Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

X-ray diffraction analysis of crystals from the human major histocompatibility antigen HLA-B*2706 in complex with a viral peptide and with a self-peptide

Anna Zawacka,[a]‡ Bernhard Loll,[b]‡§ Jacek Biesiadka,[b] Wolfram Saenger,[b] Barbara Uchanska-Ziegler[a] and Andreas Ziegler[a]*

[a]Institut für Immungenetik, Charité – Universitätsmedizin Berlin, Campus Virchow-Klinikum, Humboldt-Universität zu Berlin, Spandauer Damm 130, 14050 Berlin, Germany, and [b]Institut für Chemie und Biochemie/Kristallographie, Freie Universität Berlin, Takustrasse 6, 14195 Berlin, Germany

‡ These authors contributed equally to this work.
§ Present address: Max-Planck-Institut für Medizinische Forschung, Abteilung für Biomolekulare Mechanismen, 69126 Heidelberg, Germany.

Correspondence e-mail: andreas.ziegler@charite.de

Received 1 November 2005
Accepted 17 November 2005
Online 24 November 2005

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The human leukocyte antigen (HLA) alleles *HLA-B*2704* and *HLA-B*2706* show an ethnically restricted distribution and are differentially associated with ankylosing spondylitis, with *HLA-B*2706* lacking association with this autoimmune disease. However, the products of the two alleles differ by only two amino acids, at heavy-chain residues 114 (His in HLA-B*2704; Asp in HLA-B*2706) and 116 (Asp in HLA-B*2704; Tyr in HLA-B*2706). Both residues could be involved in contacting amino acids of a bound peptide, suggesting that peptides presented by these subtypes play a role in disease pathogenesis. Two HLA-B*2706–peptide complexes were crystallized using the hanging-drop vapour-diffusion method with PEG as precipitant. Data sets were collected to resolutions of 2.70 Å (viral peptide pLMP2, RRRWRRLTV; space group $P2_12_12_1$) and 1.83 Å (self-peptide pVIPR, RRKWRRWHL; space group $P2_1$). Using HLA-B*2705 complexed with the pGR peptide (RRRWHRWRL) as a search model, unambiguous molecular-replacement solutions were found for both HLA-B*2706 complexes.

1. Introduction

The association of most or even all autoimmune diseases with the major histocompatibility complex (HLA complex in humans) is one of the hallmarks of this genetic region (Horton *et al.*, 2004). However, the molecular details underlying these associations have only begun to be understood in a small number of cases (Rioux & Abbas, 2005). These difficulties are exemplified by the class I allele *HLA-B27*. Although it has been known since 1973 that *HLA-B27* is very strongly associated with spondyloarthropathies (Brewerton *et al.*, 1973; Schlosstein *et al.*, 1973), there is still no generally accepted explanation that could account for all clinical, biochemical and functional aspects that characterize the relationship between *HLA-B27* and these diseases. Spondyloarthropathies encompass an entire set of related diseases, with ankylosing spondylitis (AS) being one of them (Khan, 2002). A possible clue to explain the association between AS and *HLA-B27* might come from a detailed functional and biophysical comparison of *HLA-B27* subtypes that differ in their disease association but are nevertheless very closely related (Khan & Ball, 2002; Ramos & López de Castro, 2002; Kim *et al.*, 2005).

HLA class I molecules consist of a highly polymorphic heavy chain (HC) that is non-covalently associated with the non-polymorphic protein β_2-microglobulin (β_2m). Two extracellular HC domains form a groove which carries a peptide that may be derived from self- or nonself-proteins within the cell (Madden, 1995). Structural and thermodynamic comparisons of the products of *HLA-B27* subtypes in complex with several different peptides have already been carried out (see, for example, Hülsmeyer *et al.*, 2002, 2004, 2005; Fiorillo *et al.*, 2005; Rückert *et al.*, 2005). In individuals with the AS-associated *HLA-B27* subtype *B*2705*, cytotoxic T-lymphocytes with specificity for the self-antigen pVIPR [RRKWRRWHL, derived from vasoactive intestinal peptide type 1 receptor (residues 400–408)] have been observed. These cells increase in number during the development of AS (Fiorillo *et al.*, 2000) and a substantial fraction cross-reacts with the viral pLMP2 peptide [RRRWRRLTV, derived from latent membrane protein 2 (residues 236–244) of Epstein–Barr virus; Brooks *et al.*, 1993; Fiorillo *et al.*, 2000]. However, pVIPR-reactive T cells were not found in individuals with the non-AS-associated

Table 1
Data-collection statistics of HLA-B*2706–pLMP2 and HLA-B*2706–pVIPR.

Values in parentheses refer to the highest resolution shell.

	HLA-B*2706–pLMP2	HLA-B*2706–pVIPR
Space group	$P2_12_12_1$	$P2_1$
Unit-cell parameters (Å, °)	a = 50.7, b = 82.0, c = 108.9, α = 90, β = 90, γ = 90	a = 50.8, b = 81.7, c = 64.43, α = 90, β = 108, γ = 90
Solvent content (%)	49.6	57.5
Matthews coefficient† ($Å^3$ Da^{-1})	2.5	2.9
Resolution (Å)	30.00–2.70 (2.77–2.70)	30.00–1.83 (1.90–1.83)
Unique reflections	12253 (880)	43847 (4069)
Completeness (%)	99.0 (93.2)	99.1 (92.2)
$\langle I \rangle / \langle \sigma(I) \rangle$	16.5 (3.5)	34.7 (6.0)
R_{sym}‡	0.054 (0.283)	0.036 (0.209)

† According to Matthews (1968). ‡ $R_{sym} = \sum_h \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i I_{h,i}$.

*B*2709* subtype (Fiorillo *et al.*, 2000). The B*2705 and B*2709 proteins differ by only a single amino-acid substitution (Asp116His) at the floor of the binding groove, but this exchange has drastic consequences for the conformations of the pVIPR and pLMP2 peptides within the peptide-binding clefts (Hülsmeyer *et al.*, 2004; Fiorillo *et al.*, 2005).

The alleles *B*2704* and *B*2706* are also differentially associated with AS and constitute another pair of subtypes that differ only minimally from each other (Vega *et al.*, 1986; Rudwaleit *et al.*, 1996): B*2704 (His114, Asp116, as in B*2705) and B*2706 (Asp114, Tyr116) may also be expected to bind peptides distinguishably, thereby leading to differential T-cell responses (Sesma *et al.*, 2002). Peptide-elution experiments have indeed shown that 12% of the peptides bound to B*2704 cannot be eluted from B*2706 molecules, while 10% of the B*2706-bound peptides are not detectable in B*2704 (Garcia *et al.*, 1997; Sesma *et al.*, 2002; López de Castro *et al.*, 2004). Both subtypes are restricted to orientals, in particular individuals from China and Southeast Asia, and it was demonstrated that, like *B*2705*, *B*2704* is associated with AS, while *B*2706* (like *B*2709*) is not (López-Larrea *et al.*, 1995; Nasution *et al.*, 1997; Ren *et al.*, 1997; Chen *et al.*, 2002; Dhaliwal *et al.*, 2003).

A structural comparison of B*2706 with other HLA-B27 subtypes, especially with the closely related B*2704 (Loll, Zawacka, Biesiadka, Petter *et al.*, 2005), will contribute to a better understanding of the effect of amino-acid exchange at the floor of the peptide-binding groove on the repertoire of bound peptides. In particular, it will be interesting to investigate the possibility of differential subtype-dependent molecular mimicry (Lang *et al.*, 2002) between pLMP2 and pVIPR. Molecular mimicry has been observed between the two peptides when displayed by B*2705, but not by B*2709, indicating that it might be connected with the distinct AS-associations of these subtypes (Fiorillo *et al.*, 2005).

2. Materials and methods

2.1. Protein preparation

The nonapeptides pLMP2 (RRRWRRLTV) and pVIPR (RRKWRRWHL) were synthesized using standard solid-phase procedures and purified by reverse-phase HPLC (Alta Bioscience, Birmingham, England). The cDNA clone for the HC of B*2706 containing the three extracellular domains was generated by *in vitro* mutagenesis from a B*2704 clone. Human β_2m and the HC of B*2706 were separately expressed in *Escherichia coli* as inclusion bodies. They were solubilized by treatment with 50%(*w*/*v*) urea. According to a previously described reconstitution protocol (Garboczi *et al.*, 1992; Hülsmeyer *et al.*, 2002), the HLA-B27–peptide complexes were refolded for 14 d at 277 K, starting from denatured HC (12 mg), β_2m (10 mg) and 4 mg of either pVIPR or pLMP2. All three components were rapidly injected into 400 ml refolding buffer (400 m*M* arginine–HCl, 2 m*M* EDTA, 5 m*M* reduced glutathione, 0.5 m*M* oxidized glutathione, 100 m*M* Tris–HCl pH 7.5). Size-exclusion chromatography with Superdex 75 (Pharmacia) was then utilized to obtain highly pure intact complexes that eluted at a position corresponding to their calculated size. Fractions containing the ternary complexes were pooled and their composition (HC, β_2m) surveyed by sodium dodecyl sulfate gel electrophoresis. As omission of the peptides during reconstitution invariably resulted in lack of complex formation and absence of the chromatographic peak that is characteristic of the ternary complex, no further biochemical analyses regarding its composition were carried out. For crystallization experiments the protein was concentrated by Amicon Ultra-15 to 13–15 mg ml^{-1} in a buffer system containing 20 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl, 0.01% sodium azide.

2.2. Crystallization and data collection

B*2706–pLMP2 and B*2706–pVIPR were crystallized by hanging-drop vapour diffusion at 291 K on a siliconized cover slip over a 1 ml reservoir containing 1 ml precipitant solution (100 m*M* Tris–HCl pH 7.0 or pH 8.0) and varying concentrations [18–28%(*w*/*v*)] of polyethylene glycol (PEG) 8000, PEG 6000 or PEG 4000 to optimize crystal formation. 1.5 µl protein solution was mixed with 1.5 µl precipitant solution identical to that within the respective reservoir, employing conditions that have been described previously (Hülsmeyer *et al.*, 2002, 2004, 2005; Fiorillo *et al.*, 2005; Loll, Zawacka, Biesiadka, Petter *et al.*, 2005). The wells were immediately sealed, but since the initially obtained microcrystals were too small for X-ray data collection, streak-seeding was carried out after 1–1.5 h. A thick cat whisker was used to crush crystals in a crystallization drop from a previous experiment involving a similar composition of the precipitant solution and a thinner whisker was initially drawn through this drop and then through the drop with the newly prepared B*2706–pLMP2 or B*2706–pVIPR complexes. Following a second sealing of the wells, crystals of B*2706–pLMP2 were obtained after 4 d from a solution containing 24%(*w*/*v*) PEG 8000, 100 m*M* Tris–HCl pH 7.0 and B*2706–pVIPR crystallized using a precipitant solution composed of 20%(*w*/*v*) PEG 8000, 100 m*M* Tris–HCl pH 7.0. The crystals of both subtypes grew to maximum dimensions of about 80 × 20 × 10 µm in one week and had similar size and morphology. Prior to data collection, the crystals were transferred to a cryoprotectant solution by stepwise increases of the glycerol concentration to 10% and finally frozen in liquid nitrogen.

The data set from B*2706–pLMP2 was collected at beamline ID 14-2 of the European Synchrotron Radiation Facility (ESRF) in Grenoble (France) and that from B*2706–pVIPR at beamline BW6 of the Deutsches Elektronen-Synchrotron (DESY) in Hamburg (Germany). Both data sets were measured at a temperature of 100 K. The quality of B*2706–pLMP2 diffraction was slightly improved using flash-annealing (Harp *et al.*, 1998) by shielding the cryostream for about 5 s. Integration and scaling were accomplished using the *HKL* suite (Otwinowski & Minor, 1997). The crystals of B*2706–pLMP2 belonged to the orthorhombic space group $P2_12_12_1$ and diffracted to 2.70 Å, whereas crystals of B*2706–pVIPR belonged to the monoclinic space group $P2_1$ and diffracted beyond 1.83 Å (Table 1). Applying the Matthews equation (Matthews, 1968) and assuming a molecular weight of ~44 kDa for the HC–peptide–β_2m complex, solvent contents of 49.7% for B*2706–pLMP2 and 57.5%

for B*2706–pVIPR were estimated with one molecule per asymmetric unit. Crystallization data and X-ray data-collection statistics are summarized in Table 1.

The phase problem was solved using likelihood-based molecular replacement with *PHASER* (Storoni *et al.*, 2004). The atomic coordinates of the high-resolution crystal structure of B*2705–pGR (PDB code 2a83; Rückert *et al.*, 2005) served as search model (peptide and water molecules as well as the side chains of residues distinguishing B*2705 from B*2706 were omitted). Clear solutions for both structures were found using diffraction data in the resolution range 20–3 Å. Examination of the electron-density map generated from the initial phases allowed us to identify the presence of the nonapeptides as well as polymorphic HC amino-acid residues.

3. Results and discussion

The highly pure B*2706–pLMP2 and B*2706–pVIPR complexes were crystallized and crystal formation was optimized using streak-seeding techniques, resulting in well ordered crystals that diffracted to 2.70 Å (B*2706–pLMP2) and 1.83 Å (B*2706–pVIPR) resolution at synchrotron sources (ESRF and DESY, respectively). Contrary to our expectation from experiments involving the B*2704 complexes with the pLMP2 and pVIPR peptides (Loll, Zawacka, Biesiadka, Petter *et al.*, 2005), which both crystallized in space group $P2_12_12_1$, the B*2706–pLMP2 and B*2706–pVIPR complexes crystallized in space groups $P2_12_12_1$ and $P2_1$, respectively. This lack of crystal isomorphism has been previously observed in crystals of B*2703 in complex with pLMP2 and pVIPR (Loll, Zawacka, Biesiadka, Rückert *et al.*, 2005) and might indicate that the nonapeptides adopt conformations in B*2706 similar to those in B*2703 (Loll, Zawacka, Biesiadka, Rückert *et al.*, 2005; Zawacka *et al.*, manuscript in preparation). However, since B*2706 differs in six amino acids from B*2703, with five of these being part of the peptide-binding groove (Ramos & López de Castro, 2002; López de Castro *et al.*, 2004), it is impossible to predict the peptide-binding mode within the two B*2706 complexes with certainty without detailed model building.

To this end, refinement of both B*2706 crystal structures has been initiated to permit a comparison with the previously solved crystal structures of pLMP2 and pVIPR with B*2705, B*2709 and B*2703 (Hülsmeyer *et al.*, 2004; Fiorillo *et al.*, 2005; Loll, Zawacka, Biesiadka, Rückert *et al.*, 2005) and in particular with the closely related B*2704 (Loll, Zawacka, Biesiadka, Petter *et al.*, 2005).

Financial support for this work was provided by Deutsche Forschungsgemeinschaft (to BL and JB as well as to WS, BU-Z and A. Ziegler through SFB 449/B6) and by Fonds der Chemischen Industrie [to A. Zawacka (Kekulé-Fellowship) and WS]. We thank Uschi Gruber for excellent technical assistance and are grateful for allocation of beam time and support at ESRF (Grenoble) as well as DESY (Hamburg).

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