

Structural Basis for the Specific Recognition of the Major Antigenic Peptide from the Japanese Cedar Pollen Allergen Cry j 1 by HLA-DP5

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Abstract

The major allergen, Cry j 1, was isolated from Japanese cedar *Cryptomeria japonica* (Cry j) pollen and was shown to react with immunoglobulin E antibodies in the sera from pollinosis patients. We previously reported that the frequency of HLA-DP5 was significantly higher in pollinosis patients and the immunodominant peptides from Cry j 1 bound to HLA-DP5 to activate Th2 cells. In the present study, we determined the crystal structure of the HLA-DP5 heterodimer in complex with a Cry j 1-derived nine-residue peptide, at 2.4 Å resolution. The peptide-binding groove recognizes the minimal peptide with 10 hydrogen bonds, including those between the negatively charged P1 pocket and the Lys side chain at the first position in the peptide sequence. We confirmed that HLA-DP5 exhibits the same Cry j 1-binding mode in solution, through pull-down experiments using structure-based mutations of Cry j 1. We also identified the characteristic residues of HLA-DP5 that are responsible for the distinct properties of the groove, by comparing the structure of HLA-DP5 and the previously reported structures of HLA-DP2 in complexes with pDRA of the self-antigen. The comparison revealed that the HLA-DP5·pCry j 1 complex forms several hydrogen bond/salt bridge networks between the receptor and the antigen that were not observed in the HLA-DP2·pDRA complex. Evolutionary considerations have led us to conclude that HLA-DP5 and HLA-DP2 represent two major groups of the HLA-DP family, in which the properties of the P1 and P4 pockets have evolved and acquired the present ranges of epitope peptide-binding specificities.

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Introduction

Plant pollens are the most common causes of prevalent type I hypersensitivity (allergy) diseases,

which induce seasonal rhinitis and conjunctivitis in humans [1–3]. *Dactylis glomerata* and *Ambrosia artemisiifolia* (ragweed) pollens cause the two major hay fevers endured internationally, while

Cryptomeria japonica (Cry j, Japanese cedar) pollen is the most prevalent allergen in Japan [4], with respect to the number of pollinosis patients. In particular, the number of Japanese cedar pollinosis patients has rapidly increased since the 1960s in Japan [5], and presently, more than 25 million people suffer through the months from February to April, when the pollen is dispersed [6]. So far, three major allergens, Cry j 1 [7], Cry j 2 [8], and Cry j 3 [9], have been isolated from the cedar pollen and have been shown to react with immunoglobulin E (IgE) antibodies from the pollinosis patients [9,10]. Cry j 1 and Cry j 2 are 37–45 kDa basic glycoproteins [11], and they share 70–80% amino acid sequence identity with each other [11]. They were identified as a pectate lyase (isozyme) [12] and a polymethylgalacturonase (enzyme) [13], respectively.

IgE is responsible for inducing type I hypersensitivity (allergic) diseases [14]. Secretion of antibodies, including IgE, by plasma cells requires interactions between antigen-activated B cells and antigen-activated CD4⁺ type 2 helper T (Th2) cells, to allow the differentiation of plasma cells from B cells. CD4⁺ T cells are activated by the recognition of immunogenic peptides presented by class II human leukocyte antigen (HLA) molecules on the surface of antigen-presenting cells [15]. The allergen-specific IgE production completely depends on the class switch of the immunoglobulin gene in plasma cells, and this is mediated by a cytokine, interleukin-4, produced by CD4⁺ Th2 cells upon the recognition of allergen-derived peptides in the context of class II HLA molecules.

The class II HLA molecules are heterodimers of the α and β chains and are encoded by genes in the class II HLA region on the short arm of chromosome 6 [16]. The class II HLA molecules are classified into three isotypes, HLA-DR, HLA-DQ, and HLA-DP. In HLA-DR, the polymorphic variation is primarily provided by the β chain, rather than the α chain [17]. On the other hand, in HLA-DQ and HLA-DP, both the α and β chains are polymorphic, and thus, a variety of HLA-DQA/DQB and HLA-DPA/DPB haplotypes are formed [17]. The crystal structures of class II HLA complexes with non-self-peptides derived from a plant have been determined for the HLA-DQ2•deamidated-gliadin [18], the HLA-DQ2.3•Gamma-gliadin [19], and the HLA-DQ8•deamidated-glutein [20,21]. Nine specific amino acid residues in each peptide are accommodated in the peptide-binding groove, formed by the α and β chains.

We previously analyzed the statistical association between Japanese cedar pollinosis and class II HLA alleles and found that the patients possess the HLA-DP5 (*DPA1*02:02* and *DPB1*05:01*) at a rate of about 80% [22]. Furthermore, we also identified an immunodominant peptide that induces HLA-DP5-restricted Th2 cells, among 38 synthesized overlapping 13-residue peptides spanning the entire length of Cry j 1. The minimal peptide sequence for

the HLA-DP5-restricted Th cells was suggested to be nine residues, 214-KVTVAFNQF-222, of Cry j 1 [22]. Therefore, we thought that the HLA-DP5-associated susceptibility to Japanese cedar pollinosis may be explained by the following phenomenon: HLA-DP5-mediated antigen presentation of the major Cry j 1 epitope to Th2 cells producing interleukin-4, an essential cytokine for IgE production by antibody-secreting plasma cells. Subsequently, it was reported that a large number of Cry j 1 and Cry j 2 peptides were associated with various class II HLA allelic products. Among the HLA-DP family members, only HLA-DP5 and HLA-DP2 were shown to be associated with the Cry j antigenic peptides [23,24]. However, the structural mechanisms of specific peptide recognition are still unknown, for not only the peptides of the Cry j antigens but also those of other pollen antigens. The only peptide-bound HLA-DP structure available is that of HLA-DP2 (*DPA1*01:03* and *DPB1*02:01*) in complex with pDRA derived from the self-HLA-DR α chain [25].

In the present study, we determined the crystal structure of the extracellular region of the HLA-DP5 (*DPA1*02:02* and *DPB1*05:01*) heterodimer, in complex with the Cry j 1-derived nine-residue peptide, KVTVAFNQF, at 2.4 Å resolution. The peptide-binding groove specifically formed by the HLA-DP5 α and HLA-DP5 β chains captures the minimal peptide KVTVAFNQF with 10 hydrogen bonds, including those between the negatively charged P1 pocket and the Lys side chain at the first anchoring position in the peptide sequence. This binding mode was confirmed *in vitro*, by mutations of the Cry j 1 residues facing the peptide-binding groove of HLA-DP5 in the crystal structure. Furthermore, we identified the characteristic residues of HLA-DP5 that are responsible for the distinct properties of the groove, by comparing the structure of HLA-DP5 and the previously reported structure of HLA-DP2 (*DPA1*01:03* and *DPB1*02:01*) in complex with pDRA. The comparison revealed that the HLA-DP5•Cry j 1 complex forms several hydrogen bond/salt bridge networks between the receptor and the antigen that were not observed in the HLA-DP2•pDRA complex. Thus, the specific HLA-DP–antigenic peptide binding mechanism has been established.

Results

Overall structure of the complex of HLA-DP5 with the Cry j 1 peptide

We prepared a plasmid encoding the HLA-DP5 α chain extracellular region (residues 1–181) and the HLA-DP5 β chain extracellular region (residues 4–189) with the N-terminus fused to a nine-amino-acid residue

Table 1. Crystallographic data collection and refinement statistics

	HLA-DP5•Cry j 1(9-mer)
<i>Data collection</i>	
Space group	<i>P</i> 1
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	61.2, 64.4, 130.4
α , β , γ (°)	93.0, 97.5, 109.4
Resolution (Å)	64–2.4 (2.49–2.40) ^{a,b}
<i>R</i> _{sym}	0.116 (0.563)
<i>I</i> / σ <i>I</i>	12.3 (3.05)
Completeness (%)	97.4 (95.6)
Redundancy	3.5 (3.4)
<i>Refinement</i>	
Resolution (Å)	64.0–2.4
No. of reflections	70,445
<i>R</i> _{work} / <i>R</i> _{free}	0.201/0.237
No. of atoms	
Protein	12,380
Ligand	56
Water	267
<i>B</i> -factors	
Protein	29.1
Ligand	27.6
Water	29.8
rmsd	
Bond lengths (Å)	0.005
Bond angles (°)	1.07

^a One crystal was used for data collection and refinement.^b Values in parentheses are for the highest-resolution shell.

Cry j 1 peptide (KVTVAFNQF) and co-expressed the two chains by the baculovirus–insect cell expression system. It was assumed that the α and β chains of

HLA-DP5 form a “heterodimer” and that the peptide fused to the β chain is accommodated in the binding site formed by the two chains. The HLA-DP5•Cry j 1(9-mer) complex was purified and crystallized as described in [Materials and Methods](#), and the structure was determined at 2.4 Å resolution. The statistics for the data collection and the refined model is shown in [Table 1](#). The asymmetric unit of the HLA-DP5•Cry j 1(9-mer) crystal contains four complexes, *A*, *B*, *C*, and *D*. Each complex superposed on another with a root-mean-square deviation (rmsd) value of ~0.8 Å for the C α atoms. Therefore, the structure of complex *A* is described hereafter. The α and β chains consist of the α 1 and α 2 domains and the β 1 and β 2 domains, respectively ([Fig. 1a](#)). The α 1 and β 1 domains form the peptide-binding cleft, and the α 2 and β 2 domains form the heterodimeric interface ([Fig. 1a](#) and [b](#)). An *N*-acetyl-D-glucosamine moiety is attached to Asn118 (α 2 domain) in HLA-DP5•Cry j 1(9-mer) ([Fig. 1a](#)). The overall structures of the HLA-DP5 heterodimers are similar to those of the class II HLA heterodimers [26], including HLA-DP2.

In the HLA-DP5•Cry j 1(9-mer) structure, the nine residues of the accommodated Cry j 1 peptide (KVTVAFNQF) are clearly visible in the electron density map and occupy the p1-to-p9 binding pockets ([Fig. 1b](#) and [c](#)). Lys(p1), Phe(p6), and Phe(p9) are the “anchoring residues”, and their side chains are buried within their corresponding pockets formed along the peptide-binding groove of HLA-DP5 ([Fig. 1c](#)). In solution, the purified HLA-DP5•Cry j 1(9-mer) complex exists as a single

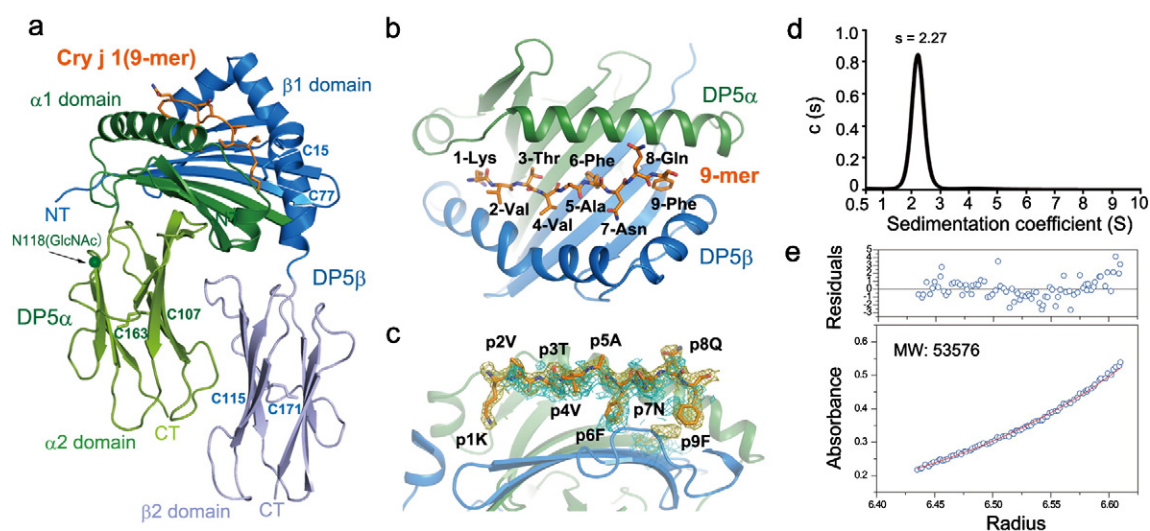


Fig. 1. Crystal structure of the HLA-DP5•Cry j 1 complex. (a–c) The HLA-DP5•Cry j 1(214–222) complex structure. Extracellular domains of the HLA-DP5α and HLA-DP5β chains are shown as ribbon models in green (α 1) and light green (α 2) and in blue (β 1) and light blue (β 2), respectively. The Cry j 1(9-mer) peptide is represented by orange sticks. (a) Overall structure. (b) Top views of the peptides in the peptide-binding groove. (c) Side views of the peptides in the peptide-binding groove. The $2F_o - F_c$ electron density maps, contoured at 1.5σ , are shown as a cyan mesh (before refinement) and an olive mesh (after refinement) within 1.0 Å of the peptide. (d and e) Analytical ultracentrifugation of the HLA-DP5•Cry j 1(9-mer) complex. (d) Distribution of sedimentation coefficients (*s*) by sedimentation velocity experiments. (e) A plot of the sedimentation equilibrium data with the residuals from the best fit to a single ideal species.

complex species, as revealed by analytical ultracentrifugation (Fig. 1d and e).

Binding mode of HLA-DP5•Cry j 1(9-mer)

The Cry j 1 peptide interacts extensively with the side chains from the HLA-DP5 groove, involving a total of 10 hydrogen bonds, with four formed by the p1, p7, and p8 side chains and six mediated by the p2, p4, p5, and p7 main chains of the peptide (Fig. 2a). Actually, in common mostly with the peptide•HLA-DR1 complexes, five hydrogen bonds are observed between the main chain of the Cry j 1 peptide and the side chains of HLA-DP5: p2 and Tyr9(α), p2 and Asn80(β), p4 and Asn62(α), p5 and Lys69(β), and p7 and Asn69(α). Moreover, the other

five hydrogen bonds are formed between the side chains of the peptide and HLA-DP5. This binding by an extensive hydrogen bonding network is in sharp contrast to the binding of the pDRA peptide with HLA-DP2 [25] (PDB ID: 3LQZ), which involves mostly hydrophobic interactions with only one hydrogen bond (Fig. 2b). A unique feature of the HLA-DP5•Cry j 1 peptide interactions is that the side chain of one of the anchoring residues, Lys(p1), hydrogen bonds with Gln31 α and Asp84 β (Fig. 2d). The main chain, but not the side chain, of Val(p2) hydrogen bonds with Tyr9 α and Asn80 β (Fig. 2d). On the other hand, the side chain of Thr(p3) forms hydrophobic interactions with Phe22 α and Phe54 α (Fig. 2e). The side chain of Val(p4) also forms hydrophobic interactions with Phe24 β , Val72 β , and Met76 β , while the main chain hydrogen bonds with Asn62 α and Gln13 β (Fig. 2e). The main chain of Ala(p5) hydrogen bonds with Lys69 β (Fig. 2e). The side chain of another anchoring residue, Phe(p6), interacts hydrophobically with Met11 α , Leu66 α , and Tyr26 β (Fig. 2e). The side and main chains of Asn(p7) hydrogen bond with Tyr28 β and Asn69 α , respectively (Fig. 2f). The side chain of Gln(p8) hydrogen bonds with Asn68 α (Fig. 2f). Finally, the side chain of Phe(p9) forms hydrophobic interactions with Leu73 α , Phe9 β , Leu35 β , Val36 β , and Trp59 β (Fig. 2f). In addition, the side chains of Val(p2), Thr(p3), and Val(p4) do not fill their corresponding pockets, indicating that some larger side chains might be accommodated in these pockets (Fig. 2c).

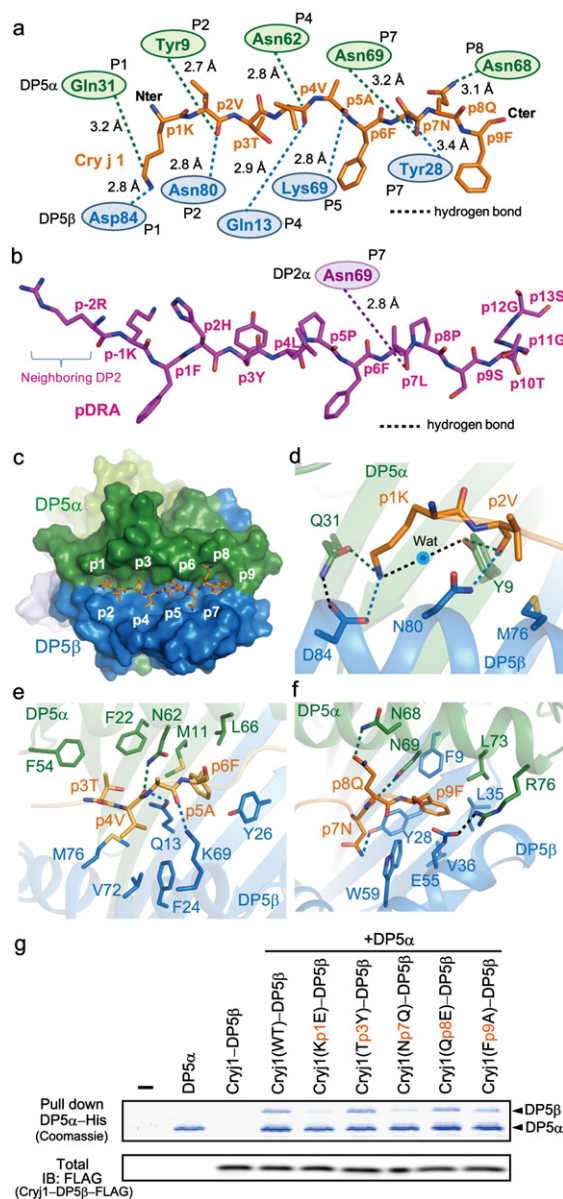


Fig. 2. Binding mode of the HLA-DP5•Cry j 1(9-mer) complex. (a) The hydrogen bonds between HLA-DP5 and the Cry j 1(9-mer) peptide. The residues of HLA-DP5 α and HLA-DP5 β involved in direct hydrogen bonding are labeled in green and blue circles, respectively. The Cry j 1(9-mer) peptide is shown as an orange stick model. (b) The hydrogen bond between HLA-DP2 and the pDRA peptide (PDB ID: 3LQZ). The pDRA peptide is shown as a purple stick model. The residue of HLA-DP2 α involved in direct hydrogen bonding is labeled in the purple circle. (c) Top view of the Cry j 1(9-mer) peptide and the nine pockets in the peptide-binding groove. HLA-DP5 α and HLA-DP5 β are shown in green and blue surface presentations, respectively. The Cry j 1(9-mer) peptide is depicted by an orange stick model. Details of the Cry j 1(p1-p2) (d), Cry j 1(p3-p6) (e), and Cry j 1(p7-p9) (f) interactions in the peptide-binding groove of HLA-DP5 are shown. (a-e) The hydrogen bonds are represented as broken lines. (g) Mutant analysis based on the HLA-DP5•Cry j 1(9-mer) complex. HLA-DP5 was prepared by co-expressing HLA-DP5 α and the Cry j 1-HLA-DP5 β fusion protein in the cell-free synthesis system. Interactions of the Cry j 1(9-mer) mutants with HLA-DP5 were analyzed: His-tagged HLA-DP5 α was captured and stained with Coomassie Brilliant Blue (top panel), and FLAG-tagged Cry j 1-HLA-DP5 β was immunoprecipitated and detected by Western blotting (bottom panel). Representative data from three independent experiments are presented.

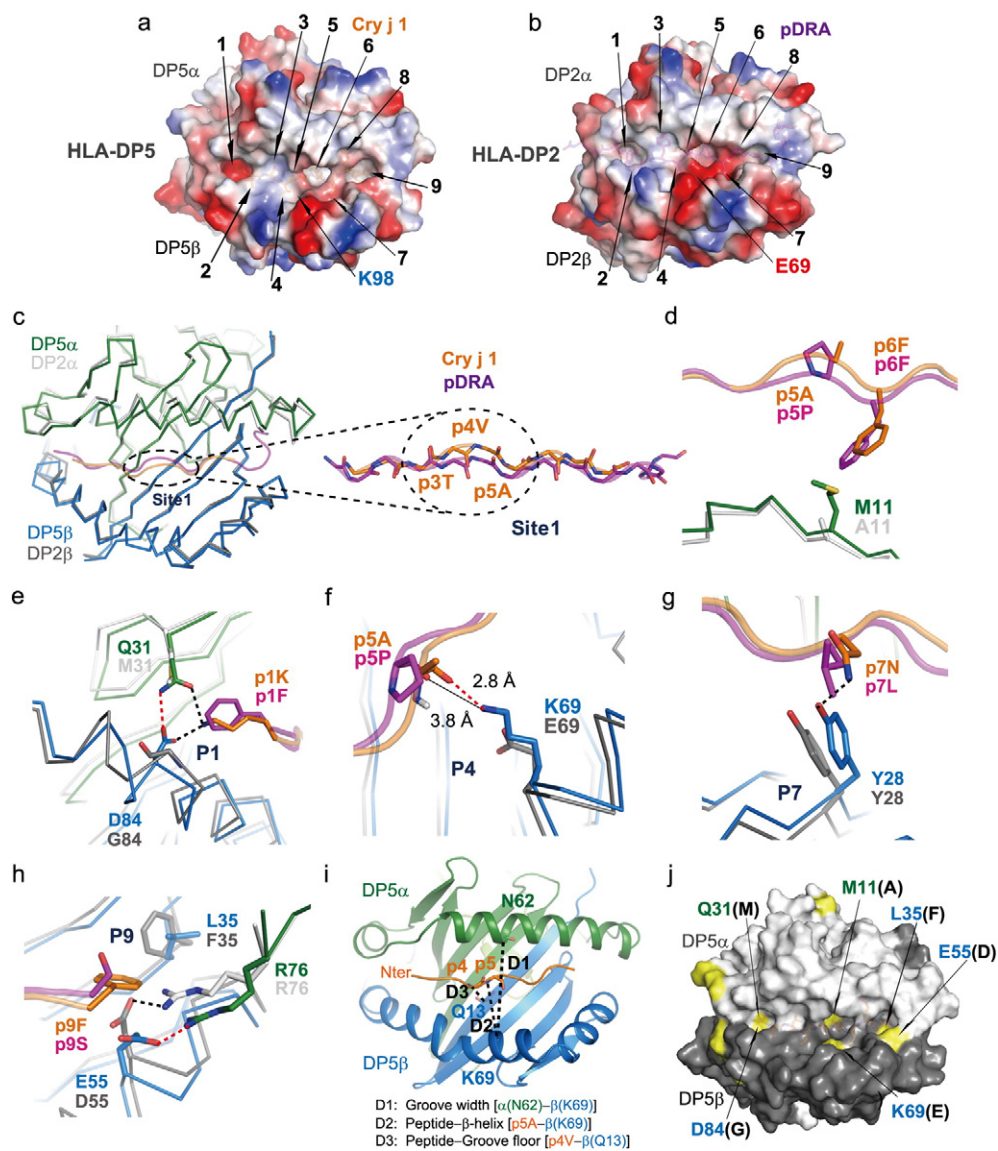


Fig. 3. Comparison of the HLA-DP5•Cry j 1(9-mer) complex with the HLA-DP2•pDRA complex. (a and b) Electrostatic potential maps of HLA-DP5 (a) and HLA-DP2 (b). Positive potential, blue; negative potential, red. The positions of the P1–P9 pockets for the Cry j 1 and pDRA peptides are indicated. (c) Superimposition of the HLA-DP5•Cry j 1(9-mer) complex on the HLA-DP2•pDRA complex. The major structural difference in the backbones of the peptides is indicated with a broken circle (Site 1). (d–h) Close-up views in stick presentations. Details of the Site 1 (d) and P1 (e), P4 (f), P7 (g), and P9 (h) pockets are shown. (c–h) The peptides and HLA molecules are colored orange (Cry j 1), purple (pDRA), green (HLA-DP5α), blue (HLA-DP5β), white (HLA-DP2α), and gray (HLA-DP2β), respectively. (i) According to Ref. [25], we compared the published human and mouse major histocompatibility complex II structures with a bound peptide with the total width of the peptide-binding groove (D1), the size of the gap between the peptide and the β chain α helix (D2), and the distance between the peptide and the floor of the binding groove (D3). (j) Non-conserved residues of the peptide-binding grooves between HLA-DP5 and HLA-DP2 are shown in yellow. The residues of HLA-DP5α and HLA-DP5β are labeled in green and blue, respectively, and those of HLA-DP2 are shown in parentheses.

To examine the contributions of the Cry j 1 side chains to the binding with HLA-DP5α and/or HLA-DP5β, we created five mutants of the Cry j 1 9-mer peptide (Fig. 2g). In this study, HLA-DP5 was prepared by co-expressing HLA-DP5α and the Cry j 1–HLA-DP5β fusion protein in the cell-free synthesis system, since the HLA-DP5α•HLA-DP5β heterodi-

mer was not formed by the co-expression of HLA-DP5α and HLA-DP5β without the Cry j 1 fusion. Using this property, we analyzed the interactions of the Cry j 1 9-mer mutants with HLA-DP5: His-tagged HLA-DP5α was captured, and the level of HLA-DP5α•Cry j 1–HLA-DP5β complex formation was monitored by the amount of co-precipitated Cry j 1–

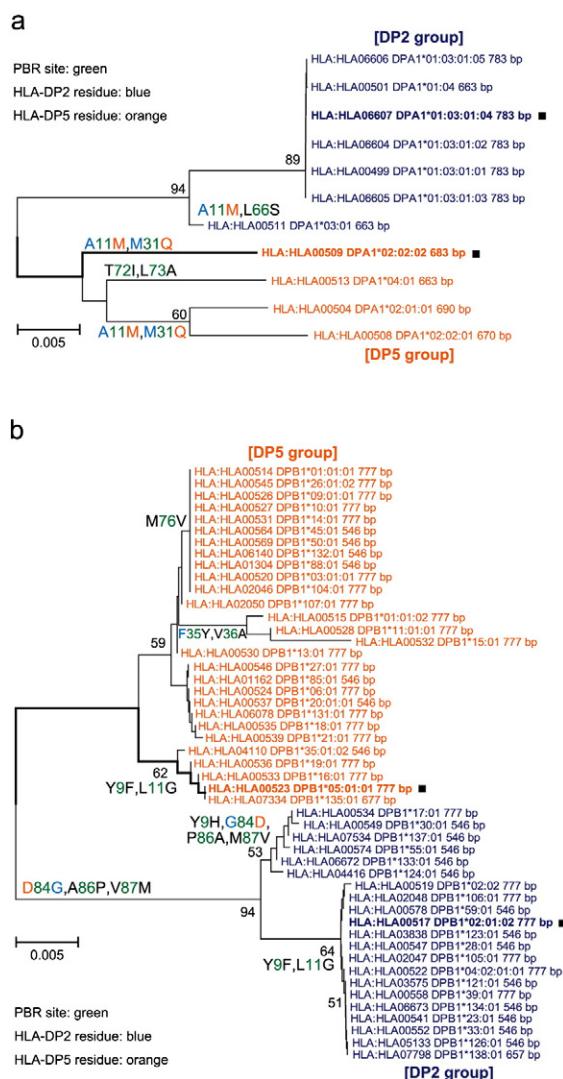


Fig. 4. Phylogenetic analyses of the HLA-DP alleles. (a) Phylogenetic tree of the HLA-DPA1 alleles. (b) Phylogenetic tree of the HLA-DPB1 alleles. (a and b) The NJ tree was constructed with only synonymous substitutions. Only the amino acid substitutions that occurred in the PBR were parsimoniously inferred and placed on or under each branch. For simplicity, not all amino acid substitutions on the main branches are shown. Particularly, position 55, 65, or 69 of the DP β chain is not listed in each branch because of high-frequency replacement. For DPA1, the ancestral state was determined by a comparison with HLA-DRA. However, no appropriate outgroup sequence was found for DPB1, and the ancestral state was inferred from the extant sequences. Green characters represent substitutions in the PBR. Blue and orange characters indicate residues of HLA-DP2 and HLA-DP5, respectively.

HLA-DP5 β . Mutations at the p1, p7, and p9 positions of the Cry j 1 peptide reduced the level of HLA-DP5 α •HLA-DP5 β complex formation (Fig. 2g). The effects of the p1 and p7 mutations were quite drastic. Lys(p1) is bound in the deep acidic pocket involving Gln31 α and

Asp84 β , and its mutation to Glu (Kp1E) caused repulsion within the pocket (Fig. 2d). The mutation of Asn(p7) to the longer Gln (Np7Q) may have impaired the hydrogen bonding with Tyr28 β (Fig. 2f). The mutation of Phe(p9) to Ala (Fp9A) probably weakened the hydrophobic interaction with the p9 pocket (Fig. 2f). In contrast, the Tp3Y and Np8E mutations exhibited negligible effects on the level of complex formation (Fig. 2g). Actually, the Tyr substitution for Thr(p3) may be accommodated well in the pocket constituted by Phe22 α and Phe54 α (Fig. 2e), while Glu at position p8 may interact with the pocket in the same manner as Gln(p8) (Fig. 2f).

Properties of the peptide-binding grooves of HLA-DPs and other class II HLA molecules

The HLA-DP5•Cry j 1 and HLA-DP2•pDRA complex structures superimposed on each other almost perfectly, with an rmsd value of 1.0 Å for the equivalent C α atoms. In the HLA-DP5 and HLA-DP2 structures, the P1, P4, P6, and P9 pockets are significantly deeper than the P2, P3, and P7 pockets (Fig. 3a and b). However, each of the four deeper pockets of HLA-DP5 has a different position, size, or charge from that of the corresponding pocket of HLA-DP2. The bottom of the P1 pocket of HLA-DP5 consists of Gln31 α and Asp84 β and is negatively charged (Fig. 3a and e). Therefore, the HLA-DP5 P1 pocket is suitable for accommodating a positively charged amino acid residue, such as Lys. In contrast, the bottom of the HLA-DP2 P1 pocket is hydrophobic (Fig. 3b and e). The acidic P1 pocket is also present in some class II HLA structures, such as HLA-DQ2 [18], HLA-DQ2.3 [19], and HLA-DQ8 [20]. On the other hand, the P4 pocket of HLA-DP5 has a positive charge (Lys69 β), whereas that of HLA-DP2 has three negative charges (Glu26 β , Glu68 β , and Glu69 β) (Fig. 3a and b) [25]. The distance between the P6 and P9 pockets of HLA-DP5 is shorter than that of HLA-DP2 (Fig. 3a and b); the Tyr28 β side chain shifts toward the P9 pocket and hydrogen bonds with Asn(p7)(Cry j 1) (Fig. 3g). Furthermore, the diameter and the depth of the P9 pocket are larger in HLA-DP5 than in HLA-DP2 [25] (Fig. 3a and b) due to the differences between Glu55 β (HLA-DP5) and Asp55 β (HLA-DP2) and between Leu35 β (HLA-DP5) and Phe35 β (HLA-DP2) (Fig. 3h).

We compared the width of the peptide-binding groove, represented by the distance between position 62 α in the α 1 domain and position 69 β in the β 1 domain (Fig. 3i) of HLA-DP5 with those of HLA-DP2 and other class II major histocompatibility complex/HLA molecules. The peptide-binding groove of HLA-DP2 is the widest (16.18 Å) [25]. By contrast, the groove width of HLA-DP5 is 15.08 Å, which is close to the average value (15.01 Å) [25] of the other structures. We also measured the distance between the peptide and the groove floor, as

Table 2. The non-conserved residues of HLA-DPs that affect the properties of the peptide-binding grooves

HLA-DP	Peptide-binding groove					
	Position 31(α)	Position 84(β)	Position 69(β)	Position 11(α)	Position 35(β)	Position 55(β)
HLA-DP5 ^a	Q (P1) (charge)	D (P1) (charge)	K (P4) (closed)	M (P6) (depth)	L (P9) (depth)	E (P9) (width)
HLA-DP2 ^b	M (P1)	G (P1)	E (P4) (open)	A (P6)	F (P9)	D (P9)

^a*DPA1*02:02* and *DPB1*05:01*.^b*DPA1*01:03* and *DPB1*02:01*.Blue boxes: The position involves divergent residues based on *DPA1* and *DPB1* four-digit allele-level typing.

Green boxes: The position involves divergent residues identified by comparing the structures of HLA-DP5•Cry j 1 and HLA-DP2•pDRA.

determined by positions p4 and 13(β) in the β 1 domain (Fig. 3i). The distances in the HLA-DP5 and HLA-DP2 structures are 8.40 Å (the longest) and 8.06 Å, respectively, while the average value in the other class II major histocompatibility complex/HLA structures is 6.54 Å [25]. A superimposition of HLA-DP5 bound with the Cry j 1 peptide onto HLA-DP2 bound with pDRA (Fig. 3c) revealed that the position of Phe(p6) of Cry j 1 is shifted up by the side chain of Met11 α , as compared with Phe(p6) of pDRA. The main chain of Ala(p5) of Cry j 1 is consequently pushed up (Fig. 3d), which facilitates its characteristic interaction with Lys69 α (HLA-DP5) (Fig. 3f). The amino acid sequence of HLA-DP5 is highly homologous to that of HLA-DP2, with a sequence identity of 95%. Six of the seventeen non-conserved residues are clustered in the peptide-binding groove: Met11 and Gln31 of the α chain; Leu35, Glu55, Lys69, and Asp84 of the β chain of HLA-DP5 (Fig. 3j). It should be emphasized here that these non-conserved residues participate in the formation of the P1, P4, P6, and P9 pockets, as described above, to achieve the distinct specificities of the peptide binding by HLA between HLA-DP5 and HLA-DP2.

Evolutionary analysis of HLA-DP alleles in the peptide-binding region

To scrutinize the non-conserved residues between HLA-DP5 and HLA-DP2, we constructed phylogenetic trees based on the synonymous substitutions under the molecular clock hypothesis (Fig. 4a and b; Supplementary Fig. 1a). In both the *DPA1* and *DPB1* trees, the extent of synonymous divergence was about 4% between HLA-DP2 and HLA-DP5, which represents divergence 20 million years ago, using a synonymous substitution rate of 10^{-9} /site/year. Thus, HLA-DP2 and HLA-DP5 diverged long ago. Since then, the *DPA1* and *DPB1* genes have accumulated further lineage-specific amino acid substitutions, and each formed the DP2 and DP5 groups with multiple members (Fig. 4a and b; Supplementary Fig. 1b and c). Especially, the

amino acid substitutions involving Met11 α and Gln31 α in the peptide-binding region (PBR) of the DP5 group occurred twice in the newly created branches (“the external branches”), and moreover, those involving Asp84 β of the DP5 group occurred in the differentiation of the DP2 and DP5 groups (Fig. 4a and b; Supplementary Fig. 1b–e). Three residues, Met11 α , Gln31 α , and Asp84 β , are “non-conserved” between HLA-DP5 and HLA-DP2 (Fig. 3j) and are also relevant to the distinct peptide-binding specificities. Notably, Gln31 α and Asp84 β form the negatively charged P1 pocket in HLA-DP5. Thus, the crystal structure, together with structural comparisons and evolutionary analyses, revealed the specific differentiation of the P1 pocket in the HLA-DP family.

Discussion

In our previous study, we observed a statistically significant association between HLA-DP5 and susceptibility to Japanese cedar pollinosis. We also identified a dominant epitope, Cry j 1(208–227), which was recognized in the context of HLA-DP5 by CD4⁺ Th2 cells in many patients with Japanese cedar pollinosis [22,23]. Therefore, we thought that the HLA-DP5-associated susceptibility to Japanese cedar pollinosis may be explained by HLA-DP5-mediated antigen presentation of the major Cry j 1 epitope to Th2 cells producing interleukin-4, an essential cytokine for IgE production by antibody-secreting plasma cells. Subsequently, the core 9-mer Cry j 1(214-KVTVAFNQF-222) peptide presented by HLA-DP5 was identified [22].

In the present study, we determined the crystal structure of HLA-DP5 bound with this 9-mer Cry j 1 core peptide. In the structure, the peptide-binding groove formed by the HLA-DP5 α and HLA-DP5 β chains recognizes the KVTVAFNQF sequence (Fig. 1), which corresponds exactly to the minimal peptide previously proposed by *in vitro* experiments [22]. Furthermore, we confirmed that HLA-DP5 exhibits the same Cry j 1-binding mode in solution,

through pull-down experiments using structure-based mutations of Cry j 1(9-mer) (Fig. 2).

Previous four-digit allele-level typing studies of the *DPA1* and *DPB1* alleles indicated that the residues at positions 31(α), 56(β), and 85–87(β) are “non-conserved” in the DP alleles (Table 2) [27]. In addition, we compared the structures of the HLA-DP5•Cry j 1 and HLA-DP2•pDRA complexes and identified the characteristic positions that are responsible for the distinct properties between HLA-DP5 and HLA-DP2, with regard to the peptide-binding mode (Table 2). In the present peptide-binding mode, the Lys residue at the Cry j 1(p1) position is a specific anchor residue, as it forms a salt bridge with the negatively charged P1 pocket of HLA-DP5. The bottom of the p1 pocket consists of the “non-conserved” residues at positions 31(α) and 84(β) and may accommodate different kinds of side chains between HLA-DP5 and HLA-DP2 (Fig. 3a, b, and e). Similarly, Leu and Glu at positions 35(β) and 55(β), respectively, of HLA-DP5 alter the shape of the p9 pocket, as compared to that of the P9 pocket involving Phe35(β) and Asp55(β) of HLA-DP2 (Fig. 3a, b, and h). Overall, the “non-conserved” residues on the peptide-binding groove of HLA-DP5 generate its distinct pocket properties from those of HLA-DP2. Therefore, the HLA-DP5•Cry j 1 complex forms 10 hydrogen bonds between the groove and the

peptide (Fig. 2a), while these features are not observed in the HLA-DP2•pDRA complex [25] (Fig. 2b).

Moreover, the Cry j 1(p5) position is pushed up by Lys at position 69(β), in collaboration with Met at position 11(α) of HLA-DP5, in the HLA-DP5•Cry j 1 complex (Fig. 3c, d, and f; Table 2). The region around the p4 and p5 positions of the peptide is frequently recognized by complementarity determining region 3 of the specific T cell receptor [28], and the conformational features at the p5 position may allow the peptide to escape from negative selection in the thymus. In the HLA-DP2•pDRA crystal, the complex adopted the auto-inhibited conformation for beryllium (Be), by association with the N-terminal Arg(p−2) of pDRA and the neighboring acidic pocket of HLA-DP2 [25]. However, in the present HLA-DP5 structure, none of the peptide residues are inserted in the pocket because the Lys at position 69(β) of HLA-DP5 occupies the space (Fig. 3f and Table 2). This is also relevant to the proposal by Dai *et al.* [25], who noted that a Lys residue at the p4 position of an HLA-DP2-binding peptide may not allow Be binding.

The evolutionary analysis revealed that the Met11 α , Gln31 α , and Asp84 β residues, which are characteristic of HLA-DP5, are responsible for its distinct peptide binding features. First, Gln31 α and Asp84 β are critical for the specificity of the P1 pocket

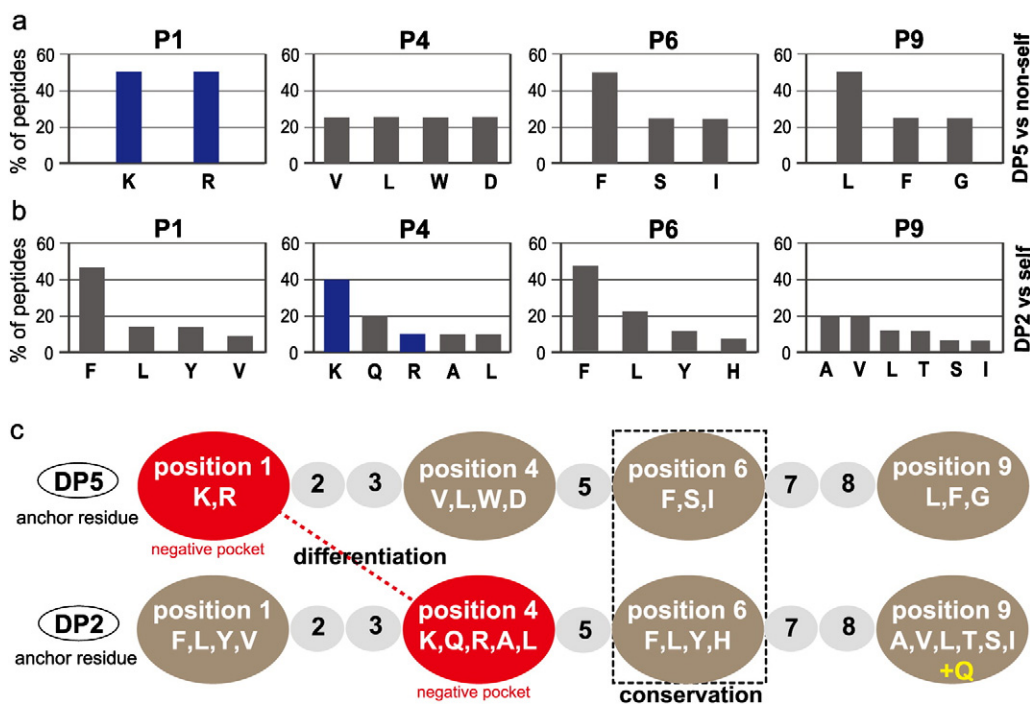


Fig. 5. Comparison of the peptide-binding motifs and the pocket properties between HLA-DP5 and HLA-DP2. Amino acid residues of the main anchor positions frequently detected in the non-self-peptides bound to HLA-DP5 (a) and the self-peptides bound to HLA-DP2 (b). (a and b) Calculations included only the longer peptide sequences from each source protein. (b) The chart constructed by using examples from a previous report [29]. (c) Relationships between the peptide-binding motifs and the pocket properties of HLA-DP5 and HLA-DP2.

for the positively charged Lys(p1) residue (Fig. 3e). Second, Met11 α is involved in the mechanism underlying the pushed-up position of the Phe(p6) residue (Fig. 3d). This finding suggested that the substitutions in the PBR could have been responsible for the diversification of the peptide-binding mode within the DP5 group. Interestingly, Met11 α of HLA-DP5 and Ala11 α of HLA-DP2 are coupled with Lys69 β and Glu69 β , respectively (Table 2), to form the haplotype-specific peptide-binding modes described above. Furthermore, Leu35 β of HLA-DP5 and Phe35 β of HLA-DP2 are coupled with Glu55 β and Asp55 β , respectively (Table 2), and these interactions are important for the formation of the different pocket sizes between HLA-DP5 and HLA-DP2. These replacements occurred when the two groups branched apart 20 million years ago.

Three other epitope peptides of Cry j 1/2 [Cry j 1 (61–75), Cry j 2 (81–95), and Cry j 2 (336–350)] reportedly interact with HLA-DP5 [23]. Considering that the negatively charged P1 pocket with Gln31 α is the major characteristic of the DP5 group, we assigned a positively charged residue, K or R, in the epitope peptides to the p1 position and then examined if residues p2–p9 are compatible with the properties of the corresponding pockets of HLA-DP5 (Supplementary Table 1a). Furthermore, we preliminarily modeled the epitope peptides within the peptide-binding groove, on the basis of the present HLA-DP5•peptide complex structure (Supplementary Fig. 2a–c). Thus, we found that these peptides fit well not only in the P1 pocket but also in the P6 and P9 pockets and form 9–10 hydrogen bonds in total, indicating that the assignment of K/R to p1 is reasonable (Fig. 5a and Supplementary Table 1a). Consequently, we concluded that the peptide-binding groove of the DP5 group can bind the epitope peptides bearing p1Lys/Arg, with a certain allowance for the other pockets.

On the other hand, 27 peptides, including Cry j 2 (185–193), reportedly associate with HLA-DP2 [23,29,30]. Considering the HLA-DP2•pDRA complex structure, we assigned the aromatic residues, F or Y, in these epitope peptides to the p1 and p6 positions and examined if their residues p2–p5/p7–p9 are compatible with the peptide-binding groove of HLA-DP2 (Supplementary Table 1b). Moreover, we constructed several preliminary structural models (Supplementary Fig. 2d and e). The p1 and p6 side chains fit well in their corresponding pockets, and 4–5 hydrogen bonds are formed in total. Interestingly, Lys(p4) is most frequently observed for the DP2 epitope peptides (Fig. 5b and Supplementary Table 1b) and fits nicely within the negatively charged P4 pocket of HLA-DP2. Actually, the epitope peptide with the putative optimal residues, Phe(p1), Lys(p4), and Phe(p6), has been found [29], whereas the other peptides have suboptimal side chains at either p1/p6 or p4. In the case of the

HLA-DP2•pDRA complex structure, the lack of Lys(p4) in pDRA, which has Leu(p4) instead, is compensated by the bound Be ion. It was previously argued that the pDRA/Be-binding mode of HLA-DP2 is probably different from the others [25,31]. Overall, the peptide-binding groove of the DP2 group can bind these epitope peptides with a certain allowance.

For both the DP5 and DP2 groups, the properties of the P6 pocket have been conserved to accommodate an aromatic side chain in the course of evolution (Fig. 5c). In contrast, the DP5 and DP2 groups differentially acquired the negatively charged pocket at the P1 and P4 positions, respectively (Fig. 5c). It is likely that this evolutionary diversification of the peptide-binding groove created the specificities of the HLA-DP family members to cover the present diversity of the epitope peptides.

The present crystal structure, together with the structural comparisons and evolutionary analyses, revealed the differentiation of the P1 pocket in the HLA-DP family. These results provide the essential structural basis for understanding the diversification of the ligand-binding specificities in the HLA-DP family, toward innovative pharmaceutical research for patients with hay fever.

Materials and Methods

Cloning, expression, and purification

The gene encoding the signal sequence and residues 1–181 of the HLA-DP5 α chain was cloned into the baculovirus transfer vector pFastBacDual (Life Technologies), as a fusion with the C-terminal tobacco etch virus (TEV) protease cleavage site and a His tag. The gene encoding residues 4–189 of the HLA-DP5 β chain was cloned into the resulting vector, as a fusion with the gp67 signal sequence followed by the nine-amino-acid residues (KVTVAFNQF) of the Cry j 1 peptide and the linker sequence (GGSLVPRGSGGGGS) at its N-terminus, as well as the TEV protease cleavage site and FLAG tag at its C-terminus. The baculovirus for the His-tagged HLA-DP5 α and FLAG-tagged Cry j 1–HLA-DP5 β production was created using the BAC-to-BAC Baculovirus Expression System (Invitrogen). *Spodoptera frugiperda* cells (5×10^5 cells/ml), derived from expresSF+® Insect Cells (Protein Sciences), were cultured in Sf-900™ III SFM (Life Technologies) and infected with the viral stock at a multiplicity of infection of 0.2. After 20 h at 27 °C in suspension culture (125 rpm), the cells were pelleted and resuspended in an equal volume of the medium and were continuously cultured at 19 °C in suspension for 5 days. The heterodimer HLA-DP5•Cry j 1(9-mer) was purified from the culture supernatants by immobilized ANTI-FLAG® M2 Affinity Gel (Sigma) and then the C-terminal tag of the heterodimer in the eluate was removed by an overnight treatment with TEV protease at 4 °C. Seven residues (GSSGSSG) from the tag remain at the C-terminus because of the location of the TEV cleavage site. The heterodimer was further purified by size-exclusion

chromatography (Superdex 200 HR 10/30; GE Healthcare) and eluted with 20 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl. The appropriate fractions were collected according to the SDS–PAGE analysis.

Analytical ultracentrifugation

All of the analytical ultracentrifugation experiments were performed at 4 °C, using an An-50 Ti rotor and a Beckman Optima XL-I analytical ultracentrifuge. Absorbance scans were recorded at 280 nm. The sample buffer was 20 mM Tris–HCl, pH 8.0, containing 150 mM NaCl. The solvent density and the protein partial specific volume ($\bar{v}$) were estimated using the program SEDNTERP[†]. The sedimentation coefficient was determined from sedimentation velocity experiments, performed at a rotor speed of 40,000 rpm. The concentrations of the protein samples were 0.32 and 0.16 mg/ml. The data were analyzed with the program SEDFIT [32]. For the sedimentation equilibrium experiments, protein samples at three different concentrations (0.40, 0.27, and 0.13 mg/ml) were centrifuged at rotor speeds of 6000, 7500, 9000, 10,500, and 12,000 rpm until the sedimentation–diffusion equilibrium was reached (16 h). The equilibrium data were fitted using the manufacturer's software (Beckman XL-A/XL-I Data Analysis Software ver. 6.03).

Crystallization and X-ray data collection

The preliminary crystals of the HLA-DP5•Cry j 1(9-mer) complex were obtained by initial crystallization screening, using the 96-well sitting-drop vapor diffusion method (Hampton Research). The crystals of the HLA-DP5•Cry j 1(9-mer) complex used for structure determination were obtained by the sitting-drop vapor diffusion method, by mixing the protein solution (0.7 mg/ml) with an equal volume of the reservoir solution [24% (w/v) polyethylene glycol 3350 and 0.2 M ammonium nitrate] and incubating the mixture at 20 °C. Plate-like crystals grew to average dimensions of 0.5 mm × 0.2 mm × 0.01 mm within 2 weeks. A single crystal of HLA-DP5•Cry j 1(9-mer) was coated with its reservoir solution, containing 22.5% polyethylene glycol 400 as cryoprotectant, and was flash-cooled in liquid nitrogen. The native diffraction data of HLA-DP5•Cry j 1(9-mer) were collected at −173 °C at a single wavelength at X06SA, Swiss Light Source, and were recorded on a MARmosaic 225 CCD detector (Rayonix, USA). All of the HLA-DP5•Cry j 1(9-mer) diffraction data were processed with the XDS program [33]. The crystallographic data and the data collection statistics of HLA-DP5•Cry j 1(9-mer) are provided in Table 1.

Structure determination and refinement

We solved the structure of the HLA-DP5•Cry j 1(9-mer) heterodimer by molecular replacement with PHASER [34], using the reported HLA-DP2 structure (PDB accession code: 3LQZ), with pDRA omitted, as a search model. This solution was subjected to one round of simulated annealing refinement in PHENIX [35]. The initial maps showed additional density in the peptide-binding groove, which indicated the binding site for Cry j 1(9-mer). The

HLA-DP5•Cry j 1(9-mer) model was subsequently completed by manual rebuilding in Coot [36] and rigid-body refinement in PHENIX, with a single *B*-factor per domain, using local non-crystallographic symmetry restraints throughout. The last round of refinement was performed without non-crystallographic symmetry. There are four HLA-DP5•Cry j 1(9-mer) complexes in the asymmetric unit. Refinement statistics are provided in Table 1. The programs PROCHECK [37] and MolProbity [38] were used to assess the geometry of the final model. Ramachandran statistics are as follows [favored/disallowed (%]): HLA-DP5•Cry j 1(9-mer), 98/0.1. Alignments were calculated using CLUSTAL W [39]. Figures were produced using PyMOL[‡].

In vitro protein binding assay

The DNA fragments encoding the full-length ectodomain of HLA-DP5α (residues 1–181) and that of HLA-DP5β (residues 4–189) as an N-terminal fusion with the nine-amino-acid residue Cry j 1 peptide (KVTVAFNQF) were cloned into the TA vector pCR2.1TOPO (Invitrogen). HLA-DP5α and HLA-DP5β are fusion proteins containing C-terminal histidine and FLAG tags, respectively, following the TEV protease cleavage site. The Kp1E, Tp3Y, Np7Q, Qp8E, and Fp9A mutants of Cry j 1(9-mer) were prepared by site-directed mutagenesis, using a QuikChange Site-Directed Mutagenesis kit (Stratagene). These variants were synthesized using the small scale dialysis mode of the *Escherichia coli* cell-free reaction [40,41] in the presence of disulfide isomerase and GSH/GSSG. Equal amounts of HLA-DP5 in complex with Cry j 1(WT) and Cry j 1(Mutants) were incubated with Ni-NTA agarose (Qiagen) beads. The beads were washed 10 times with washing buffer [20 mM Tris–HCl buffer (pH 8.0), containing 150 mM NaCl and 20 mM imidazole]. The bound proteins were eluted with 20 mM Tris–HCl buffer (pH 8.0), containing 150 mM NaCl and 500 mM imidazole, resolved by SDS–PAGE and stained by Coomassie Brilliant Blue. Simultaneously, equal amounts of these variants were incubated with ANTI-FLAG® M2 Affinity Gel (Sigma) beads. The beads were washed five times with washing buffer [20 mM Tris–HCl buffer (pH 8.0), containing 150 mM NaCl and 1 mM ethylenediaminetetraacetic acid]. The bound proteins were eluted with 20 mM Tris–HCl buffer (pH 8.0), containing 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, and 0.1 mg/ml 3× FLAG® Peptide (Sigma), and the fractions were analyzed by SDS–PAGE and immunoblotted with an ANTI-FLAG® M2 Monoclonal Antibody (Sigma). Immunoblotting images were obtained using a Luminescent Image Analyzer (LAS-4000; GE Healthcare).

Phylogenetic analysis

The NJ tree [42] of synonymous substitutions was constructed by using a program implemented in MEGA5 [43]. On the NJ tree, amino acid substitutions within the PBRs of the DPα and DPβ chains were manually placed parsimoniously. For *DPA1*, *DRA1* sequences were used as outgroups, but for *DPB1*, outgroup sequences were not used. Instead, the root was placed on an inter-nodal branch of the DP2 and DP5 groups.

Accession number

The atomic coordinates and structure factors of the HLA-DP5•Cry j 1(9-mer) complex have been deposited in the PDB, with the accession code [3WEX](#).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jmb.2014.06.020>.

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† <http://www.jphilo.mailway.com>.

‡ <http://www.pymol.org/>.

Abbreviations used:

IgEimmunoglobulin E; HLAhuman leukocyte antigen;

PBRpeptide-binding region; TEVtobacco etch virus.

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