Identification of a dominant self-ligand bound to three HLA B44 alleles and the preliminary crystallographic analysis of recombinant forms of each complex

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Abstract **A naturally processed and presented ligand that is shared by human leukocyte antigen (HLA) B*4402, B*4403 and B*4405 molecules has been identified in peptides isolated from immunoaffinity purified HLA B44 complexes. This peptide derived from HLA DPα residues 46–54, an endogenous product of HLA DP expressed in the cell line Hmy2.C1R, is a prominent peptide in the mass spectra of species isolated as bound peptides from each allele when the three HLA B44 subtypes were introduced as transfected gene products. Recombinant truncated forms of HLA B*4405$_{1-276}$, HLA B*4403$_{1-276}$, HLA B*4402$_{1-276}$ and β_2-microglobulin have been prepared as inclusion bodies in *Escherichia coli* and refolded in the presence of the DPα$_{46-54}$ peptide and purified by a combination of size exclusion and anion exchange chromatography. This material was determined to be correctly folded based on detection of a conformational epitope recognized by the W6/32 monoclonal antibody. Large, plate-like crystals of the three complexes were produced using polyethylene glycol as the precipitant. All the crystals belong to the space group $P2_12_12_1$ with unit cell dimensions of approximately $a = 51$, $b = 82$, $c = 110$ Å. The crystals of three B44/DPα complexes diffracted to a resolution of 1.9 Å or better. For the first time, using this natural, high abundance ligand of the HLA B44 molecules we have successfully expressed and refolded the three HLA B44 molecules and produced crystals amenable to structural studies.** © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: X-ray crystallography; HLA; Mass spectrometry; Protein structure; Polymorphism; Peptide ligand

1. Introduction

Human leukocyte antigen (HLA) molecules are cellular receptors that bind peptide antigens within specialized intracellular environments, prior to transport to the cell surface where they can interact with the T cell receptor (TcR) on T cells. Class I HLA molecules typically bind short peptides 8–11 amino acids in length in the lumen of the endoplasmic reticulum (ER) as heterodimers of class I heavy chain and the monomorphic β_2-microglobulin light chain. This complex of class I heterodimers and peptide is then transported via the Golgi apparatus to the cell surface where the peptide antigen is presented to the TcR on the surface of cytotoxic T lymphocytes (CTLs). The recognition of this class I complex by CTLs can result in the destruction of the antigen presenting cell (APC). Since class I molecules are expressed by all nucleated cells and the source of peptides that bind to class I molecules is typically from endogenous sources, CTL-mediated killing of APC is most relevant in the context of anti-tumor and anti-viral immunity as well as transplantation (both graft versus host disease and solid graft rejection).

The overall structure of class I molecules has been well documented; however, high resolution structures that reveal the different modes of peptide binding and peptide specificity continue to provide critical information regarding the structure and function of these molecules. The HLA B44 family of alleles is an important family of HLA molecules that are found in around 24% of Caucasian individuals [1]. There is no structure for any members of this family nor a structural explanation for the selection of ligands (P2 Glu, P9 Phe/Tyr) by these molecules [2,3]. HLA B*4405 differs from other members of the HLA B44 family by 1–2 amino acids, yet it displays very different behavior in terms of antigen presentation. The amino acid residues that differ between these alleles occur at position 156 (Asp in HLA B*4402 and B*4405 and Leu in HLA B*4403) and position 116 (Asp in HLA B*4402 and B*4403 and Tyr in HLA B*4403) and have previously been linked to differential association to the transporter associated with antigen presentation (TAP) molecule, a translocon responsible for the transport of antigenic peptide precursors from the cytoplasm to the lumen of the ER [4]. Thus, compared to HLA B*4402 and B*4403 (the two most prevalent alleles of this family), HLA B*4405 is able to present antigen more rapidly [5] and exhibits tapasin independence [6] for antigen loading and surface expression. This does not appear to be due to the acquisition of higher affinity ligands or en-

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hanced thermostability of the complex [6]. We therefore sought evidence for a structural basis of these important immunological features of HLA B*4405 relative to the other alleles and investigated the role of the polymorphic amino acids at positions 116 and 156 in this context. In this report we document the successful isolation of a common peptide ligand from three HLA B44 alleles (4402, 4403, 4405), the use of this peptide to direct the refolding of recombinant HLA B*4402, B*4403 and B*4405 in vitro, as well as the purification, crystallization and preliminary analysis of each complex.

2. Materials and methods

2.1. Expression, refolding and purification

Recombinant HLA B44 was expressed in *Escherichia coli* as inclusion bodies as described in [7]. The class I heavy chain was modified by the removal of the leader sequence, transmembrane region and cytosolic tail (amino acids 1–276 of the mature protein sequence). cDNA encoding this region was ligated into the bacterial expression vector pET, and transformed into the BL21 (RIL) strain of *E. coli*. At an A_{600} of 0.6, cultures were induced with 1 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 12 h, bacteria were lysed in 50 mM Tris–HCl pH 8.0, 1% Triton X-100, 1% sodium deoxycholate, 100 mM NaCl and 10 mM dithiothreitol (DTT). Inclusion bodies were isolated by centrifugation then washed with 50 mM Tris–HCl, 0.5% Triton X-100, 100 mM NaCl, 1 mM NaEDTA, 1 mM DTT pH 8.0, and washing in 50 mM Tris–HCl, 1 mM NaEDTA, 1 mM DTT pH 8.0, and then solubilized in 25 mM MES, 8 M urea, 10 mM NaEDTA pH 6.0 with the protease inhibitors 1 μg/ml Pepstatin A and 200 μM phenylmethylsulfonyl fluoride (PMSF). Recombinant protein (60 mg HLA B44 heavy chain and 20 mg β_2m) was refolded with 30 mg of the DPα_{46-54} peptide (EEFGRAFSF) in the presence of 3 M guanidine–HCl, 10 mM Na acetate, and 10 mM NaEDTA pH 4.2, over 24 h in 0.1 M Tris, 2 mM EDTA, 400 mM L-arginine–HCl, 0.5 mM oxidized glutathione, 5 mM reduced glutathione pH 8.0 at 4°C. Following refolding, protein was dialyzed overnight against Milli Q using a 6–8000 kDa MWCO dialysis membrane (Spectrum, CA, USA). Protein was purified as a single peak by fast protein liquid chromatography using ion exchange on a DE52 column (Whatman, Maidstone, Kent, UK), size exclusion on a Superdex 75 pg gel filtration column (Amersham Pharmacia Biotech, Uppsala, Sweden), and a final ion exchange on a Mono Q HR column (Amersham Pharmacia Biotech). Following each purification step, protein was concentrated using Millipore 5K NMWL membrane centrifugal filter devices (Millipore, Bedford, MA, USA) and sterile-filtered. Quantitative analysis was based on comparisons to bovine serum albumin protein standards using SDS–PAGE. Protein was concentrated to 3 mg/ml for use in crystallization trials.

2.2. Qualitative analysis of B44 peptide complexes by capture enzyme-linked immunosorbent assay (ELISA)

The conformational integrity of the B44 peptide complexes was probed using a β_2-microglobulin-dependent, conformation-sensitive monoclonal antibody, W6/32 [8]. Refolded complexes of HLA B*4402, B*4403 and B*4405 were verified by use in a capture ELISA with W6/32 and a monoclonal antibody for human β_2-microglobulin as described previously [9]. Briefly, 96-well round-bottom Maxisorp immunoplates (Nunc, Denmark) were coated overnight at 4°C with 20 μg/ml of W6/32 in 0.5 M carbonate buffer, then washed in 0.05% Tween 20 in phosphate-buffered saline (PBS) and blocked for 1 h with 1% BSA at 37°C. After washing, refolded complexes for each of the three alleles were then serially diluted in 0.1 M Tris pH 8.0 and bound to the plate for 1 h at 37°C. Plates were washed and incubated with horseradish peroxidase-labeled anti-human β_2m (Dako, Carpinteria, CA, USA; 1:5000; 0.5% BSA) for 1 h. Plates were developed using the substrate *o*-phenylenediamine in 0.05 M citrate buffer, pH 5.0 for 30 min at 37°C. Plates were read at $OD_{492\,nm}$, and results were calculated as the mean of triplicate analyses.

2.3. Crystallization

All crystallization trials were conducted using the hanging drop vapor diffusion technique using 24-well tissue culture plates. The crystals were grown by mixing equal volumes of 3 mg/ml HLA B44–peptide complex with the reservoir buffer (20–30% PEG 4K, pH 5.2–5.9, 0.1 M citrate, 0.2 M ammonium acetate) at room temperature. Each well contained 1 ml of reservoir buffer. Large plate-like crystals appeared within 1–5 days. The crystals were flash-frozen prior to data collection using 10% glycerol as the cryoprotectant.

2.4. Crystallography

All data were collected in-house on an R-Axis IV++ detector with CuKα X-rays generated by a Rigaku RU-H3RHB rotating anode generator and focused using Osmic mirrors. The frozen crystals were collected using the inverse-φ geometry. The diffraction data were processed and analyzed using D*TREK [10] and programs from the CCP4 suite [11].

2.5. Cell lines and culture

The B-lymphoblastoid cell line Hmy2.C1R was generated by γ-irradiation of a fast growing lymphoma LICR.LON.Hmy2 [12] and selected with antibodies against HLA A and HLA B alleles and complement. This resulted in a cell line with no detectable HLA A or B gene products, but with intact antigen processing and presentation pathways [13]. Thus, these cells are able to support high level expression of individually transfected HLA A, B or C gene products [13]. The individual transfection of HLA B*4402, B*4403 or B*4405 into C1R was performed by electroporation (975 μF, 200 V) using a plasmid encoding the cDNA of each of the B44 molecules and a selectable marker (pREP7) at a ratio 10:1. All cells were grown in RF-10 (RPMI 1640, Life Technologies, Gaithersburg, MD, USA) supplemented with 2 mM glutamine (Life Technologies), antibiotics, and 10% FBS (Commonwealth Serum Laboratories, Melbourne, Australia). For the peptide elution experiments cells were grown in 1 l roller bottles in RF-10 with the appropriate selection. Flow cytometry was used to establish that HLA B44 was stably expressed in the transfected cell lines during expansion.

2.6. Purification of cell surface-associated HLA B44 complexes and peptide analysis

Purification of HLA B44 complexes was performed as described previously [14]. Briefly, C1R.B*4402, C1R.B*4403 or C1R.B*4405 cells were grown in roller bottles to a density around 1×10^6 cells/ml. Approximately 5×10^9 cells were lysed at 4°C in 0.5% Nonidet P-40, 20 mM Tris, and 150 mM NaCl (pH 7.4) supplemented with Complete™ protease inhibitor cocktail (Roche). Cell lysates were clarified by centrifugation and the supernatant filtered and passed over a Tris-blocked Sepharose 4B precolumn. The precleared lysates were then applied to a column containing 2 ml of W6/32 affinity matrix (prepared as described previously [14]) and the column was washed in buffer containing 50 mM Tris, 150 mM NaCl, and 0.005% Nonidet P-40 (pH 8.0). The column was subsequently washed extensively with 50 mM Tris and 150 mM NaCl (pH 8.0), a high salt buffer (50 mM Tris and 500 mM NaCl (pH 8.0), and finally with 50 mM Tris (pH 8.0). Bound HLA B44 peptide complexes were eluted with 10% acetic acid, which also facilitates dissociation of the B44-bound peptides. The eluate was then passed through a Centricon 3 membrane (Millipore), and the flow-through was concentrated by vacuum centrifugation to a final volume of approximately 300 μl. This material was then subjected to further purification with reversed-phase high-performance liquid chromatography (RP-HPLC) using a Smart system HPLC (Pharmacia Biotech, Uppsala, Sweden). Peptides were separated using a μRPC C2/C18 (2.1 mm (inside diameter)×10 cm column; Pharmacia Biotech). Eluted peptides were resolved from contaminating detergent polymers by employing a rapid gradient from 0 to 60% acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA; 12%/min, 200 μl/min). Further purification of the eluted peptides was afforded using an optimized linear gradient from buffer A (0.1% TFA) to 40% B (acetonitrile/0.09% TFA; 1.3%/min), then 40% B to 60% B (4%/min) at a flow rate of 200 μl/min was used to separate peptides. Fractions (150 μl) were retained and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; as described in Section 2.7).

2.7. Peptide synthesis, sequencing and mass spectrometry

Synthetic peptides were purchased from Mimotopes (Clayton, Australia) and synthesized with free carboxyl and amino termini using

PIN-based techniques (30). MALDI-TOF MS was performed using a Bruker Reflex mass spectrometer (Bruker, Bremen, Germany) operated exclusively in the reflectron mode as described previously [15]. Aliquots of fractions (1–2 μl) were mixed with an equal volume of α-cyano-4-hydroxycinnamic acid (10 mg/ml in acetonitrile/ethanol, 1/1, v/v), spotted onto a target, and dried for analysis. Post-source decay (PSD) experiments were performed using 14 stepwise decrements in the reflectron potential and increasing the laser irradiance to optimize the production of fragment ions at each voltage. Identification of fragmented ion species was determined by assigning C- and N-terminal ion series and comparing parent *m/z* and fragmentation data to database entries using MS-FIT routines available through the protein prospector program (http://prospector.ucsf.edu) [16]. Accurate parent ion mass and fragmentation data allowed assignment of peptide sequences, which were confirmed by fragmenting a synthetic version of the peptide under identical conditions and comparison of the mass-spectral fingerprints.

3. Results and discussion

3.1. Isolation of HLA B44-bound peptides and analysis of a common ligand

Peptides have previously been isolated from HLA B*4402 and HLA B*4403 and exhibit a canonical glutamic acid P2 motif, with either phenylalanine or tyrosine at PΩ (i.e. C-terminal amino acid residue) [2,17]. HLA B*4405 is a rarer subtype of this common Caucasian and Asian allelic family that displays novel biochemical and immunochemical behavior, including very rapid intracellular trafficking [5] and lack of dependence for the chaperone tapasin for antigen loading

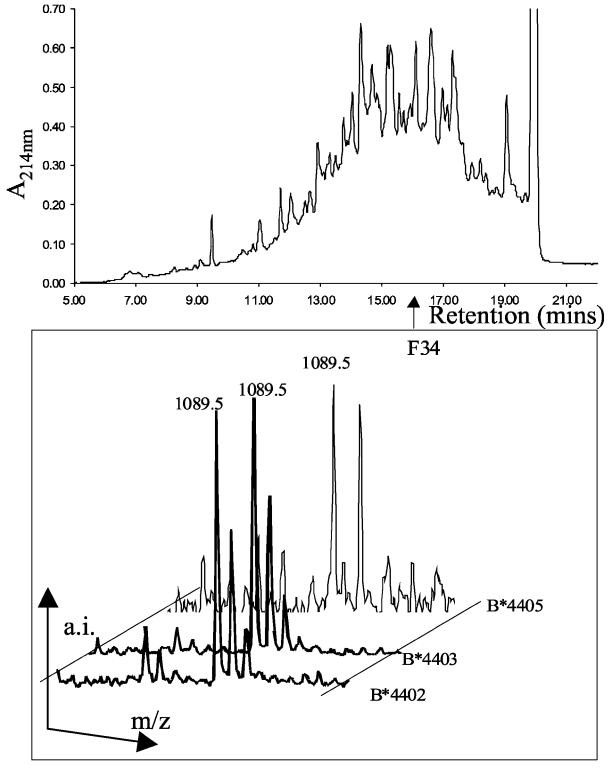


Fig. 1. An example of the RP-HPLC separation of peptides isolated from HLA B*4402 using μRPC C18 narrowbore (2.1 i.d. mm × 10 cm) column and a linear gradient of 0–60% acetontrile in 0.1% TFA is shown (upper figure). Below is a region of the mass spectra from a fraction containing a dominant species of *m/z* 1089.5 amu that is common to eluates from all three B44 molecules (an expanded region of the MALDI-TOF spectra of eluates is shown for each allele).

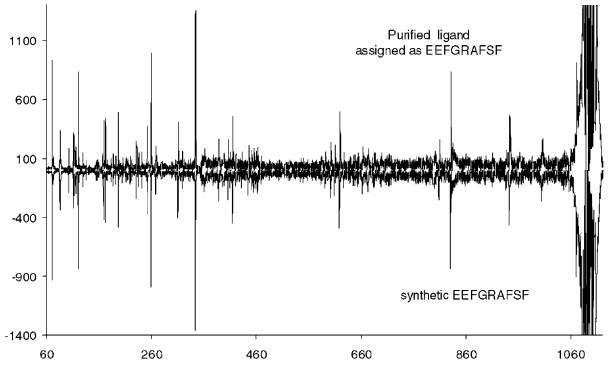


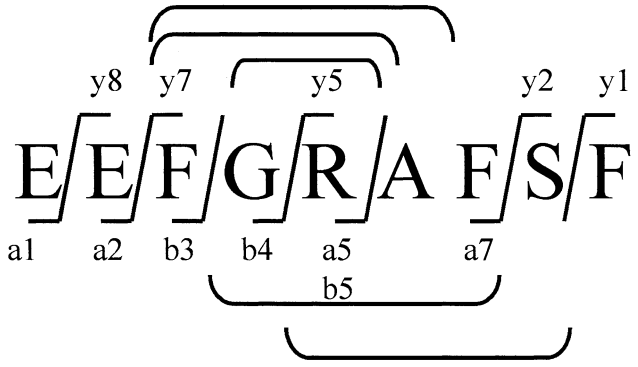
Fig. 2. PSD analysis of the species with *m/z* = 1089.5 amu isolated from the three HLA B44 molecules (the species from B*4402 eluates is shown as the spectra of positive polarity and was typical for the PSD spectra for all three eluates). PSD in MALDI-TOF MS was performed using 14 stepwise decrements in the reflectron potential and increasing the laser irradiance to optimize the production of fragment ions at each voltage. Accurate parent ion mass and fragmentation data allowed assignment of peptide sequences, which were confirmed by fragmenting a synthetic version (spectra of negative polarity) of the peptide under identical conditions and comparison of the mass-spectral fingerprints.

([6] and Purcell et al., manuscript in preparation). Studies of T cell cross-reactivity on these closely related HLA B44 subtypes suggest that HLA B*4405 binds to an overlapping subset of peptides compared to B*4402 and B*4403; however, some differences between these alleles are also evident ([1,5,18,19] and our unpublished data). In order to assess the similarity in peptide repertoire of B*4405 to previously reported ligands of B*4402 and B*4403 we isolated HLA B*4405 complexes from the surface of transfected APC, eluted peptides and analyzed the peptides by MALDI-TOF MS. The peptide pools derived from each purified allele contained a dominant species at $(M+H)^+ = 1089.4$ amu, which was contained within a single RP-HPLC fraction (Fig. 1). This peptide was subsequently sequenced by PSD in MALDI-TOF MS and identified as an abundant self-peptide from HLA DPα amino acid residues 46–54 (Fig. 2, spectra of positive polarity), the class II molecules also expressed by the C1R cell line. A complex fragmentation pattern was observed by MALDI-TOF PSD analysis due to the internal arginine residue, which included the formation of characteristic internal ions that can complicate automated sequence assignment [15]. To confirm the sequence assignment the fragmentation of the retrospectively synthesized DPα peptide EEFGRAFSF (Fig. 2, negative spectra) was compared to the natural ligand, as shown in Fig. 2 (comparison of positive and negative spectra) and Table 1; identical fragmentation patterns were observed. This confirms unambiguously the sequence assignment, since such fragmentation patterns act as molecular fingerprints [20,21].

3.2. Expression and refolding of HLA B44 – peptide complexes

Given the apparent abundant nature of the DPα peptide and the lack of any crystal structures for the B44 family of alleles we decided to express and refold recombinant HLA B44 complexed to this peptide (DPA1*0201 residues 46–54). We hypothesized that an abundant natural ligand that survives the cell surface extraction and purification procedure is most likely a high affinity ligand suitable for structural stud-

Table 1
Assignment of fragment ions of a natural HLA B*4405 ligand



Ion type	Predicted m/z	Detected m/z	Ion type	Predicted m/z	Detected m/z
Immonium & related ions			**b Series**		
R	70.00	70.1	b_2	259.09	259.2
E	102.06	102.1	b_3	406.16	406.7
R	112.00	112.1	b_4	463.18	463.5
F	120.08	120.2	b_5-H_2O	602.26	602.9
			b_5	619.28	619.4
Internal fragment ions			**y Series**		
GR-NH_3	197.10	197.1	y_1	166.09	166.3
FGR-NH_3	344.17	344.5	y_2	253.12	253.3
GRAF-NH_3 or FGRA-NH_3	415.21	415.5	y_5	627.33	627.4
RAFS-NH_3	445.22	445.5	y_7	831.42	831.5
a Series			y_8-NH_3	943.43	943.5
a_1	102.06	102.1			
a_2	231.10	231.3	**other**		
a_5	591.29	591.5	MH^+-H_2O	1071.49	1071.6
a_7	809.39	809.6			

ies. cDNA for each HLA B44 allele was cloned into pET-based vectors and a truncated form (amino acids 1–276) of these heavy chains expressed as insoluble inclusion bodies in *E. coli* strain BL21 (RIL). Cultures were induced with 1 mM of IPTG for 12 h and inclusion bodies prepared according to the method of Garboczi [7]. Recombinant protein (60 mg HLA B44 heavy chain and 20 mg β_2m) was refolded with 30 mg of the DPα_{46-54} peptide (EEFGRAFSF) over 24 h in a redox refolding buffer. Following refolding, protein was dialyzed overnight against Milli Q water and purified to homogeneity by DEAE anion exchange chromatography, size exclusion (Superdex 75) and a final Mono Q anion exchange chromatographic step. Quantitative analysis of the purified complex was based on comparisons to BSA protein standards using SDS-PAGE (see Fig. 3A,B). The conformational integrity of the refolded complexes was assessed by a capture ELISA incorporating the conformationally sensitive monoclonal

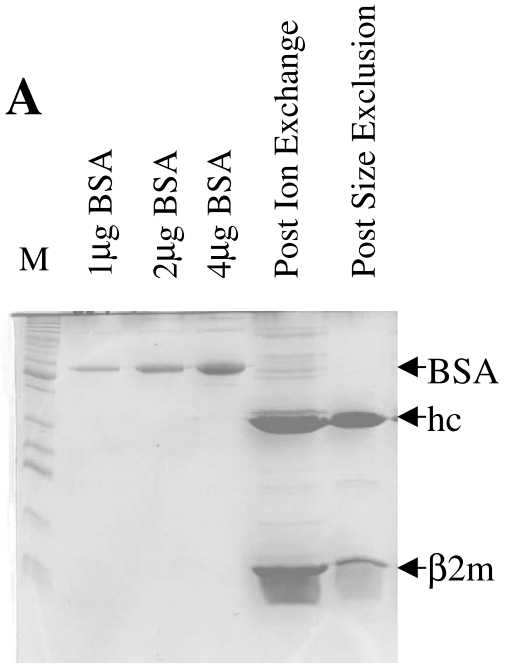
Table 2
Data collection statistics

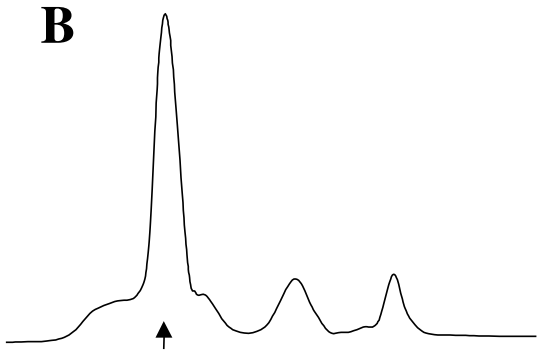
B*4405	
Temperature	100 K
Space group	$P2_12_12_1$
Cell dimensions (Å) (*a*,*b*,*c*)	50.77, 82.23, 110.00
Resolution (Å)	1.7
B*4402	
Temperature	100 K
Space group	$P2_12_12_1$
Cell dimensions (Å) (*a*,*b*,*c*)	50.8, 81.8, 110.1
Resolution (Å)	1.6
B*4403	
Temperature	100 K
Space group	$P2_12_12_1$
Cell dimensions (Å) (*a*,*b*,*c*)	50.7, 82.1, 109.1
Resolution (Å)	1.9

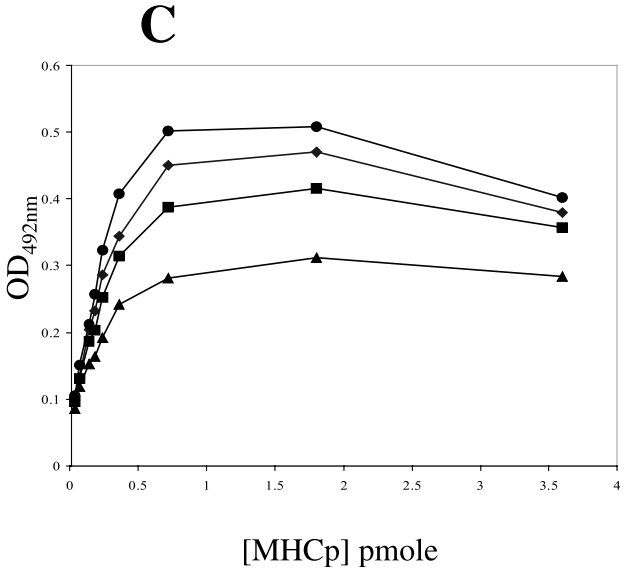
→

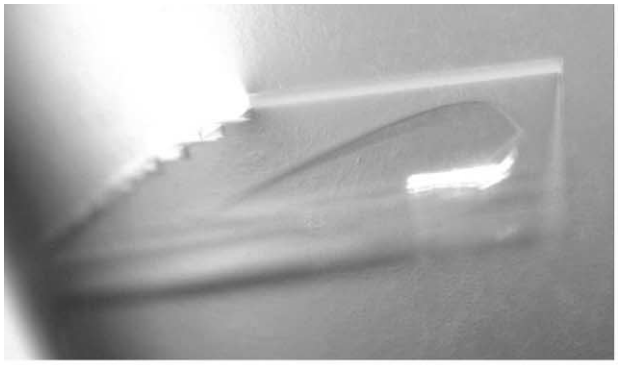
Fig. 3. A: SDS–PAGE separation of components of the recombinant B*4402 complexes following ion exchange and size exclusion purification steps. B: Size exclusion chromatogram of ion exchange-purified recombinant HLA B*4402. The elution position of HLA B*4402 complex was consistent with the elution position of standards, including other HLA–peptide complexes, indicating an apparent molecular weight of approximately 45 kDa. C: Capture ELISA using a conformation-sensitive monoclonal antibody reveals correct conformation of refolded and purified HLA B44 complexes. Key: ◆ = B*4402/DPα, ▲ = B*4403/DPα, ■ = B*4405/DPα, ● = B8/FLRGRAYGL.

antibody W6/32 [8]; as shown in Fig. 3C, each complex possessed the monoclonal antibody determinant and displayed dose-dependent recognition, indicating that the recombinant B44 complexes were in a native conformation. Protein was concentrated to 3 mg/ml for use in crystallization trials.

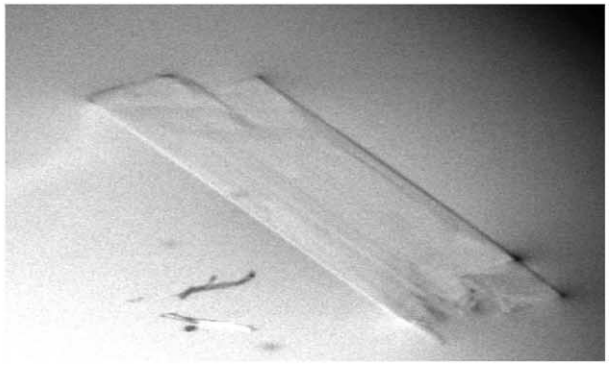








HLA B*4402-DPα47-54



HLA B*4403-DPα47-54

HLA B*4405-DPα47-54

Fig. 4. Photo microscopy of the crystals formed for the three B44–DPα complexes. Each allele formed large plate-like crystals under the conditions described in Section 2.3.

3.3. *Crystallization of three allelic variants of the HLA B44–DPα$_{46-54}$ complex*

In order to make structure-based observations regarding the nature of allelic micropolymorphism and its influence on ligand binding, each HLA B44 allele was individually refolded and subjected to crystallization trials. Crystals were success-

fully grown by the hanging drop method using PEG 4000 as the precipitant for each complex. The crystals were grown by mixing equal volumes of 3 mg/ml HLA B44–peptide complex with the reservoir buffer (20–30% PEG 4000, pH 5.2–5.9, 0.1 M citrate, 0.2 M ammonium acetate) at room temperature. Large plate-like crystals appeared within 1–5 days (see Fig. 4). The crystals were flash-frozen prior to data collection using 10% glycerol as the cryoprotectant. The crystals belong to space group $P2_12_12_1$ with unit cell dimensions approximately $a = 51$, $b = 82$, $c = 110$ (see Table 2 for individual statistics). The unit cell volume is consistent with one complex in the asymmetric unit. The three crystals diffract to a resolution of 1.9 Å or better. Structure determination is currently underway.

The high resolution structures of these three alleles will represent the first report of the same peptide bound to multiple human HLA molecules in a serological subfamily, differing from each other by 1–2 amino acids. We suggest that the structures will provide insights into the role these subtypes play in transplant rejection and also provide structural bases for the disparate intracellular behavior of these molecules. Moreover, the HLA DPα peptide is derived from a polymorphic human gene, and the region spanning amino acids 46–54 contains a dimorphism (R50Q) depending on the HLA DP allele. This suggests that this peptide may also play a role as a minor histocompatibility antigen, one of the major causes of chronic rejection in transplantation. Thus, these structures have additional general interest and the methods described herein for the production of recombinant forms of these molecules have potential use in diagnostics that use HLA tetramer-based approaches for example [22].

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