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Crystallization and preliminary X-ray structural studies of a Melan-A pMHC–TCR complex

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Melanocytes are specialized pigmented cells that are found in all healthy skin tissue. In certain individuals, diseased melanocytes can form malignant tumours, melanomas, which cause the majority of skin-cancer-related deaths. The melanoma-associated antigenic peptides are presented on cell surfaces *via* the class I major histocompatibility complex (MHC). Among the melanoma-associated antigens, the melanoma self-antigen A/melanoma antigen recognized by T cells (Melan-A/MART-1) has attracted attention because of its wide expression in primary and metastatic melanomas. Here, a preliminary X-ray crystal structural study of a soluble cognate T-cell receptor (TCR) in complex with a pMHC presenting the Melan-A peptide (ELAGIGILTV) is reported. The TCR and pMHC were refolded, purified and mixed together to form complexes, which were crystallized using the sitting-drop vapour-diffusion method. Single TCR–pMHC complex crystals were cryocooled and used for data collection. Diffraction data showed that these crystals belonged to space group $P4_1/P4_3$, with unit-cell parameters $a = b = 120.4$, $c = 81.6$ Å. A complete data set was collected to 3.1 Å and the structure is currently being analysed.

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1. Introduction

Melanocytes are specialized pigmented cells that are widespread in skin and eye tissue (Gray-Schopfer *et al.*, 2007). The degree of pigment production manifests as skin 'phototype' (skin colour and ease of tanning) and is the most useful predictor of human skin-cancer risk in the general population (Maresca *et al.*, 2006). The main contributors to pigmentation are melanins, which are produced by melanocytes (Gray-Schopfer *et al.*, 2007). The skin provides photoprotection and thermoregulation by using melanin. Thus, melanocytes play a key role in protecting our skin from the damaging effects of UV radiation and in preventing skin cancer, which occurs at an estimated 2–3 million cases across the world each year.

The first identified melanoma antigen recognized by T cells (MART) presented by class I MHC was MAGE (van der Bruggen *et al.*, 1991). Molecular characterizations of the antigens Melan-A/MART-1, gp100 and tyrosinase were subsequently reported (Coulie *et al.*, 1994; Kawakami, Eliyahu, Delgado, Robbins, Rivoltini *et al.*, 1994; Kawakami, Eliyahu, Delgado, Robbins, Sakaguchi *et al.*, 1994). In order to increase the weak immunogenicity of these antigens, attempts were made to modify them: for example, Melan-A/MART-1 26–35 and gp100 209–217 (Parkhurst *et al.*, 1996; Valmori *et al.*, 1998). Some modifications generated stronger T-cell responses to both wild-type and modified peptides, whilst other modifications resulted in an increased response to modified peptides only. How T-cell receptor (TCR) molecules recognize these presented antigens remains unclear. Previous work has focused on the mechanisms of melanoma-antigen presentation by MHC complexes (Sliz *et al.*, 2001; Hülsmeyer *et al.*, 2005).

Among the many melanoma-associated antigens, Melan-A/MART-1 has attracted attention because of its wide expression in primary and metastatic melanomas. It is recognized by about 90% of tumour-infiltrating lymphocytes (TILs) originating from HLA-A*0201 patients (Parmiani, 2001). We therefore isolated a TCR from

a Melan-A-specific CD8+ T-cell clone that recognizes the ELAGIGILTV peptide in complex with HLA-A*0201 and crystallized the trimolecular complex.

2. Materials and methods

2.1. Protein expression

The cDNAs of the TCR α- and β-chains from Melan-A CD8+ T-cell clone Mel5 were isolated using reverse transcription (Vα12-2, TRAJ27 and Vβ30, TRBJ2-2). The TCR chains were amplified by polymerase chain reaction and cloned into the pGMT7 *Escherichia coli* expression system as described previously, with mutations to cysteine at Cysα48 and Cysβ57 and mutation of alanine to Cysβ75 (Boulter *et al.*, 2003). The extracellular domain of HLA-A*0201 and β2m were cloned into the pGMT7 system as described previously (Gao *et al.*, 1997).

The plasmids were transformed into *E. coli* Rosetta DE3 competent cells and transformed cells were used to inoculate 1 l TYP media containing ampicillin (100 µg ml^{-1}). 1 m*M* IPTG (isopropyl β-D-thiogalactopyranoside) was used to induce expression for 5 h before harvesting the cells by centrifugation. The cell pellets were sonicated in lysis buffer (10 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 10 m*M* EDTA, 2 m*M* DTT, 0.5 m*M* PMSF, 100–400 µg ml^{-1} lysozyme, 10% glycerol, 20 µg ml^{-1} DNAse I) and centrifuged to remove soluble debris. The resulting inclusion bodies were washed with Triton buffer (0.5% Triton X-100, 50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 0.1% sodium azide, 10 m*M* EDTA, 2 m*M* DTT) and further washed with resuspension buffer (50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 10 m*M* EDTA, 2 m*M* DTT). The washed TCR inclusion bodies were then dissolved in guanidine buffer (6 *M* guanidine, 50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 10 m*M* EDTA and 10 m*M* DTT). MHC and β2m inclusion bodies were separately dissolved in a solution of 10 m*M* Tris–HCl pH 8.0, 8 *M* urea and 10 m*M* DTT. The purity of the inclusion bodies was assessed by SDS–PAGE.

2.2. Protein refolding and purification

pMHC and TCR protein refolding was carried out as described previously (Gao *et al.*, 1997; Boulter *et al.*, 2003). Briefly, 60 mg guanidine-solubilized α- and β-chain inclusion bodies were mixed in a 1:1 molar ratio and refolded by rapid dilution into 1 l refolding buffer (5 *M* urea, 0.4 *M* L-arginine, 100 m*M* Tris pH 8.1, 3.7 m*M* cystamine, 6.6 m*M* β-mercaptoethylamine) at 277 K. For pMHC refolding, the peptide was dissolved in DMSO. HLA-A*0201 heavy chain, β2m and peptide were mixed in a 1:1:3 molecular ratio with a rapid dilution into 0.4 *M* L-arginine, 100 m*M* Tris pH 8.0, 2 m*M* EDTA, 3.7 m*M* cystamine and 6.6 m*M* β-mercaptoethylamine. Solutions were mixed at 277 K for at least 1 h. Dialysis was conducted against 10 m*M* Tris–HCl pH 8.1 until the conductivity of the two refolds was less than 1 mS cm^{-1}.

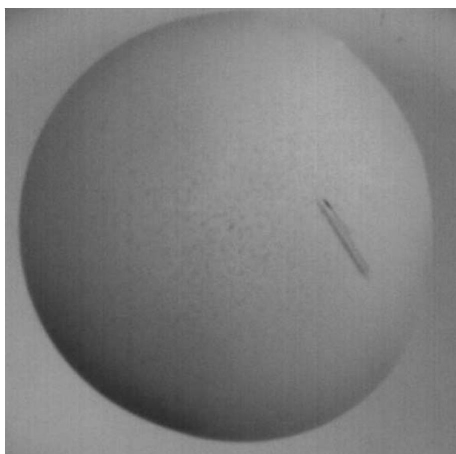

Figure 1
Typical appearance of crystals in the sitting drops.

Table 1
Diffraction data statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P4_1/P4_3$
Unit-cell parameters (Å)	$a = b = 120.4$, $c = 81.6$
Wavelength (Å)	0.979
Resolution (Å)	29–3.1 (3.27–3.1)
Measured/unique reflections	81690/21338 (11943/3090)
Completeness (%)	99.8 (100)
Multiplicity	3.8 (3.9)
$I/\sigma(I)$	7.4 (2.1)
R_{merge} (%)	20.0 (66.2)

The refolded proteins were filtered (0.2 µm) and purified by ion-exchange chromatography (POROS 50HQ column, PerSeptive Biosystems Inc.) and gel-filtration chromatography (Superdex-75HR, GE Healthcare) into crystallization buffer (10 m*M* Tris–HCl pH 8.1, 10 m*M* NaCl). The proteins were then separately concentrated to approximately 10 mg ml^{-1} using 10 kDa centrifugal concentrators prior to mixing to form the complex for crystallization.

2.3. Crystallization

Concentrated TCR and pMHC were mixed in a 1:1 molecular ratio. Crystal screening was initiated using Hampton Research Crystal Screens 1, 2 and Cryo 1 with drops consisting of 1 µl protein solution and 1 µl crystallization buffer using the hanging-drop method. Plates were incubated at 293 K and were analysed after 24 h, 48 h and one week. Further crystal screens were automated at the Structural Biology Laboratory, Daresbury using the Innovadyne Screenmaker 96+8 with Qiagen Nextal crystallization coarse screen solutions. Needle-shaped crystals were observed in a variety of conditions from Nextal PEGs Suite and Cryos suite after 5 d incubation at 293 K, but with poor diffraction of up to 30 Å resolution.

Optimized crystallization conditions were obtained using an array of PEG solutions (10–30% PEG 550 MME and PEG 400 pH 6.5–8.5, 15% glycerol) prepared using a Hamilton Microlab STAR^{LET} liquid-handling robot. Crystals (Fig. 1) were harvested from 23% PEG 550 MME, 0.1 *M* Tris pH 7.4 and 15% glycerol after incubation at 293 K for three weeks.

2.4. Data collection and processing

Data were collected using the rotation method at SRS Station 14.2, Daresbury, UK with an ADSC Quantum 4 CCD-detector system. The wavelength was set to 0.979 Å. A total of 200 frames were recorded, each covering 0.5° of rotation. The crystal was maintained at 100 K in an Oxford Cryostream. Reflection intensities were estimated using the *MOSFLM* package (Leslie, 1992) and the data were scaled, reduced and analysed with *SCALA* and the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). Crystal data and relevant statistics are given in Table 1.

3. Results and discussion

The pMHC–TCR complex crystals were analysed by X-ray diffraction and found to belong to space group $P4_1/P4_3$, as deduced from

systematic absences, with unit-cell parameters $a = b = 120.4$, $c = 81.6$ Å (Table 1). One molecule of the complex can be accommodated per asymmetric unit, giving a V_M value of 3.1 $Å^3 Da^{-1}$ and a solvent content of 60.5%. A full data set was collected to 3.1 Å resolution. A total of 81 690 observations were measured, including 21 332 unique reflections. The completeness was 99.8%, with a multiplicity of 3.8 and an R_{sym} of 20.0%. The R_{sym} statistic is relatively high, but the resolution cutoff was determined by an $I/\sigma(I)$ value of 2.1 in the outermost shell, which is acceptable for most applications, particularly with the reasonably high multiplicity of 3.9 in that shell. Considering that the present work is the result of two rounds of optimization and screening of a number of crystals, it may be considered an achievement to obtain this quality of data.

Structure determination and refinement is currently under way.

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