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Crystallization and preliminary X-ray crystallographic studies of swine CD8α

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CD8αα homodimers or CD8αβ heterodimers form on the T-cell surface, where they are essential as co-receptors for MHC class I molecules in activation of the CTL response. To date, swine have been found to show the highest percentage of lymphocytes with surface expression of CD8α. Crystallographic analysis of swine CD8α (sCD8α) to 1.8 Å resolution revealed that the crystals belonged to space group $P3_221$, with unit-cell parameters $a = 80.97$, $b = 80.97$, $c = 95.19$ Å. The Matthews coefficient and the solvent content were calculated to be 3.23 Å^3 Da^{-1} and 61.89%, respectively. These results may aid further structural and functional analyses of sCD8α.

1. Introduction

$CD8^+$ cytotoxic T lymphocytes (CTLs) recognize antigenic peptides bound to major histocompatibility complex class I (MHC I) molecules on the surface of target cells and play a pivotal role in host defence towards intracellular pathogens and tumours (van der Merwe & Davis, 2003). CD8 attaches to peptide–MHC I (pMHC I) bound to a specific T-cell receptor (TCR) and forms an immunological synapse which results in the proliferation of CTL and clearance of the target cells (O'Rourke & Mescher, 1993). CD8 is expressed on the T-cell surface as dimers in two forms: CD8αα homodimers and CD8αβ heterodimers. Both dimers are composed of an extracellular immunoglobulin-like (Ig-like) domain, a stalk region, a transmembrane domain and an intracellular tail (Chang *et al.*, 2005; Gao & Jakobsen, 2000). Crystal structures of human and mouse CD8αα and CD8αβ show the extracellular Ig-like domain is a typical Ig variable domain and is involved in the binding to MHC I (Leahy *et al.*, 1992; Chang *et al.*, 2005; Gao *et al.*, 1997). Although both CD8αα and CD8αβ can serve as co-receptors for enhancing antigen presentation, they are different in several aspects. CD8αβ is expressed mainly by αβ T cells in the thymus and peripheral blood, while CD8αα is distributed in γδ T cells, intestinal intraepithelial lymphocytes, dendritic cells (DCs) and natural killer (NK) cell subpopulations (Norment & Littman, 1988; Chang *et al.*, 2005). These findings suggest that CD8αβ is more sensitive than CD8αα in the activation and differentiation of T cells (Wheeler *et al.*, 1992). In addition, CD8αα also binds to the nonclassical MHC I molecule (TL) with greater affinity than to classical MHC I molecules (Liu *et al.*, 2003).

The swine cellular immune system has many unique species characteristics, which are particularly reflected in $CD8^+$ T cells (Gerner *et al.*, 2009). Swine $CD8^+$ T cells include αβ T cells, γδ T cells, NK cells and a unique subset of extrathymic $CD4^+CD8^+$ double-positive cells (Yang & Parkhouse, 1997). The frequency of double-positive cells in peripheral blood is variable, but can reach as high as 80% (Saalmüller *et al.*, 1989; Pescovitz *et al.*, 1985). In comparison to other species, the expression of the CD8α chain on swine lymphocytes is much higher and is mainly in the CD8αα homodimer form (Gerner *et al.*, 2009; Saalmüller *et al.*, 1987). Furthermore, these CD8αα homodimers may be co-expressed together with CD8αβ heterodimers on αβ T lymphocytes (Gerner *et al.*, 2009). The abundant expression of swine CD8α (sCD8α) chain on lymphocytes indicates a possible important role for sCD8α in the immune response which has yet to be fully described. Here, we report the purification and crystallization of the

swine CD8$\alpha\alpha$ homodimer. These results may aid further structural and functional analyses of the role of sCD8α in the swine cellular immune system.

2. Materials and methods

2.1. Preparation of the sCD8α protein

The gene fragment encoding sCD8α mature peptide residues 1–125 (extracellular Ig-like domain) of *Sus scrofa* was chemically synthesized by the Shanghai Generay Biotechnology Company according to the sequence in GenBank (NM_001001907). The gene product was ligated into the pET21a vector (Novagen) using a pair of primers (forward primer, CCAACATATGagcttgttccggacgtcgccgg; reverse primer, CCGCTCGAGTTAgccgcagaaccacccgaaac). The plasmid was transformed into *Escherichia coli* strain BL21 (DE3). 0.5 m*M* IPTG was used to induce the expression of sCD8α inclusion bodies (Cole *et al.*, 2005). The bacteria were harvested by centrifugation at 6000*g* for 10 min and then resuspended in cold phosphate-buffered saline (PBS). After sonication, the sample was centrifuged at 16 000*g* and the pellet was washed three times with a solution consisting of 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT and 0.5% Triton X-100. Finally, the inclusion bodies were dissolved in guanidinium chloride (Gua–HCl) buffer [6 *M* Gua–HCl, 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 concentration of 30 mg ml^{-1}.

2.2. Protein refolding and purification

Soluble sCD8α was prepared essentially as described previously by Gao *et al.* (1998) with some modifications. The dissolved sCD8α inclusion bodies were gradually added to 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 pH 7.4) to 60 mg l^{-1}. After incubation for 24 h at 277 K, the soluble portion was concentrated and purified by chromatography on a Superdex 75 10/300 column (GE Healthcare).

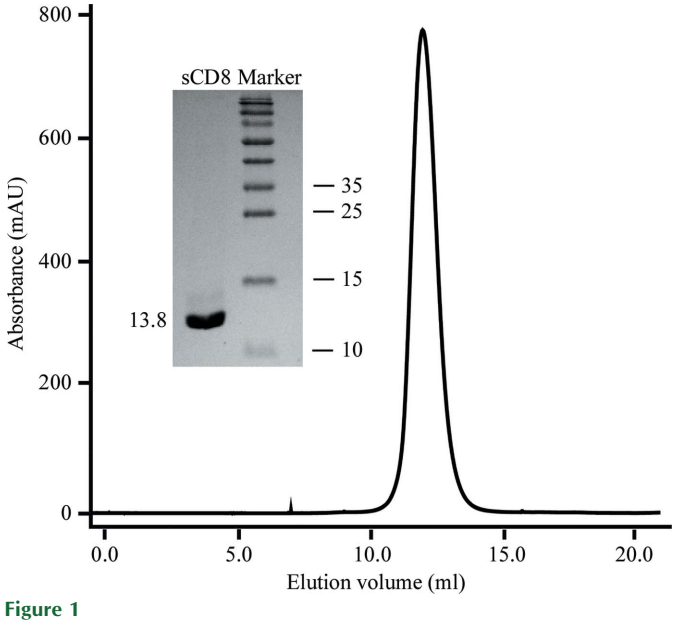

Figure 1
Purification of refolded sCD8α by FPLC Superdex 75 10/300 column chromatography (GE Healthcare). The peak represents the correctly refolded sCD8$\alpha\alpha$ homodimer (~27 kDa). Inset: reduced SDS–PAGE gel (15%) for the peak. The right column contains molecular-weight markers (labelled in kDa).

2.3. Crystallization of sCD8α

Purified sCD8α was concentrated to 6 mg ml^{-1} in a buffer consisting of 20 m*M* Tris pH 7.4 and 50 m*M* NaCl for crystallization. After mixing it with reservoir buffer in a 1:1 ratio, the sCD8α was crystallized by the hanging-drop vapour-diffusion technique at 291 K. The Index kit (Hampton Research, Riverside, California, USA) was used for crystal screening. After 7 d, crystals of sCD8α were obtained using solution No. 32 [1.0 *M* ammonium sulfate, 0.1 *M* Bis-Tris pH 5.5, 1%(*w*/*v*) polyethylene glycol 3350].

2.4. Data collection and processing

Diffraction data were collected to 1.8 Å resolution on beamline NE3A in the KEK synchrotron facility (Tsukuba, Japan) at a wavelength of 1.0 Å using an ADSC Q270 imaging-plate detector. In each case, the crystal was first soaked in reservoir solution containing 15% glycerol as a cryoprotectant for several seconds and then flash-cooled in a stream of gaseous nitrogen at 100 K (Parkin & Hope, 1998). The collected intensities were indexed, integrated, corrected for absorption, scaled and merged using *HKL*-2000 (Otwinowski & Minor, 1997).

2.5. Alignment of sCD8α with human, monkey and mouse CD8α molecules

The amino-acid sequence of sCD8α was aligned with CD8α sequences from human, monkey and mouse CD8α crystal structures. Sequence alignment was performed using the *DNAMAN* program v.5.2.2 (Lynnon Biosoft). The GenBank accession Nos. or PDB entries of the used sequences are as follows: sCD8α, ABK30934; human CD8α, 1akj (Gao *et al.*, 1997); monkey CD8α, 2q3a (L. Zong, Y. Chen, H. Peng, D. K. Cole, F. Gao, Y. Liu & G. F. Gao, unpublished work); mouse CD8α, 3dmm (Wang *et al.*, 2009).

3. Results and discussion

The sCD8α protein was expressed as inclusion bodies in *E. coli* as described previously for human and monkey CD8α (Zong *et al.*, 2009; Gao *et al.*, 1998). Soluble sCD8α was obtained by *in vitro* refolding with approximately 5% efficiency and was purified to homogeneity

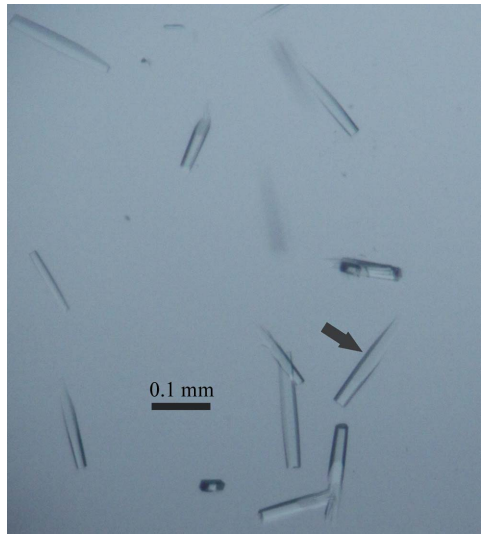

Figure 2
Photograph of the crystals used for diffraction analysis.

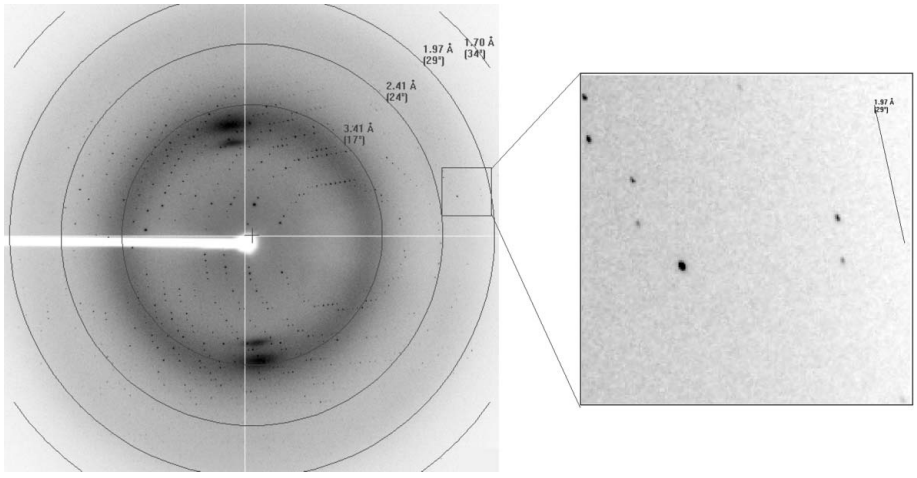

Figure 3
Diffraction pattern of sCD8α; spots corresponding to diffraction to high resolution are highlighted in the box.

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Swine  CD8α  SLFRTSPEMVQASLGETVKLRCEVMHSNTLTSCSWLYQ.KPGAASKPIFLMYLSKTRNKT..A    60
Mouse  CD8α  PEL-IF-KKMD-E--QK-D-V---LG-.VSQG----F-NSSSKLPQ-T-VV-MASSH--ITWD    62
Monkey CD8α  NQ--V--LGRTWN-----E-K-Q-LL--PTSG----F-.PR-T-AR-T--L---QNKP-A..-    60
Human  CD8α  -Q--V--LDRTWN-----E-K-Q-LL--PTSG----F-.PR---AS-T--L---QNKP-A..-    60

Swine  CD8α  EGLDT.RYISGYK.ANDNFYLILHRFREEDQGYYFCSFLSNSVLYFSNFMSVFLPAKPTKTP    120
Mouse  CD8α  -K-NSSKLF-AMRDT-NKYV-T-NK-SK-NE------VI----M---SVVP-LQKVSSALV-    124 34.40%
Monkey CD8α  -----.QRF--KR.LG-T-V-T-RD--Q-NE------A----IM---H-VP--------T--    120 55.83%
Human  CD8α  -----.QRF--KR.LG-T-V-T-SD--R-NE------A----IM---H-VP--------T--    120 57.50%
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Figure 4
Amino-acid sequence alignment of sCD8α with CD8α molecules from human, monkey and mouse for which structures have been determined. The sequence identities between sCD8α and the CD8α molecules from other species are listed on the right. Dashes represent identical residues to sCD8α. Two conserved cysteines form an intramolecular disulfide bond and are highlighted by shading. GenBank accession Nos. and PDB codes are as follows: sCD8α, ABK30934; human CD8α, 1akj; monkey CD8α, 2q3a; mouse CD8α, 3dmm.

Table 1
X-ray diffraction data and processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P3_221$
Unit-cell parameters (Å)	a = 80.97, b = 80.97, c = 95.19
Resolution range (Å)	50−1.80 (1.86–1.80)
Total reflections	516532
Unique reflections	33859
Completeness (%)	99.9 (99.9)
$R_{\rm merge}$† (%)	10.7 (58.9)
Average $I/\sigma(I)$	30.24 (5.48)
Average multiplicity	15.2 (14.4)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$is the observed intensity and $\langle I(hkl)\rangle$ is the average intensity from multiple measurements.

by Superdex 75 10/300 size-exclusion chromatography (Fig. 1). The chromatographic elution profile shows a smooth, homogeneous and symmetrical peak at 12 ml (∼28 kDa). Considering the molecular weight of sCD8α (13.8 kDa), the result shows that the soluble sCD8α exists in a homodimeric form. SDS–PAGE analysis shows a pure band corresponding to the expected molecular weight of sCD8α (see inset in Fig. 1).

The purified sCD8α was concentrated to 6 mg ml^{-1} for crystal screening. Crystals (Fig. 2) appeared after 7 d under the initial conditions. The crystal used for data collection belonged to space group $P3_221$, with unit-cell parameters a = 80.97, b = 80.97, c = 95.19 Å, and diffracted to 1.8 Å resolution (Fig. 3). The crystal was estimated to contain two sCD8α molecules in the asymmetric unit and the Matthews coefficient $V_{\rm M}$ was 3.23 Å^3 Da^{-1}, with a calculated solvent content of 61.9%. Selected data statistics are shown in Table 1.

A multiple amino-acid sequence alignment is shown in Fig. 4. The similarity of the residues and the identity of two cysteine residues indicate that sCD8α should contain an extracellular Ig-like domain similar to those of CD8α molecules from other species. However, sCD8α only has 57.50, 55.83 and 34.40% amino-acid sequence identity to human, monkey and mouse CD8α molecules, respectively. The low homology indicates that sCD8α could have unique structural characteristics. These results provide the basis for the further study of the functions of sCD8α in the swine cellular immune system.

Structure determination and refinement is currently under way by molecular replacement with human CD8$\alpha\alpha$ (PDB entry 1akj; Gao *et al.*, 1997) as the search model.

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