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# Expression, purification, crystallization and preliminary X-ray diffraction analysis of rhesus macaque CD8*αα* homodimer

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As a T-cell co-receptor, CD8 binds to MHC class I molecules and plays a pivotal role in the activation of cytotoxic T lymphocytes. To date, structures of CD8 have been solved for two different mammals: human and mouse. The infection of rhesus macaques (*Macaca mulatta*) by simian immunodeficiency virus (SIV) is the best animal model for studying HIV. In this study, the rhesus macaque CD8 (rCD8) *αα* homodimer was obtained and rCD8*α* exodomain protein crystals were successfully obtained for further structural analysis. Diffraction data were collected to a resolution of 2.4 Å. The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters $a$ = 46.52, $b$ = 56.28, $c$ = 82.40 Å. These data will facilitate further studies on the structural differences between these CD8 structures and the cellular immune responses of rhesus macaque.

## 1. Introduction

The infection of rhesus macaques (*Macaca mulatta*) with simian immunodeficiency virus (SIV) provides one of the best animal models for investigating the basis of protective immunity against human immunodeficiency virus (HIV; Allen *et al.*, 1998; Johnson, 1996; Sauermann, 2001). Host cytotoxic T lymphocyte (CTL) responses provide a powerful defence to the virus post-infection and CTL-based immunization is currently the most promising approach towards vaccine development (Kaufmann & McMichael, 2005). A number of clinical and experimental observations have implicated virus-specific CD8+ T lymphocyte responses as critically important for controlling viral replication in humans infected with HIV-1 and in rhesus macaques infected with SIV (Kuroda *et al.*, 1999; Stott & Almond, 1995; Walker *et al.*, 1986).

As a co-receptor of the T-cell receptor (TCR), CD8 molecules stabilize the interaction of the T-cell receptor with MHC class I peptides. In the absence of CD8 interaction, MHC class I-restricted immune responses, including cytokine production and cytotoxic effector function, are hampered (Fung-Leung *et al.*, 1991). CD8 is encoded by two distinct genes that are termed CD8*α* and CD8*β* and are expressed on the cell surface as CD8*αα* homodimers or CD8*αβ* heterodimers (Norment & Littman, 1988).

The function of CD8 in the immune response in humans has been studied extensively (Norment *et al.*, 1988; Gao & Jakobsen, 2000; Gao *et al.*, 2002) as well as the main features of the binding of CD8 to many MHC molecules (Wyer *et al.*, 1999; Gao *et al.*, 2000). Functional analysis of mouse CD8*αα* and CD8*αβ* dimers has been carried out (Kern *et al.*, 1999). Human and mouse CD8 have also been studied structurally: crystal structures have been determinated of the human HLA-A*02–CD8*αα* complex (Gao *et al.*, 1997, 1998) and of murine MHC H-2Kb complexed with CD8*αα* (Kern *et al.*, 1998), as well as of the nonclassical MHC class I-like molecule TL antigen complexed with CD8*αα* (Liu *et al.*, 2003) and of the murine CD8*αβ* heterodimer (Chang *et al.*, 2005). However, there is no experimental structure for macaque monkey CD8 [95% and 45% amino-acid sequence identities to human CD8*α* (hCD8) and murine CD8*α* (mCD8), respectively]. Here, we report the crystallization of the rhesus macaque CD8*α* exodomain protein homodimer (rCD8*αα*) and describe the expression, refolding and purification of the CD8*αα* exodomain protein.

## 2. Materials and methods

### 2.1. Cloning of rhesus macaque CD8α (rCD8α) exodomain protein

Rhesus macaque CD8 α-chain nucleotides covering amino acids 1–120 (the exodomain protein without the signal peptide) plus an ATG start codon were synthesized based on the Indian-origin rhesus macaque sequence (GeneBank ID 698329) with *Nde*I and *Xho*I recognition sites. The DNA fragments were ligated into pET21a vector (Novagen). Plasmids were transformed into *Escherichia coli* strain BL21 (DE3) pLysS.

### 2.2. Preparation of rCD8α exodomain as inclusion bodies

Inclusion bodies of rCD8α exodomain protein were expressed and prepared using previously described protocols (Gao *et al.*, 1997). Briefly, the inclusion bodies were isolated from cell pellets by sonication and washed with wash buffer [0.5% Triton X-100, 50 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 10 m*M* EDTA, 10 m*M* β-mercaptoethanol (β-ME) and 0.1% $NaN_3$] three times. The inclusion bodies were then washed with resuspension buffer (50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 10 m*M* EDTA, 10 m*M* β-ME and 0.1% $NaN_3$). The inclusion bodies were dissolved overnight in guanidine buffer [6 *M* guanidine hydrochloride, 50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 10 m*M* EDTA, 10%(*v*/*v*) glycerol, 10 m*M* DTT] using about 1 ml guanidine buffer per 10 mg protein.

The rCD8α exodomain protein was renatured by dilution refolding using the refolding buffer 100 m*M* Tris–HCl, 2 m*M* EDTA, 400 m*M* L-arginine–HCl, 0.5 m*M* oxidized glutathione, 5 m*M* reduced glutathione, 0.1 m*M* PMSF and 0.1 m*M* $NaN_3$; the pH was adjusted to 8.0.

Approximately 30 mg protein was added to 500 ml refolding buffer. The refolding solution was incubated overnight and then concentrated using Stirred Cell and Ultra centrifugal filter devices (Millipore). Refolded protein was purified by gel filtration using a Superdex 75 10/300 GL column on an ÄKTA FPLC system (Amersham Biosciences).

### 2.3. Crystallization of rhesus macaque CD8α exodomain protein

After concentration in final buffer (20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl), the protein concentration of rCD8αα was adjusted to 10 mg $ml^{-1}$ using a BCA Protein Assay Kit (Thermo Scientific; Stoscheck, 1990). All crystallization attempts were performed at 291 K by hanging-drop vapour diffusion. Initial conditions were established using Crystal Screens I and II (Hampton Research; Chen *et al.*, 2007). Initial conditions were established with a 1 µl:1 µl mixture of protein solution with solution from Crystal Screens I and II (Hampton Research).

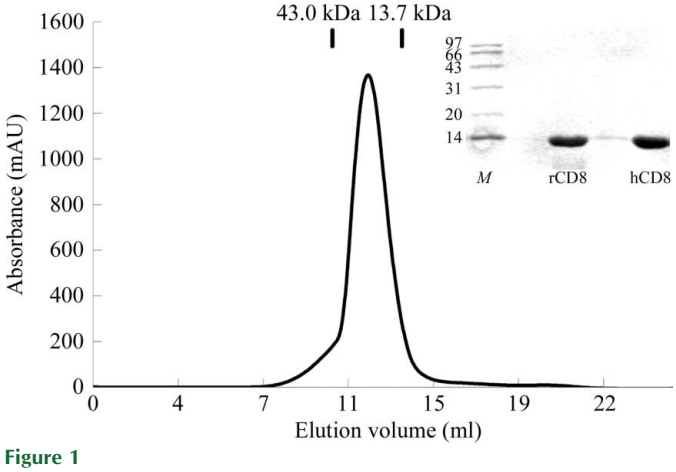


**Figure 1**
Size-exclusion chromatography and SDS–PAGE analysis of the refolded rCD8α proteins. The size-exclusion chromatography profile of rCD8α exodomain protein using Superdex 75 is presented as a black line; the approximate positions of molecular-weight standards (43 and 13.7 kDa) are marked. Inset, SDS–PAGE analysis. Lanes *M*, rCD8 and hCD8 contain protein markers (labelled in kDa), rhesus macaque CD8 and human CD8, respectively.

**Table 1**
Data-collection statistics.

Values in parentheses are for the outer shell.

| | |
|---|---|
| Space group | $P2_12_12_1$ |
| Unit-cell parameters | |
| *a* (Å) | 46.52 |
| *b* (Å) | 56.28 |
| *c* (Å) | 82.40 |
| $\alpha = \beta = \gamma$ (°) | 90 |
| Resolution range (Å) | 50–2.40 (2.49–2.40) |
| Total No. of reflections | 58674 (6158) |
| Unique reflections | 9027 (919) |
| Average redundancy | 6.5 (6.7) |
| Completeness (%) | 99.9 (100.0) |
| $R_{merge}$† (%) | 12.7 (46.5) |
| Average $I/\sigma(I)$ | 22.6 (5.1) |

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th measurement of reflection *hkl* and $\langle I(hkl)\rangle$ is the mean intensity of all symmetry-related reflections.

### 2.4. Data collection and X-ray diffraction analysis

For data collection, crystals were transferred to 15%(*v*/*v*) glycerol in reservoir buffer for about 1 min before being flash-cooled to 100 K in a nitrogen-gas stream. Data were collected using a Rigaku MicroMax007 rotating-anode X-ray generator operated at 40 kV and 20 mA (Cu *Kα*; λ = 1.5418 Å) and equipped with an R-AXIS VII$^{++}$ image-plate detector. The data-collection parameters were as follows: a total of 180 images were collected with 1° rotation per image, 2 min exposure time and a crystal-to-detector distance of 180 mm. Data were processed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The Matthews coefficient and solvent content were calculated with *MATTHEWS_COEF* (Matthews, 1968).

## 3. Results and discussion

### 3.1. Refolding and purification of rCD8αα homodimer

The inclusion bodies were isolated from 1 l LB culture containing about 6 g wet weight of cells. After sonication and centrifugation, the total mass of inclusion bodies was measured to be ∼2.5 g.

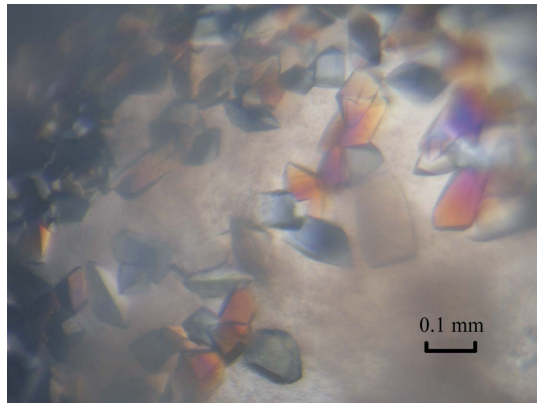


**Figure 2**
Typical appearance of rhesus macaque CD8α exodomain protein crystals.

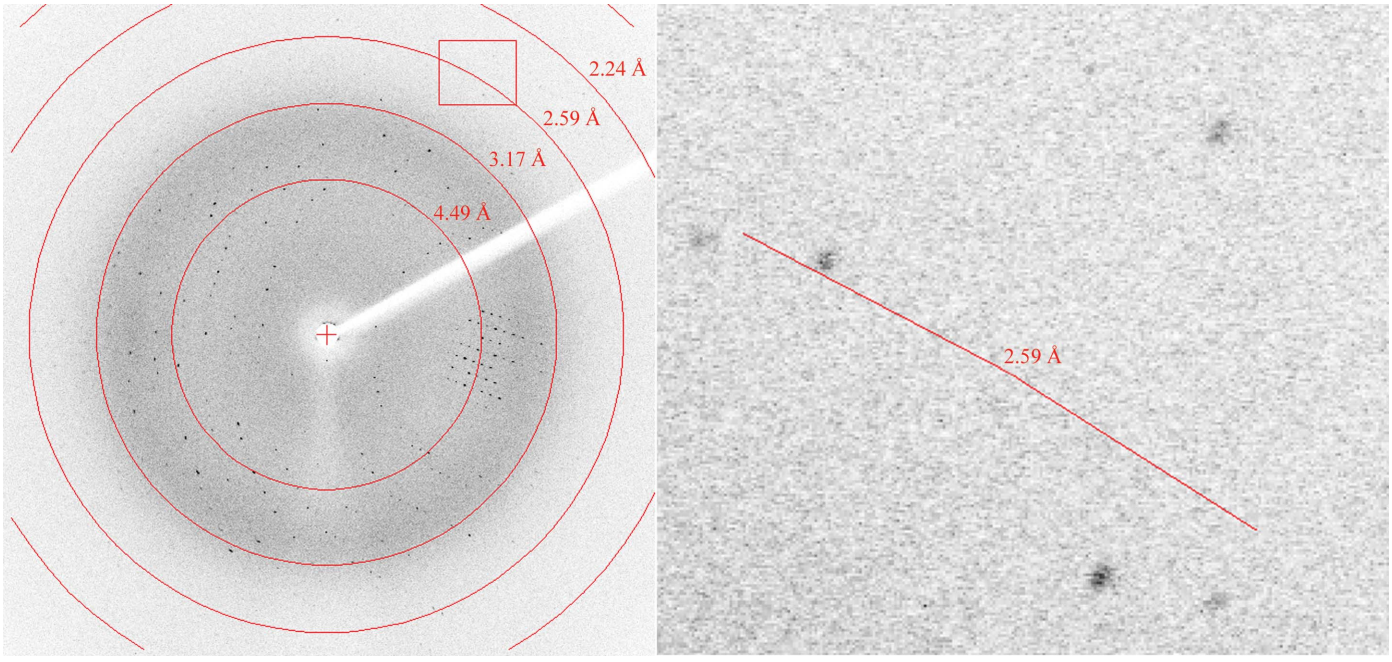


**Figure 3**
Diffraction pattern of an rCD8$\alpha$ exodomain protein crystal. The image on the right is an enlargement of the area framed on the left.

Fig. 1 shows the size-exclusion chromatogram and SDS–PAGE of the rCD8$\alpha$ exodomain protein after refolding. The rCD8$\alpha$ exodomain protein eluted as a single peak positioned between the 43 and 13.7 kDa protein markers, indicating that most of the CD8$\alpha$ exodomain protein forms an $\alpha\alpha$ homodimer (25 kDa) in solution without obvious equilibrium. Subsequent SDS–PAGE analysis showed one band corresponding to the expected molecular weight of the CD8$\alpha$ exodomain monomer (13 kDa), similar to human CD8$\alpha$ (hCD8$\alpha$; Fig. 1).

### 3.2. Crystals suitable for data collection and structure determination

It typically took 10 d for rCD8$\alpha\alpha$ crystals to grow from a 1:1 mixture of the protein solution with a crystallization reagent consisting of 0.05 *M* potassium phosphate monobasic, 20%(*w*/*v*) polyethylene glycol 8000 (Crystal Screen condition No. 42, Hampton Research). The largest crystals were about 300 × 100 × 150 µm in size (Fig. 2). The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a$ = 46.52, $b$ = 56.28, $c$ = 82.40 Å, $\alpha = \beta = \gamma = 90.00^\circ$, and diffracted to 2.4 Å resolution (Fig. 3). Diffraction data statistics and crystal parameters are given in Table 1.

Calculation of the Matthews coefficient suggested the presence of a dimer in the asymmetric unit ($V_M$ = 2.05 Å$^3$ Da$^{-1}$, solvent content of 39.99%). Further molecular replacement using human CD8$\alpha\alpha$ as the model confirmed that the asymmetric unit contained dimeric rCD8$\alpha$ molecules. The $R$ and $R_{free}$ after initial rigid-body refinement were 0.26 and 0.31, respectively.

Structure determination of the rhesus macaque CD8$\alpha$ exodomain protein is in progress and should provide detailed insights into the biochemical properties of this molecule in an important model organism.

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