</sup>, D<sup>d</sup>, L<sup>d</sup>), which have a hotspot that causes intra-MHC recombination between the K and A regions. Mating of F<sub>1</sub> progeny to NOD mice resulted in an intra-MHC recombinant (K<sup>wm7</sup>, A<sup>q7</sup>, D<sup>p</sup>), which was then backcrossed to NOD for five generations, at which time the non-MHC T1D susceptibility loci were confirmed to be homozygous for NOD DNA. Mice homozygous for the recombinant MHC haplotype had a markedly reduced incidence of T1D, whereas heterozygotes were partially protected. In this study, the protective effect was localized to within 4.4 cM centromeric to the Lmp2 gene, a region including the H-2K gene. To explore H-2K as a candidate gene, NOD mice transgenically expressing H-2K<sup>wm7</sup> were subsequently generated (12). Multiple transgenic lines were established, and the ratio of the expression of H-2K<sup>wm7</sup> to H-2K<sup>d</sup> was measured using allele-specific antibodies. In the lines in which this ratio was greatest, significant protection from T1D was observed, demonstrating the T1D-protective effect of H-2K<sup>wm7</sup>. It is important to note that expression of class I MHC transgenes in NOD mice does not uniformly lead to protection from T1D, as HLA-A*0201-transgenic mice show marked disease acceleration (17), whereas transgenic expression of H-2K<sup>b</sup> has no effect on T1D development (18).

To investigate the mechanism by which H-2K<sup>wm7</sup> mediates its T1D-protective effect in NOD mice, we used multiple complementary techniques, including purification and sequencing of H-2K<sup>wm7</sup>-bound peptides and crystallographic analysis of H-2K<sup>wm7</sup> molecules. Our results suggest a mechanism for the disease-protective effect of H-2K<sup>wm7</sup> that has not been previously reported for an MHC molecule of either class. We have found that H-2K<sup>wm7</sup> is predominantly occupied by a single self-peptide derived from histone H2B, suggesting that its inability to support T1D development could be due, at least in part, to the failure of peptides from critical β-cell antigens to compete for binding and be presented to T cells. The potential relevance of our findings to humans is suggested by the intriguing observation that the T1D-protective HLA-A<sup>*31</sup> (11) binds six major peptides, one of which is histone derived (19).

### Methods

**Cloning of the H-2K<sup>wm7</sup> complementary DNA**

Total RNA was prepared from the spleen of a 20-week-old female B10.A(R209) mouse (20) and reverse transcribed into single-strand complementary DNA (cDNA) using oligo dT as primer. The short and long forms of the H-2K<sup>wm7</sup> heavy chain cDNA (containing the short or long forms of exon 8, respectively) were amplified by PCR using KOD hotstart DNA polymerase and sense (5'-ATGGAATTCCTGCATGCACGTC-3') and antisense (5'-TTATTCATCTATCATTTATTTCTTC-3') primers. PCR products were cloned into pPCR-Script Amp SK<sup>+</sup> and sequenced from both directions at the DNA Sequencing Facility of the Albert Einstein College of Medicine. The long form of the H-2K<sup>wm7</sup> cDNA was subsequently cloned into pcDNA3.1<sup>+</sup> for expression in mammalian cells. The portion of the H-2K<sup>wm7</sup> cDNA encoding the putative extracellular domain was cloned into pET-3a for expression in bacteria.

**Generation of a cell line stably expressing H-2K<sup>wm7</sup>**

Hygromycin-resistant human C1R-A2.1 cells (21), kindly provided by Dr. V. Engelhard, were electroporated with linearized H-2K<sup>wm7</sup>/pcDNA3.1<sup>+</sup> using a Bio-Rad Gene Pulser (0.21 kV, 960 μF). Stable C1R-A2.1/K<sup>wm7</sup> transfectants were selected for growth in 2 mg ml<sup>-1</sup> G418. Growing cells were analyzed for H-2K<sup>wm7</sup> expression by flow cytometry using the mouse mAb HD25 (22) and FITC-conjugated goat anti-mouse IgG<sub>1</sub> + IgM. For the experiments depicted in Fig. 5, surface H-2K<sup>wm7</sup> expression was monitored by flow cytometry using the H-2K<sup>wm7</sup>-specific mouse mAbs, HD25 (IgG<sub>2b</sub>), HD24 (IgG<sub>2a</sub>) or HD42 (IgM) (22), and FITC-conjugated rat anti-mouse IgG<sub>2b</sub>, IgG<sub>2a</sub> or IgM, respectively.

**Immunoadfinity purification of H-2K<sup>wm7</sup>**

H-2K<sup>wm7</sup> molecules were immunoadfinity purified from 1 x 10<sup>9</sup> C1R-A2.1/K<sup>wm7</sup> cells using mAb HD25 (22) and their associated peptides were extracted as described in ref. (23).

**Mass spectrometry**

Aliquots of peptides extracted from H-2K<sup>wm7</sup> molecules were loaded onto capillary precolumns (360 μm o.d. x 75 μm i.d.; Polymeric Technologies, Phoenix, AZ, USA) packed with irregular C<sub>18</sub> resin (5–20 μm, YMC, Inc.) and washed with 0.1% acetic acid (Sigma–Aldrich Chemical Co., St Louis, MO, USA) and then loaded onto analytical columns (360 μm o.d. x 50 μm i.d.; Polymeric Technologies) packed with regular C<sub>18</sub> resin (5 μm, YMC, Inc.) and constructed with an integrated electrospray emitter tip as described previously (24). Samples were analyzed by nanoflow HPLC (nHPLC)–microelectrospray ionization (μESI) coupled to a home-built Fourier-transform (FT) ion cyclotron resonance mass spectrometer or a hybrid linear quadrupole ion trap LTQ-FT mass spectrometer (Thermo Scientific, San Jose, CA, USA). The gradient used on an Agilent (Palo Alto, CA, USA) 1100 series HPLC solvent delivery system consisted of 0–100% B in 17 min [A, 0.1 M acetic acid (Sigma–Aldrich Chemical Co., St Louis, MO, USA); B, 0.1 M acetic acid in 70% acetonitrile (Malinckrodt, Inc., St Louis, MO, USA)].
In vitro assembly and purification of H-2Kwm7 kindly provided by Dr T. Shiroishi. Hybridoma cell lines producing HD25, HD24 and HD42 were mouse mAbs HD25 (IgG2b), HD24 (IgG2a) or HD42 (IgM). Peptides (VNDIFERI, IQQSIERL and VNDIFEAI) were purchased from Mimotopes (Raleigh, NC, USA) and dissolved in DMSO. β2m and peptide were added rapidly into 1 l of refolding buffer composed of 100 mM Tris–HCl, 0.4 M arginine–HCl, 2 mM EDTA, 5 mM reduced glutathione and 0.5 mM oxidized glutathione, pH 8.5. Heavy chain was added to the refold buffer in three aliquots 8–12 h apart. Heavy chain, β2m and peptide were used at a molar ratio of 1:2:10. The refolded material was concentrated and then purified on a Superdex G75 gel filtration column with 10 mM Tris–HCl, 50 mM NaCl, pH 8.5 as the running buffer. The purified material was concentrated to 4–6 mg ml⁻¹ for crystallization trials.

Crystallization and data collection

Diffraction quality crystals of H-2Kwm7 were obtained through sitting drop vapor diffusion by mixing 1 μl of complex at 4–6 mg ml⁻¹ with 1 μl crystallization buffer (15% PEG 3350, 100 mM HEPES, pH 7.0 for VNDIFERI complex; 25% PEG 3350, 200 mM ammonium sulfate, 100 mM Tris, pH 8.5 for IQQSIERL and 20% PEG 3000, 100 mM HEPES, pH 7.5, 200 mM NaCl for VNDIFEAI). Each drop was equilibrated against 100 μl of crystallization buffer at 18°C resulting in crystal formation within 1 day. Crystals of the first two complexes were dehydrated over 50% PEG 3350 for 3 days, harvested through paraffin oil and flash cooled in liquid N₂. Crystals of the H-2Kwm7–VNDIFEAI complex were first transferred into the mother liquor containing 20% sucrose and then flash cooled in liquid N₂. Diffraction data for the H-2Kwm7–IQQSIERL and H-2Kwm7–VNDIFEAI complexes were collected at 2.1 and 2.04 Å resolution, respectively, at beamline X-29A, National Synchrotron Light Source (Upton, NY, USA) using an ADSC-315 detector (Area Detector Systems Corporation, Poway, CA, USA). For the VNDIFERI complex, diffraction data to 2.5 Å were recorded on a Rigaku R-Axis IV++ image plate detector coupled to a RU-H3R rotating anode X-ray generator operating at 50 kV and 100 mA and equipped with Osmino Blue optics. Data were integrated and scaled with the HKL-2000 suite (29). Anisotropy in the diffraction data was assessed and corrected with the Diffraction Anisotropy Server (30). Diffraction from crystals of all three complexes was consistent with the monoclinic space group C2₁.

The structure of H-2Kwm7–VNDIFERI was determined by molecular replacement with MOLREP (31), using the 90% identical H-2Kβ-VSV complex (PDB identification number 1KPU) as a search model. The structures of the H-2Kwm7–IQQSIERL and H-2Kwm7–VNDIFEAI complexes were determined using the refined H-2Kwm7–VNDIFERI structure as an initial search model. Refinement was carried out by iterative cycles of rebuilding in COOT (32) and refinement by REFMAC5 (33). The refined structures of the H-2Kwm7–VNDIFERI, H-2Kwm7–IQQSIERL and H-2Kwm7–VNDIFEAI complexes were deposited with the Protein Data Bank (http://www.rcsb.org/pdb/) with PDB entry codes 3F0L, 3FOM and 3FON, respectively.

Peptide synthesis

Peptides were prepared by standard fluorenylmethoxycarbonyl chemistry using an AAPPTEC (model APEX 396, Louisville, KY, USA) peptide synthesizer. Peptides were purified to >90% by reversed-phase HPLC, 0–80% solvent B in 10 min [A, 0.1% trifluoroacetic acid (Applied Biosystems, Warrington, UK); B, 0.085% trifluoroacetic acid in 100% acetonitrile], on a Poros R2/H 4.6 × 100 mm column (PerSeptive Biosystems, Framingham, MA, USA). Sequences and purity of all synthetic peptides were confirmed by matching MS/MS spectra acquired on a LTQ (Thermo Scientific) by searching against Human Protein Reference Database (http://www.hprd.org) (26).

Cell-based peptide-binding assay

A previously reported cell-based peptide-binding assay (27) was modified for use with H-2Kwm7. C1R-A2.1/Kwm7 cells were washed twice with PBS (pH 7.2) and then resuspended in 1 ml ice-cold citric acid solution (0.131 M citric acid, 0.066 M Na₂HPO₄, pH 3.0). After 1 min, 49-ml serum-free DMEM was added, and cells were pelleted and washed once with serum-free DMEM. Cells were seeded in serum-free medium in the presence of 15 μg ml⁻¹ human β2m-microglobulin (β2m) and 1 μM peptide. Cells were incubated at 4°C overnight, and surface H-2Kwm7 expression was monitored by flow cytometry using the H-2Kwm7–specific mouse mAbs HD25 (IgG₂b), HD24 (IgG₂a) or HD42 (IgM) (22) and FITC-conjugated goat anti-mouse IgG + IgM. The hybridoma cell lines producing HD25, HD24 and HD42 were kindly provided by Dr T. Shiroishi.

In vitro assembly and purification of H-2Kwm7

H-2Kwm7 was refolded as previously described for HLA-A2 with minor modifications (28). Briefly, inclusion bodies for the extracellular domain of the H-2Kwm7 heavy chain and for β2m were produced in Escherichia coli and solubilized separately at 1 mg ml⁻¹ in buffer composed of 6 M guanidine-hydrochloride, 10 mM sodium acetate (pH 4.6), 5 mM EDTA. Peptides (VNDIFERI, IQQSIERL and VNDIFEAI) were purchased from Mimotopes (Raleigh, NC, USA) and dissolved in DMSO. β2m and peptide were added rapidly into 1 l of refolding buffer composed of 100 mM Tris–HCl, 0.4 M arginine–HCl, 2 mM EDTA, 5 mM reduced glutathione and 0.5 mM oxidized glutathione, pH 8.5. Heavy chain was added to the refold buffer in three aliquots 8–12 h apart. Heavy chain, β2m and peptide were used at a molar ratio of 1:2:10. The refolded material was concentrated and then purified on a Superdex G75 gel filtration column with 10 mM Tris–HCl, 50 mM NaCl, pH 8.5 as the running buffer. The purified material was concentrated to 4–6 mg ml⁻¹ for crystallization trials.

Predominant occupation of H-2Kwm7 with one peptide
Solvent accessibility, gap volume index and hydrogen bond interactions were calculated with the Protein–Protein Interaction Server (http://www.bioinformatics.sussex.ac.uk/protorip/). Structures were superimposed by Lsqkab (34), sequence alignments were carried out at the Biology WorkBench (http://workbench.sdsc.edu) (35) and van der Waals contacts were calculated with Contact (34). Figures were generated with PyMOL (http://www.pymol.org).

Results

Characterization of peptides presented by H-2K\textsuperscript{wrm7}

We first used a biochemical approach to characterize the T1D-protective H-2K\textsuperscript{wrm7} molecule. H-2K\textsuperscript{wrm7} was immunoaffinity purified from C1R-A2.1/K\textsuperscript{wrm7} cells using the H-2K\textsuperscript{wrm7}-specific mAb HD25 (22) and extracted with acid as described in Methods. CAD MS/MS spectra of class I peptides from 7.5 × 10\textsuperscript{7} cells were obtained on the most abundant precursor ions within the range of 300–600 m/z. Figure 1(A) shows a CAD spectrum for the most abundant of these, the doubly protonated peptide ion 503.2704 m/z. The sequence was identified as VNDXFERX, where X represents either I or L. Since the isobaric amino acids I and L are identical in mass and indistinguishable by our mass spectrometry method, X was assigned to represent either of these two amino acids at first. When searched against the human RefSeq database, the sequence matched a peptide derived from histone H2B and having the sequence VNDIFERI. Thus, both Xs were assigned as I. Using this approach, 13 H-2K\textsuperscript{wrm7}-associated peptides were identified (Table 1). Three of these (VNDIFERI, IQOSIERL and VNIPFVRL) were chosen for synthesis. The MS/MS spectra and HPLC retention times of these H-2K\textsuperscript{wrm7} peptides compared favorably with their synthetic versions (Fig. 1A and data not shown). We also confirmed that these three peptides were indeed capable of binding H-2K\textsuperscript{wrm7} by adapting a previously described cell-based peptide-binding assay (27) for use with this MHC molecule. Surface H-2K\textsuperscript{wrm7} molecules were denatured by a brief treatment of C1R-A2.1/K\textsuperscript{wrm7} with citric acid (pH 3.0). H-2K\textsuperscript{wrm7} molecules were then allowed to refold at 4°C upon incubation with β2m and one of the three test peptides and detected by flow cytometry using the H-2K\textsuperscript{wrm7}-specific mAb HD25 (22). All of the peptides examined were found to bind H-2K\textsuperscript{wrm7} in this cell-based assay (Fig. 1B).

An aliquot of a synthetic peptide (DRVYIHPFHL) was added to the extracted H-2K\textsuperscript{wrm7}-associated peptides and analyzed by MS so that a correlation between ion current signal and peptide quantity could be defined (38). Although VNDIFERI had a strong ion current signal corresponding to 367 fmol, all other peptides were present in far lower amounts, ranging in abundance from 0.5–10 fmol. We calculated that within the 7.5 × 10\textsuperscript{7} cells analyzed, VNDIFERI was present at 2949 copies per cell, whereas the other peptides identified were present at <100 copies per cell (Fig. 1C; Table 1). Thus, H-2K\textsuperscript{wrm7} binds mainly the histone-derived peptide VNDIFERI.

Twelve of the thirteen H-2K\textsuperscript{wrm7}-associated peptides sequenced were 8mers, and the single 9mer represented an N-terminal-extended variant of VNDIFERI. Thus, the preferred length of peptides presented by H-2K\textsuperscript{wrm7} is eight residues, as is the case for H-2K\textsuperscript{b}, which is 90% identical to H-2K\textsuperscript{wrm7}.

### Table 1. Sequences of H-2K\textsuperscript{wrm7}-associated peptides

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<th>Copies Experimental m.w. (Da)</th>
<th>Calculated Sequence m.w. (Da)</th>
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<th>Accession number</th>
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Peptide sequences were determined by accurate mass (within 3 p.p.m.). CAD peptide assignment and database protein matches are listed. Bolded peptides were synthesized for sequence confirmation. Leu or Ile were assigned according to a match to a sequence in the database.

aFor assessment of copies per cell, an aliquot of the synthetic peptide DRVYIHPFHL (Sigma–Aldrich) was spiked into the H-2K\textsuperscript{wrm7}-associated peptides extracted from 7.5 × 10\textsuperscript{7} cells before the MS run. One hundred fmoles DRVYIHPFHL correlates to 9.7E6 ion current signal by peak height.
Fig. 1. Characterization of H-2K<sup>wm7</sup>-associated peptides. (A) Mass spectrometric sequence determination of peptide VNDIFERI. From H-2K<sup>wm7</sup>-eluted peptides, the doubly charged ion at 503.27 m/z was selected for CAD fragmentation, and a CAD mass spectrum was obtained (upper panel). Calculated masses for type b and type y ions are shown above and below the deduced sequence, respectively. The b ions originate from the N-terminus of the peptide and the y ions originate from the C-terminus. Ions observed in the spectrum are underlined. X represents I or L, which were later assigned based on a database match to peptide VNDIFERI derived from human histone H2B. The CAD mass spectrum of the corresponding synthetic peptide (lower panel) confirms the predicted sequence. (B) Synthetic versions of three of the H-2K<sup>wm7</sup>-eluted peptides were tested for binding to H-2K<sup>wm7</sup> using citric acid-stripped C1R-A2.1/K<sup>wm7</sup> cells. Stripped cells were incubated in serum-free medium with human β<sub>2</sub>m and peptide at 4°C overnight. Surface H-2K<sup>wm7</sup> expression was monitored by flow cytometry using the H-2K<sup>wm7</sup>-specific mAb HD25 and FITC-conjugated goat anti-mouse IgG + IgM. The irrelevant peptide used as a negative control was the H-2K<sup>d</sup>-binding peptide VNR-V7 (KYNKANVFL) (36). (C) An aliquot of a synthetic peptide was added to the extracted H-2K<sup>wm7</sup>-associated peptides and analyzed by MS so that a correlation between ion current signal and peptide quantity could be defined. Peptide abundances are plotted as copies per cell. The peptides are numbered in the order in which they appear in Table 1. Peptide 1 corresponds to VNDIFERI. (D) A sequence logo (http://www.cbs.dtu.dk/~gorodkin/appl/plogo.html) (37) was generated using the sequences of the twelve 8mer H-2K<sup>wm7</sup>-eluted peptides (Table 1). At each position of the peptide, the sequence logo arranges the amino acids in order of predominance from top to bottom (i.e., the amino acid found most frequently is at the top). The relative heights of the amino acids at a given position reflect their relative frequencies (i.e., a large letter indicates a high-frequency amino acid).
As depicted by the sequence logo (37) in Fig. 1(D), alignment of the 8mer peptides revealed several conserved residues, suggesting the presence of multiple anchor positions. The most notable conserved residues were R at peptide position 7 (P7), which was found in 11 of 12 sequences, and L or I at P8, found in all of the peptides sequenced. Conservation was also observed at P5, which was always occupied by the hydrophobic residues F, I or L. We hypothesized that these conserved positions would function as the anchor residues for peptides binding to H-2K\(^{wm7}\).

Crystal structure of H-2K\(^{wm7}\)

As reviewed in refs (39, 40), crystallographic analysis of class II MHC molecules associated with autoimmune disease susceptibility or resistance has been very useful in suggesting mechanisms to account for their disease-inducing or protective effects. To determine whether crystallography would similarly provide an explanation for the diabetes-protective effect of the class I MHC molecule H-2K\(^{wm7}\) and also to determine whether P5, P7 and P8 were indeed the anchor residues for H-2K\(^{wm7}\)-binding peptides, the structure of H-2K\(^{wm7}\)-VNDIFERI was determined to 2.5 Å resolution. The structure showed good stereochemistry with 99.4% of all residues within the allowed region of the Ramachandran plot (Table 2). H-2K\(^{b}\) and H-2K\(^{d}\), the allele naturally present in NOD mice, share 90 and 85% sequence identity with H-2K\(^{wm7}\), respectively (Fig. 2A and B). Structural comparison of H-2K\(^{b}\) (PDB accession number 1KPU) and H-2K\(^{d}\) (PDB accession number 1VGK) with H-2K\(^{wm7}\)-VNDIFERI shows that the quaternary structure of H-2K\(^{wm7}\) conforms to the canonical MHC fold (Fig. 3A) with root mean square (r.m.s.) deviations between all Ca atoms of the entire complex of 1.4 Å and 1.1 Å, respectively. The r.m.s. deviations based on superposition of individual domains are similar for both H-2K\(^{b}\) and H-2K\(^{d}\) and do not exceed 1.0 Å (for heavy chains), suggesting that the larger r.m.s. deviation seen for the entire H-2K\(^{b}\) complex is due to the slight rearrangement of the domains relative to each other.

In the case of H-2K\(^{wm7}\)-VNDIFERI, we found that the peptide is anchored to the MHC molecule primarily by the P2, P5 and P8 residues, with their side chains deeply buried inside the peptide-binding groove (Fig. 3B). P1 lies horizontally within a relatively shallow ‘A pocket’, with its main chain atoms making van der Waals contacts (4 Å cutoff) with L5, Y7, Q63, Y159 and Y171, and its side chain contacting Q63, W167 and Y171. The shallowness of the A pocket and the modest steric restrictions present at the N-terminal end of the binding groove would potentially allow peptides longer than 8mers to bind. Evidence for extended peptides was provided by our MS analysis of H-2K\(^{wm7}\)-binding peptides (Table 1), where a variant of the VNDIFERI peptide
extended at the N-terminus was observed. In contrast, P8 is the most buried residue, fitting into a deep ‘F pocket’. The buried side chain interacts with D77, R97, T143 and K146. The main chain also makes multiple contacts with T80, Y84, T143, W147 and a water molecule. The depth and extent of burial would appear to preclude extensions of a peptide epitope in the C-terminal direction.

The aromatic side chain of P5 fills the ‘C pocket’ and makes van der Waals contacts with V9, N70, R97, R99, Q114 and two water molecules. Though the phenyl ring lies sandwiched between the guanidinium groups of R97 and R99 with all three groups almost coplanar, it does not appear to form strong cation–π interactions with these residues.

Along with hydrophobic interactions, a network of 22 hydrogen bonds holds the peptide within the peptide-binding groove, with the majority of these involving the peptide main chain. The most interesting components of the hydrogen-bonding network are provided by R97 and R99, as these residues are altered in the closely related H-2Kb. Though R97 accounts for several van der Waals contacts, it only makes a single hydrogen bond to the main chain of P6. R99, on the other hand, is present in the center of the binding groove and forms potential hydrogen bonds with the main chain at P3 and P4, as well as with the Asn side chain at P2. Within the binding pocket, the amide group of the P2 side chain also forms a hydrogen bond with the E24 carboxylate.

The peptide-binding groove of H-2Kwm7 is formed by contributions from 36 residues, but VNDIFERI makes direct contacts with only 24 of these. Due to the high sequence identity and structural similarity with H-2Kb, many of the interfacial residues are also conserved; however, nine residues are different. The most notable change in the binding groove is the absence of the P6 hydrophobic ridge created by F74 and Y116 in H-2Kb, which is replaced by a large positively charged ridge created by R97 and R99 flanking P5 in H-2Kwm7. Also, K66 and T163 in the P3 region are changed to I and A, respectively, thus increasing the hydrophobic nature of this region.

Our crystallographic analysis of H-2Kwm7–VNDIFERI provided an explanation for the conservation observed at P5 and P8 of H-2Kwm7-eluted peptides (Table 1; Fig. 1D), as these residues were found to function as anchors (along with P2) (Fig. 3). Surprisingly, however, crystallographic analysis did not provide an explanation for the

### Table 2. Crystallographic data collection and refinement statistics

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<th>H-2Kwm7–VNDIFERI</th>
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<td>237 115</td>
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<td>27 824 (908)</td>
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*aParentheses indicate statistics for the high-resolution data bin for x-ray and refinement data.

*bRsym = Σhk|Ihk|−|Ihk|/Σhk|Ihk|.

*cRcryst = Σhk|Fhk| − |Fc|/Σhk|Fc|, where Fhk and Fc are the observed and calculated structure factors for the working set of reflections, respectively. Rfree is calculated from the test set of reflections.
remarkable conservation of Arg at P7 of the peptides purified from H-2K\textsuperscript{wm7} molecules (Table 1; Fig. 1D), as this residue was found to be solvent exposed rather than serving as an anchor (Fig. 3B and C). This finding was confirmed by our structure of the H-2K\textsuperscript{wm7}–IQQSIERL complex (Table 2), which also revealed P7 to be solvent exposed (Fig. 3B).

Structural and binding studies using Ala-substituted peptides

The guanidinium group of Arg at P7 of VNDIFERI forms a hydrogen bond with the side chain hydroxyl of S73 of the H-2K\textsuperscript{wm7} heavy chain. Thus, although Arg at P7 is considerably solvent exposed, it could still make contributions to the binding of peptides to H-2K\textsuperscript{wm7}, thus accounting for its conservation among H-2K\textsuperscript{wm7}-binding peptides. To examine this possibility, we generated complexes of H-2K\textsuperscript{wm7} bound to VNDIFEAI, the P7-substituted Arg\textrightarrow{}Ala version of VNDIFERI, and determined its structure. Notably, the structure of H-2K\textsuperscript{wm7}/VNDIFEAI (Fig. 3D) demonstrated that the peptide was bound in a manner similar to the VNDIFERI complex (Fig. 3C), which suggested that the presence of Arg at P7 was not an important determinant for H-2K\textsuperscript{wm7} binding.

We next examined whether Arg\textrightarrow{}Ala-substituted versions of VNDIFERI and IQQSIERL were capable of stabilizing H-2K\textsuperscript{wm7} in our cell-based assay. Surprisingly, using HD25 to detect H-2K\textsuperscript{wm7} complex formation, it appeared that the Ala-substituted peptides were unable to stabilize this...
the H-2K\textsuperscript{wm7} epitope recognized by the HD25 antibody. If so, this would explain the failure of the antibody to detect complexes formed with the Ala-substituted peptides in the cell-based assay (Fig. 4, left panel). To test this hypothesis, we repeated the cell-based peptide-binding experiments, but this time using two other H-2K\textsuperscript{wm7}-specific antibodies. Although one of these (HD24) gave identical results to that obtained with HD25 (Fig. 4, left and center panels), a third antibody (HD42) detected H-2K\textsuperscript{wm7} complexes formed with either the Arg or Ala peptides (Fig. 4, right panel). Taken together, our results suggest that the Arg at position 7 of the peptide is indeed part of the H-2K\textsuperscript{wm7} epitope recognized by the H-2K\textsuperscript{wm7}-specific antibodies HD24 and HD25.

Our peptide analysis (Table 1) was conducted using H-2K\textsuperscript{wm7} molecules purified using the HD25 antibody. Thus, it was possible that the peptides identified were representative of only a small fraction of the entire repertoire. If this were the case, one would expect poor binding of HD25 to H-2K\textsuperscript{wm7}-expressing cells compared with that observed with HD24. To address this issue, we used flow cytometry to compare the binding of these antibodies with C1R-A2.1/K\textsuperscript{wm7} cells. Although only semi-quantitative comparisons can be made due to potential differences in the affinities of each of the primary and secondary reagents, we found robust and similar staining with HD25 and HD42 (Fig. 5). These findings demonstrate that a considerable fraction of H-2K\textsuperscript{wm7} molecules is indeed occupied by peptides having Arg at P7. Our biochemical analysis suggests the predominance of VNDIFERI, the histone H2B peptide found to be a mechanism for its diabetes-protective effect, as it has been accepted for some time that expression of particular class I MHC molecules to bind T1D-inciting peptides that other alleles cannot (68). Transgenic expression of the class II MHC molecules I-A\textsuperscript{g7} and related molecules to bind T1D-inciting \( \beta \)-cell peptides that other alleles cannot (68). Transgenic expression of the class II MHC molecules I-A\textsuperscript{g7} and related molecules to bind T1D-inciting \( \beta \)-cell peptides that other alleles cannot (68). Transgenic expression of the class II MHC molecules I-A\textsuperscript{g7} and related molecules to bind T1D-inciting \( \beta \)-cell peptides that other alleles cannot (68). Transgenic expression of the class II MHC molecules I-A\textsuperscript{g7} and related molecules to bind T1D-inciting \( \beta \)-cell peptides that other alleles cannot (68). Transgenic expression of the class II MHC molecules I-A\textsuperscript{g7} and related molecules to bind T1D-inciting \( \beta \)-cell peptides that other alleles cannot (68).

Predominant occupation of H-2K\textsuperscript{wm7} with one peptide

**Discussion**

A variety of investigations using the NOD mouse model have demonstrated that CD8\textsuperscript{+} T cells are essential for T1D development (41–50). The study of these class I MHC-restricted \( \beta \)-cell cytotoxic T cells has been greatly facilitated in the past decade by the discovery of some of the antigens that they recognize (51–53), which include insulin (53) and islet-specific glucose-6-phosphatase catalytic subunit-related protein (51). Importantly, islet-infiltrating CD8\textsuperscript{+} T cells are also prevalent in pancreatic biopsies from graft-recurrent and new-onset T1D patients (54, 55), and CD8\textsuperscript{+} T cells specific for these same antigens have been detected in the peripheral blood of patients (56–64). These findings, combined with the recent mapping of T1D susceptibility to particular human class I MHC alleles (11), suggest the need to elucidate the mechanisms by which certain class I MHC molecules can positively or negatively influence the development of T1D. Although this is an understudied area, multiple groups have investigated the mechanisms by which class II MHC molecules can determine susceptibility or resistance to T1D, as it has been accepted for some time that expression of particular class II MHC molecules is a major determinant of T1D susceptibility in both NOD mice and humans.

NOD mice express a single class II MHC molecule, I-A\textsuperscript{g7}, which is unusual in that residue 57 of the \( \beta \) chain is Ser, instead of the far more common Asp. Remarkably, human class II molecules that confer susceptibility to T1D are also characterized by the absence of Asp at \( \beta \)-7. Crystal structure analyses indicate that this results in a P9 pocket that is basic (65–67), perhaps allowing I-A\textsuperscript{g7} and related molecules to bind T1D-inciting \( \beta \)-cell peptides that other alleles cannot (68). Transgenic expression of the class II MHC molecules I-A\textsuperscript{g7} (69), I-A\textsuperscript{d7} (70) or I-E (71, 72) in NOD mice results in protection from disease, despite the co-expression of I-A\textsuperscript{g7} in these strains. Multiple mechanisms have been proposed to explain this protection, including positive selection of regulatory T cells (70), thymic deletion of autoreactive CD4\textsuperscript{+} T cells (73), alterations in the cytokines produced by \( \beta \) cell-autoreactive T cells (71), occupation of the I-A\textsuperscript{g7} peptide-binding groove with peptides...
HD25  HD24  HD42

![Fig. 5. Both peptide-dependent and peptide-independent anti-H-2K\textsuperscript{wm7} antibodies show robust binding to C1R-A2.1/K\textsuperscript{wm7} cells. Surface H-2K\textsuperscript{wm7} expression in C1R-A2.1/K\textsuperscript{wm7} cells was monitored by flow cytometry using the mAbs HD25 (IgG\textsubscript{2b}), HD24 (IgG\textsubscript{2a}) or HD42 (IgM) and FITC-conjugated rat anti-mouse IgG\textsubscript{2b}, IgG\textsubscript{2a} or IgM, respectively. C1R-A2.1 cells were used as a negative control.](image)

Derived from the protective transgenic MHC molecules (74) and the capture or ‘stealing’ of T1D-relevant peptide epitopes by the protective molecules (75, 76).

In contrast, our work suggests a novel mechanism for the T1D-protective effect conferred by the class I MHC molecule H-2K\textsuperscript{wm7}, as multiple lines of evidence indicate that this MHC molecule is predominantly occupied by a single self-peptide. First, analysis of peptides eluted from H-2K\textsuperscript{wm7} molecules revealed the presence of a peptide derived from histone H2B that is one to three orders of magnitude more abundant than all other H-2K\textsuperscript{wm7}-eluted peptides (Fig. 1C; Table 1). Second, the Arg present at P7 of H-2K\textsuperscript{wm7}-eluted peptides appears to be part of the epitope for the two different H-2K\textsuperscript{wm7}-specific antibodies HD24 and HD25. These antibodies were obtained from immunization of B10 × B10.A mice (H-2K\textsuperscript{b}, H-2K\textsuperscript{k}) with spleen and lymph node cells from B10.MOL-SGR mice (H-2K\textsuperscript{wm7}). The finding that both antibodies see the Arg at P7 of the peptide as part of their epitope suggests that the cells used as the immunogen were abundantly displaying one particular peptide in the context of H-2K\textsuperscript{wm7}. Third, despite their peptide dependence, both antibodies were previously characterized as being H-2K\textsuperscript{wm7}-specific using a complement-dependent microcytotoxicity test that employed lymph node cells from H-2K\textsuperscript{wm7}-expressing mice as targets (22). Finally, both antibodies show robust binding to C1R-A2.1/K\textsuperscript{wm7} cells (Fig. 5), further supporting the idea that H-2K\textsuperscript{wm7} is predominantly occupied by a single histone-derived peptide. Interestingly, the mice that were immunized to make the HD24 and HD25 antibodies expressed H-2K\textsuperscript{b} and H-2K\textsuperscript{k}, both of which are related to H-2K\textsuperscript{wm7} (Fig. 2A). Thus, they would have been tolerant to regions of H-2K\textsuperscript{wm7} that were similar in H-2K\textsuperscript{b} or H-2K\textsuperscript{k}, leaving the immune system to focus instead on an epitope comprised of both P7 of the histone H2B-derived peptide and residues of the H-2K\textsuperscript{wm7} heavy chain. In contrast, the B10 mice that were immunized to make the HD42 antibody (which recognizes H-2K\textsuperscript{wm7} complexes with peptides having Arg or Ala at P7 and does not seem to require a particular peptide sequence for recognition of H-2K\textsuperscript{wm7}) expressed only H-2K\textsuperscript{b}, presumably increasing the regions of the H-2K\textsuperscript{wm7} heavy chain that did not appear as self. Taken together, our work supports the idea that H-2K\textsuperscript{wm7} presents a limited array of peptides in vivo, due to its preferential occupation by VNDIFERI.

Although further investigation is indicated, our results suggest that H-2K\textsuperscript{wm7} exerts its T1D-protective effect by primarily binding the histone H2B-derived peptide VNDIFERI. This could have multiple consequences that could influence the development of T1D. For example, it is possible that peptides derived from β-cell autoantigens cannot adequately compete with the abundant histone H2B peptide for binding to H-2K\textsuperscript{wm7} and, thus, cannot be presented to T cells in quantities sufficient to elicit a destructive autoimmune response. The partial protection observed in H-2K\textsuperscript{d}/H-2K\textsuperscript{wm7} heterozygotes (13), which presumably express half the normal amount of H-2K\textsuperscript{d}, is consistent with the idea that H-2K\textsuperscript{d} can present peptides from disease-relevant antigens, whereas H-2K\textsuperscript{wm7} cannot. Though it is not technically feasible to purify and sequence H-2K\textsuperscript{wm7}-binding peptides from islets, as the quantity of material required would be prohibitive, we can say with certainty that histone H2B is present in islets, as it is one of the four core histones that comprise the nucleosome, and proteomic analysis of pancreatic islets has indeed confirmed its presence there (77). Although histones are generally thought of as being synthesized during the S phase of the cell cycle, certain variants of the histones, including H2B, are expressed in a cell cycle-independent manner and in differentiated cells (78, 79). In mice, histone H2B is encoded by 16 non-allelic genes, and in all of these, histone H2B can be ubiquitinated, which presumably express half the normal amount of H-2K\textsuperscript{d}, is consistent with the idea that H-2K\textsuperscript{d} can present peptides from disease-relevant antigens, whereas H-2K\textsuperscript{wm7} cannot. Though it is not technically feasible to purify and sequence H-2K\textsuperscript{wm7}-binding peptides from islets, as the quantity of material required would be prohibitive, we can say with certainty that histone H2B is present in islets, as it is one of the four core histones that comprise the nucleosome, and proteomic analysis of pancreatic islets has indeed confirmed its presence there (77). Although histones are generally thought of as being synthesized during the S phase of the cell cycle, certain variants of the histones, including H2B, are expressed in a cell cycle-independent manner and in differentiated cells (78, 79). In mice, histone H2B is encoded by 16 non-allelic genes, and in all of these, the H-2K\textsuperscript{wm7}-binding peptide VNDIFERI is conserved (80). Furthermore, histone H2B can be ubiquitinated, which should facilitate its entrance into the class I MHC antigen processing pathway (81).

In addition to influencing presentation of autoantigenic peptides by β cells, predominant occupation of H-2K\textsuperscript{wm7} by a single peptide would also be expected to have an impact on thymic T-cell selection. For example, using fetal thymic organ culture and thymi from TAP-1-deficient mice, Ashton-Rickardt et al. (82) demonstrated that self-peptides do indeed help to shape the repertoire of positively selected CD8\textsuperscript{+} T cells. Thus, one hypothesis to potentially explain the diabetes-protective effect of H-2K\textsuperscript{wm7} is that it is unable to select a diabetogenic CD8\textsuperscript{+} T-cell repertoire. Finally, it should be acknowledged that effects on other lymphocyte
populations, such as NK cells, could also be responsible for the T1D-protective properties of H-2K\textsuperscript{wm7}. Certain human and mouse NK cell inhibitory receptors have been shown to exhibit peptide-dependent recognition of class I MHC molecules (83, 84). Thus, predominant occupation of H-2K\textsuperscript{wm7} by a single peptide could influence NK cell activity, and these cells have been shown to influence the pathogenesis of T1D in multiple animal model systems (85–87).

In humans, T1D susceptibility can be localized to the HLA-A and HLA-B genes, and certain alleles are protective (11). These findings should stimulate further investigation of the biochemical and structural characteristics of protective class I MHC alleles. As has been found for class II MHC alleles, it is likely that multiple mechanisms of protection will ultimately be identified.

**Funding**

National Institutes of Health (AI033993 to D.F.H., AI007289 to S.G.N. and S.C.A., DK064315 and DK052956 to T.P.D., P60DK020541 to Albert Einstein College of Medicine's Diabetes Research and Training Center); Juvenile Diabetes Research Foundation (to T.P.D.); Irma T. Hirschl/Monique Weill-Caulier Trust (to T.P.D.); the flow cytometry facility at Albert Einstein College of Medicine is supported by National Institutes of Health Cancer Center (P30CA013330).

**Acknowledgements**

The authors thank Qingrong Yan for the preparation of Figure 3.

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