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Expression, refolding and preliminary X-ray crystallographic analysis of equine MHC class I molecule complexed with an EIAV-Env CTL epitope

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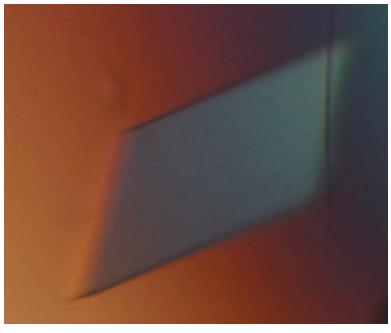

In order to clarify the structure and the peptide-presentation characteristics of the equine major histocompatibility complex (MHC) class I molecule, a complex of equine MHC class I molecule (ELA-A1 haplotype, 7-6 allele) with mouse β_2-microglobulin and the cytotoxic T lymphocyte (CTL) epitope Env-RW12 (RVEDVTNTAEYW) derived from equine infectious anaemia virus (EIAV) envelope protein (residues 195–206) was refolded and crystallized. The crystal, which belonged to space group $P2_1$, diffracted to 2.3 Å resolution and had unit-cell parameters a = 82.5, b = 71.4, c = 99.8 Å, β = 102.9°. The crystal structure contained two molecules in the asymmetric unit. These results should help to determine the first equine MHC class I molecule structure presenting an EIAV CTL epitope.

1. Introduction

Major histocompatibility complex (MHC) class I molecules are glycoproteins which play a crucial role in the processes of cellular immunity (Yewdell *et al.*, 2003). Antigenic peptides derived from pathogens are specifically presented by MHC class I molecules and subsequently recognized by T-cell receptors (TCRs) on $CD8^+$ T cells (Garboczi *et al.*, 1996). This process was not well visualized until the first structure of an MHC molecule was determined by Wiley and coworkers in 1987 (Bjorkman *et al.*, 1987). Three essential components compose an MHC class I complex: a membrane-anchored heavy chain with extremely diverse polymorphism (including external domains α1, α2 and α3), β_2-microglobulin (β2m) and a short antigenic peptide (usually 8–12 residues) which is bound in the cleft formed by the α1 and α2 domains of the heavy chain termed the peptide-binding groove (Achour *et al.*, 1998). To date, studies of MHC class I molecules have mainly focused on those from human and mouse (Liu *et al.*, 2010; Turner *et al.*, 2005). The features of equine MHC class I molecules remain poorly explored. The equine MHC class I molecule termed equine leukocyte antigen (ELA) is localized to positions q14–q22 on chromosome 20 (Ansari *et al.*, 1988). Approximately 15 ELA-A haplotypes of equine MHC class I molecules have been defined through serological assays (Lazary *et al.*, 1988). At least three loci may exist according to gene, serological and family studies (Chung *et al.*, 2003). However, the structure of the equine MHC class I molecule remains unclear.

Equine infectious anaemia virus (EIAV) is a macrophagetropic lentivirus that causes persistent infections in horses (Mealey *et al.*, 2005). Horses infected by the virus can develop recurrent febrile episodes and undergo plasma viraemia. Interestingly, in contrast to other lentiviral infections, most EIAV-infected horses can eventually control the disease process and become perpetual inapparent carriers (Mealey *et al.*, 2006). It has been proved that CTL plays an important role in eliminating the virus (Mealey *et al.*, 2003). In the 1970s, an EIAV donkey-leukocyte attenuated vaccine was developed in China and is the only effective vaccine to be developed against a lentivirus (Cohen, 2004). Therefore, determination of the equine MHC class I complex structure will help in understanding the mechanism of immune response towards EIAV and might shed light on a lentiviral vaccine strategy. In this article, utilizing *in vitro* refolding and crys-

tallization-condition screening, the equine major histocompatibility complex (ELA) class I molecule was successfully crystallized, making it possible to define the structure of the ELA-A1 haplotype 7-6 molecule (ELA-A1*7-6) complexed with an EIAV CTL epitope.

2. Materials and methods

2.1. Preparation of ELA-A1*7-6 and β2m proteins

The extracellular domain of the ELA-A1 haplotype 7-6 allele coding for 274 amino acids (GenBank accession No. AY225155) was synthesized by Beijing Sunbiotech Co. Ltd and ligated into cloning vector pMD18-T [Takara Biotechnology (Dalian) Co. Ltd]. For convenience of cloning, *Nde*I and *Xho*I restriction sites were added to the termini. After digesting the pMD18-T vector with *Nde*I and *Xho*I for about 10 h, the target 7-6 gene was recovered and inserted into the prokaryotic expression vector pET21a(+) (Novagen Merck KGaA, Darmstadt, Germany). The prokaryotic expression vector pET21a(+) containing the mouse β2m (mβ2m) gene had previously been constructed by our group (Li *et al.*, 2011). The two recombinant plasmids were transformed into *Escherichia coli* strain BL21 (DE3) and the proteins were both expressed as inclusion bodies. The process of extracting inclusion bodies was carried out as follows (Zhang *et al.*, 2010). The expression strain was inoculated into 2 l Luria–Bertani medium (LB) at 310 K. When the OD_{600} value reached 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the LB medium to a final concentration of 1 m*M* to induce protein expression. 5 h later, the bacteria were harvested, lysed and centrifugated for 10 min at 11 000 rev min^{-1}. The precipitate was then washed three times with a solution consisting of 1 m*M* EDTA, 1 m*M* DTT, 20 m*M* Tris–HCl, 100 m*M* NaCl, 0.5% Triton X-100. Finally, the inclusion bodies were dissolved in guanidinium chloride buffer [6 *M* guanidinium chloride, 50 m*M* Tris–HCl pH 8.0, 10 m*M* EDTA, 100 m*M* NaCl, 10%(*v*/*v*) glycerine, 10 m*M* DTT] to a protein concentration of 30 mg ml^{-1}.

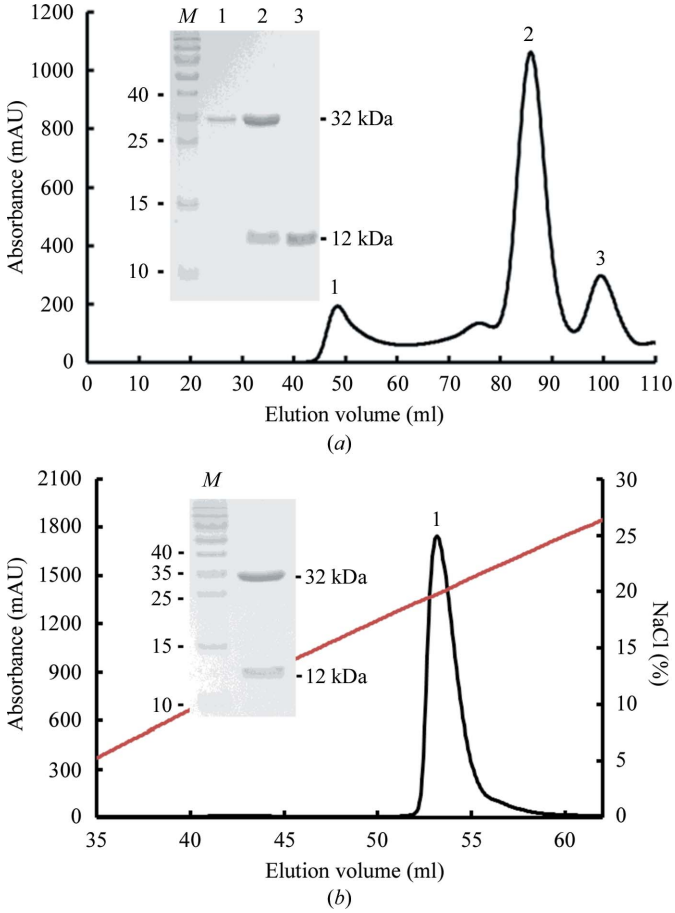

Figure 1
Elution profiles of purification of the refolded equine MHC I complex (ELA-A1*7-6, mouse β2m and EIAV Env-RW12 epitope) by Superdex 200 16/60 HiLoad gel-filtration and Resource Q anion-exchange chromatography (GE Healthcare). (*a*) Gel-filtration profile of the refolded products. Peak 1 contains aggregated heavy chain, peak 2 contains the correctly refolded complex (44 kDa) and peak 3 contains the abundant β2m. Insert: reduced SDS–PAGE gel (15%) of the corresponding purified protein from each peak. Lane *M* contains molecular-weight markers (labelled in kDa). (*b*) Results of further purification of the refolded complex by anion-exchange chromatography. The complex was eluted at an NaCl concentration of 19.0–22.5%. Insert: reduced SDS–PAGE gel (15%) of protein from the peak.

2.2. ELA-A1*7-6 complex preparation

The complex was prepared as previously described by Garboczi *et al.* (1996) with some modifications introduced in our laboratory (Zhang *et al.*, 2010). Briefly, 1 ml mβ2m protein solution was added to refolding buffer (100 m*M* Tris pH 8.0, 400 m*M* L-Arg, 2 m*M* EDTA, 5 m*M* GSH, 0.5 m*M* GSSG) using the gradual solution method at 277 K. After about 8 h, the peptide Env-RW12 (about 5 mg) was dissolved in dimethyl sulfoxide (DMSO) and added to the solution. Half an hour later, a 3 ml solution of the equine MHC I heavy chain was added dropwise to the refolding buffer. After incubation at 277 K for a further 8 h, the solution was concentrated and purified by chromatography on a Superdex 200 16/60 HiLoad size-exclusion column (GE Healthcare, USA) followed by Resource Q anion-exchange chromatography (GE Healthcare).

2.3. Crystallization of the ELA-A1*7-6 complex

The purified protein (44 kDa) was dialyzed against crystallization buffer (50 m*M* NaCl, 20 m*M* Tris–HCl pH 8.0), transferred into centrifugal filter devices with a nominal molecular-weight limit of 10 kDa (Millipore Corporation, USA) and centrifuged at 4000*g* for approximately 30 min. The concentration of protein in the filtrate was then measured using the BCA Protein Assay Kit (Novagen, USA). Finally, the protein was diluted to concentrations of 6 and 12 mg ml^{-1} for crystallization. Crystallization trials were carried out using the Index kit (Hampton Research, Laguna Hills, California, USA) at 277 and 291 K by the hanging-drop method (Li *et al.*, 2011). Two drops containing equal volumes of protein solution (at 6 and 12 mg ml^{-1}) and reservoir crystallization buffer (1 µl each) were placed over a well containing 150 ml reservoir solution in a VDX plate (Hampton Research). The best crystals were obtained in 5–7 d using Index solution No. 59 [0.02 *M* magnesium chloride hexahydrate, 0.1 *M* HEPES pH 7.5, 22%(*w*/*v*) poly(acrylic acid) sodium salt 5100] at 277 K.

2.4. Data collection and processing

Data collection from the crystal was carried out using a Rigaku MicroMax-007 rotating-anode X-ray generator operated at 40 kV and 20 mA (Cu $K\alpha$; λ = 1.5418 Å). The crystal was first immersed in reservoir solution containing 20% glycerol for a few seconds and then flash-cooled in liquid nitrogen. Diffraction data were collected to 2.3 Å resolution and were indexed and scaled using *DENZO* and the *HKL*-2000 software package (Otwinowski & Minor, 1997).

Table 1
X-ray diffraction data and processing statistics.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 82.518, b = 71.441, c = 99.874, α = 90.0, β = 102.9, γ = 90.0
Volume of the unit cell (Å^3)	573821.6
Solvent content (%)	61.67
Matthews coefficient† (Å^3 Da^{-1})	3.21
Resolution range (Å)	50.00–2.30 (2.38–2.30)
Total No. of reflections	268632
No. of unique reflections	48752
Completeness (%)	99.5 (100.0)
Average $I/\sigma(I)$	13.0 (3.9)
$R_{\rm merge}$‡ (%)	12.0 (46.4)
Average multiplicity	5.3 (5.3)

† According to Kantardjieff & Rupp (2003). ‡ $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl)\rangle$ is the average intensity from multiple measurements (Diederichs & Karplus, 1997).

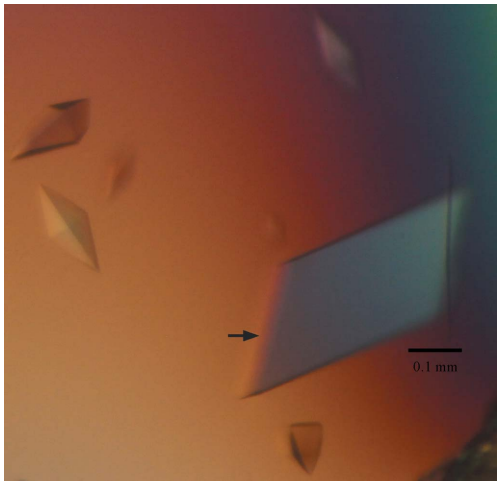

Figure 2
Diffracting crystal of the equine MHC class I molecule grown using Index condition No. 59 at 277 K. The different colours show the optical rotation of the crystals.

2.5. Comparison of the ELA-A1*7-6 sequence to those of other MHC class I molecules

Sequence alignment was performed using *DNAMAN* v.5.5.2 (Raiola *et al.*, 2004). The accession numbers of the sequences used are as follows: ELA-A1*7-6, AY225155; ELA-N*01401, ADK02607; SLA-1*0101, FJ952938; BoLA-N*01301, CAA56909; H-2Kb, V00746; Mamu-A*02, AAA96050; HLA-B*3501, 1A1_N.

3. Results and discussion

ELA-A1*7-6 was refolded together with mβ2m and the Env-RW12 epitope and resulted in yields of MHC class I molecule of about 10–15%. The complex was initially purified by Superdex 200 16/60 HiLoad size-exclusion chromatography (Fig. 1*a*). The elution profile shows three peaks corresponding to the refolded complex (44 kDa; peak 2), non-native aggregated products (peak 1) and redundant β2m (peak 3). The refolded complex was then collected and purified by Resource Q anion-exchange chromatography (Fig. 1*b*). The purity was sufficient for protein crystallization. SDS–PAGE was used to confirm the presence of the refolded complex and showed two bands corresponding to the molecular weights of the heavy chain (32 kDa) and mβ2m (12 kDa).

Crystallization trials were initially carried out using the Index kit (Hampton Research, America) at a temperature of 291 K. Protein crystals appeared in many conditions and grew quickly, but failed to diffract. We performed the trials again at a temperature of 277 K. The best crystals (Fig. 2) appeared 7 d later using Index condition No. 59 [0.02 *M* magnesium chloride hexahydrate, 0.1 *M* HEPES pH 7.5, 22%(*w*/*v*) poly(acrylic acid) sodium salt 5100]. The complex crystal belonged to space group $P2_1$, with unit-cell parameters a = 82.5, b = 71.4, c = 99.8 Å, β = 102.9°, and diffracted to 2.3 Å resolution (Fig. 3). The Matthews coefficient $V_{\rm M}$ was calculated to be about 3.21 Å^3 Da^{-1}, corresponding to 61.67% solvent content (Kantardjieff & Rupp, 2003; Matthews, 1968). Other selected data are listed in

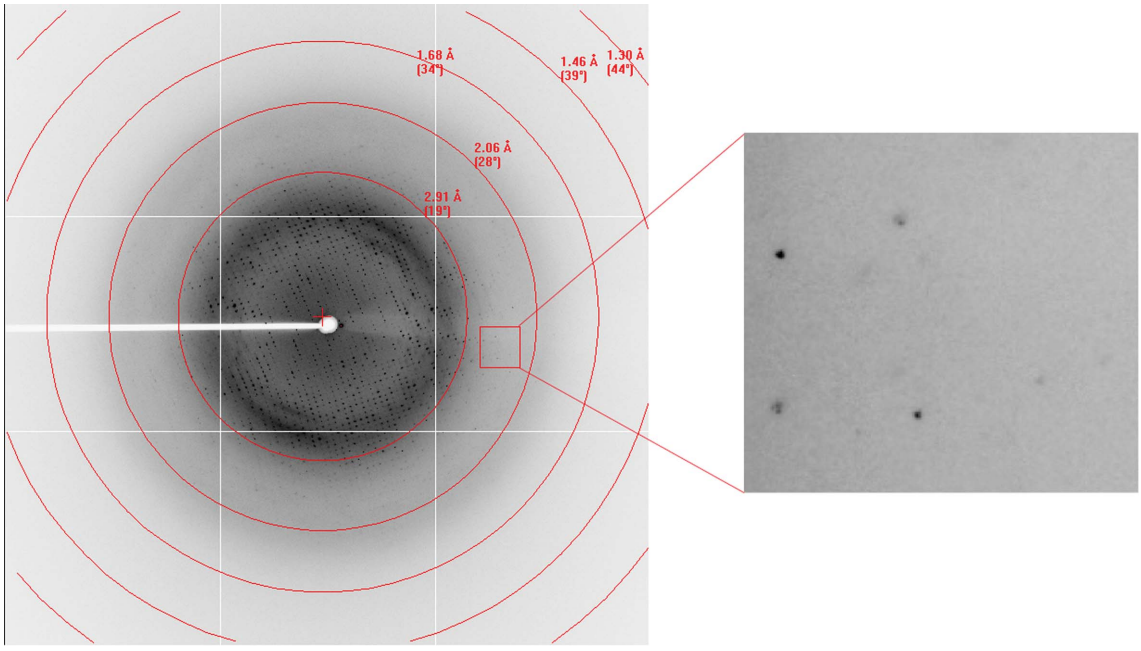

Figure 3
Diffraction pattern of the equine MHC class I molecule. The high-resolution spots are highlighted by the enlargement.

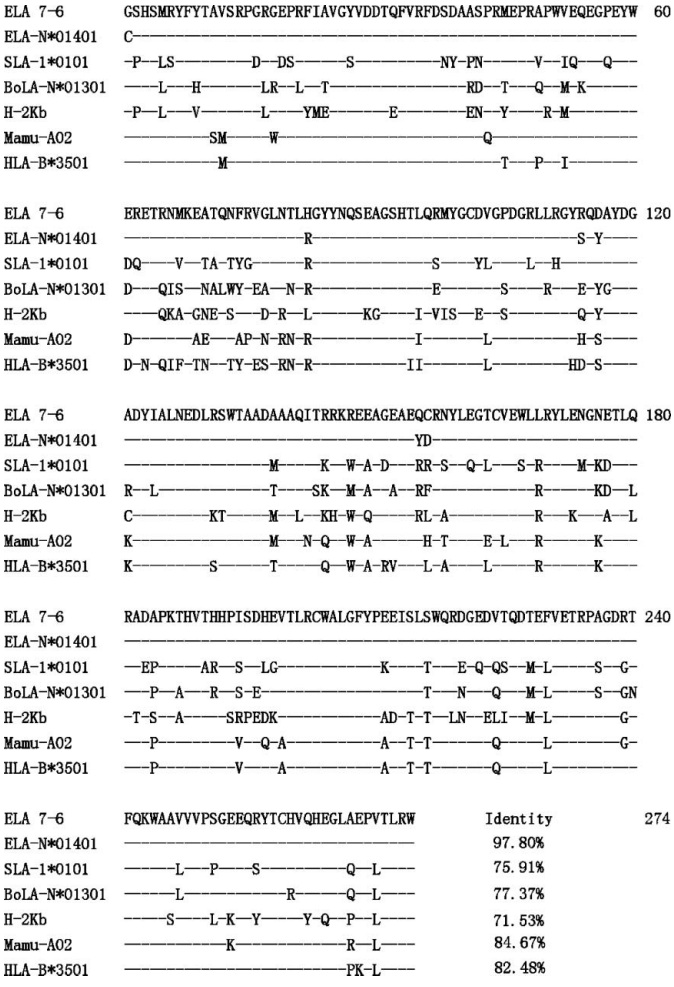

Figure 4
Alignment of the ELA-A1*7-6 amino-acid sequence with those of other MHC molecules. The alignment was performed using the *DNAMAN* program. The accession numbers of the sequences used are as follows: ELA 7-6, AY225155; ELA-N*01401, ADK02607; SLA-1*0101, FJ952938; BoLA-N*01301, CAA56909; H-2Kb, V00746; Mamu-A*02, AAA96050; HLA-B*3501, 1A1N_A. The divided domains of the heavy chain are aligned.

Table 1. It is obvious that the temperature affects not only the shape and growth rate of the crystals but also the quality.

The results of the sequence alignment of MHC class I molecules are shown in Fig. 4. Both the ELA 7-6 and 01401 alleles belong to the A1 haplotype and share 97.8% identity. ELA-A1*7-6 has 71.53, 75.91 and 77.37% identity to MHC class I molecules from mouse (H-2Kb), swine (SLA-1*0101) and cattle (BoLA-N*01301) and shares 82.48 and 84.67% identity with those from human (HLA-B*3501) and monkey (Mamu-A*02). From the results, we can see that the equine MHC class I molecule has higher homology to those from primates than to those from other animals.

Although the number of horses worldwide is decreasing, studies of their immune system are still significant. As the infection of horses with EIAV is one of the models for studying lentiviral control, the first equine MHC I molecule structure will clarify the characteristics of the horse MHC I molecule and provide information for lentiviral vaccine design.

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