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Crystallization and preliminary X-ray diffraction analysis of a single variable domain of the immunoglobulin superfamily in amphioxus, *Amphi-IgSF-V*

Amphioxus is regarded as an essential animal model for the study of immune evolution. Discovery of new molecules with the immunoglobulin superfamily (IgSF) variable (V) domain in amphioxus would help in studying the evolution of IgSF V molecules in the immune system. A protein was found which just contains only one IgSF V domain in amphioxus, termed *Amphi-IgSF-V*; it has over 30% sequence identity to the V domains of human immunoglobulins and mammalian T-cell receptors. In order to clarify the three-dimensional structure of this new molecule in amphioxus, *Amphi-IgSF-V* was expressed, purified and crystallized, and diffraction data were collected to a resolution of 1.95 Å. The crystal belonged to space group $P3_221$, with unit-cell parameters $a = b = 53.9$, $c = 135.5$ Å. The Matthews coefficient and solvent content were calculated to be $2.58 \text{ Å}^3 \text{ Da}^{-1}$ and 52.38%, respectively. The results will provide structural information to study the evolution of IgSF V molecules in the immune system.

1. Introduction

The immunoglobulin superfamily (IgSF) is a large group of proteins that are found in organisms from bacteria to humans. These proteins share a similar immunoglobulin fold but differ in biological functions (Halaby & Mornon, 1998). The basic evolutionary unit of IgSF contains two β -sheets packed as a sandwich structure. Based on the β -strand topology, IgSF domains are classified as variable (V), constant (C), strand-switched (S) or hybrid (H) (Teichmann & Chothia, 2000). IgSF V domains are regarded as a pivotal structural basis of the immune system and exhibit a broad range of immunological functions (Cannon *et al.*, 2010). However, when the immune system employed these IgSF V domains remains unknown (Hernández Prada *et al.*, 2006).

Amphioxus is regarded as an essential animal model to study immune evolution (Han *et al.*, 2010). To date, two molecules termed VCBP (variable-region-containing chitin-binding protein) and VCP (V- and C-domain-bearing protein) containing two IgSF domains have been identified in amphioxus (Yu *et al.*, 2005; Litman *et al.*, 2005). However, only the structures of V domains of VCBP have been determined (Hernández Prada *et al.*, 2006). Thus, further discovery of new molecules containing the IgSF V domain from amphioxus will be helpful in understanding the evolution of IgSF molecules in the immune system (Litman *et al.*, 2010). In this article, based on the published amphioxus genome sequence (Putnam *et al.*, 2008), a new molecule termed *Amphi-IgSF-V* that just contains one IgSF V domain was found in amphioxus. The *Amphi-IgSF-V* possesses 101 residues including the so-called 'eight invariant residues' in the IgSF V domain. In order to clarify the three-dimensional structure of this molecule, *Amphi-IgSF-V* was expressed, purified and crystallized, and X-ray diffraction data were collected to 1.95 Å resolution. The result will provide an important clue in the study of the evolution of IgSF V molecules.

2. Materials and methods

2.1. Macromolecule production

The V domain of human Ig light chain (AAC26733) was used as the query sequence to search for Ig superfamily molecules in

Table 1
Amphi-IgSF-V production information.

Source organism	Florida amphioxus (<i>B. floridae</i>)
DNA source	Synthesized according to the sequence released in GenBank (XM_002587790)
Cloning vector	pMD18-T
Expression vector	pET-21a(+)
Expression host	<i>E. coli</i> strain BL21 (DE3)
Complete amino-acid sequence of the construct produced	QIFEEAGKSVTLPCDYSFSGARYTIDWHRGAERLLNFFSEDS-NPTFDSTDLGRLSLQDGKKNLVISNLQTTDDGRYYCGVSE-IGGNPPGDGSDKNLVTVTD

amphioxus. The *BlastP* program was used to screen the database of the nonredundant protein sequence (nr) of amphioxus (taxid: 7736) and BLOSUM62 was selected for the scoring matrix in NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). As a result, the *Amphi-IgSF-V* (XM_002587790) from amphioxus (*Branchiostoma floridae*) was found.

The *Amphi-IgSF-V* gene encoding 101 amino acids was synthesized by Invitrogen Life Technologies (Shanghai) according to the

Table 2
Crystallization conditions of *Amphi-IgSF-V*.

Method	Sitting-drop vapour diffusion
Plate type	48-well VDX plate
Temperature (K)	291
Protein concentration (mg ml ⁻¹)	8
Buffer composition of protein solution	20 mM Tris-HCl pH 8.0, 50 mM NaCl
Composition of reservoir solution	0.2 M sodium chloride, 0.1 M sodium acetate trihydrate pH 4.6, 30% (v/v) 2-methyl-2,4-pentanediol
Volume and ratio of drop	2 µl, 1:1 protein solution:reservoir solution
Volume of reservoir (µl)	150

sequence released in GenBank (XM_002587790, nucleotides 58–360) and ligated into cloning vector pMD18-T [Takara Biotechnology (Dalian) Co. Ltd]. A unique *Nde* I restriction site was added to the 5'-terminus; a stop codon and a specific *Xho*I restriction site were added to the 3'-terminus. After digestion, the gene was subcloned into the pET-21a(+) vector (Novagen, Merck KGaA, Darmstadt, Germany) and transformed into *Escherichia coli* strain BL21 (DE3). When the OD₆₀₀ reached 0.6, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium to induce protein expression. After 5 h, the cells were harvested by centrifugation at 6000g for 10 min at 277 K and then resuspended in cold phosphate-buffered saline (PBS). After sonication, the sample was centrifuged and washed three times with a solution consisting of 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100 (Lu *et al.*, 2012). The inclusion bodies were dissolved to a concentration of 30 mg ml⁻¹ in guanidinium chloride (Gua-HCl) buffer [6 M Gua-HCl, 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 mM NaCl, 10% (v/v) glycerol, 10 mM DTT].

The soluble *Amphi-IgSF-V* protein was prepared as described previously (Chen *et al.*, 2010) with some modifications. The *Amphi-IgSF-V* inclusion bodies were gradually dissolved in the refolding buffer (100 mM Tris-HCl, 2 mM EDTA, 400 mM L-arginine-HCl, 0.5 mM oxidized glutathione, 5 mM reduced glutathione pH 8.0). After incubation at 277 K for 12 h, the soluble *Amphi-IgSF-V* was concentrated and purified by gel filtration using a Superdex 200 size-exclusion column (GE Healthcare) and anion-exchange chromatography using Resource-Q (GE Healthcare). Finally, 10 mg protein was obtained after refolding from 90 mg *Amphi-IgSF-V* inclusion

Figure 1
Purification of *Amphi-IgSF-V* by Superdex 200 16/60 HiLoad gel-filtration and Resource-Q anion-exchange chromatography (GE Healthcare). (a) Gel-filtration profile of the *Amphi-IgSF-V*. Inset, reduced SDS-PAGE gel (15%) of the corresponding purified protein (~11 kDa). Lane M contains molecular-weight markers (labelled in kDa) and lane S contains the purified sample (*Amphi-IgSF-V* protein). (b) Results of further purification of the *Amphi-IgSF-V* by anion-exchange chromatography. The peak was eluted at an NaCl concentration of 20–24%. Inset, reduced SDS-PAGE gel (15%) of the corresponding purified protein. Lane M contains molecular-weight markers (labelled in kDa) and lane S contains the purified sample.

Figure 2
Picture of the crystal used for diffraction analysis.

Figure 3
Diffraction pattern of the *Amphi-IgSF-V* crystal. High-resolution diffraction spots are highlighted in the box.

Table 3
Data-collection statistics for *Amphi-IgSF-V*.

Values in parentheses are for the outer shell.

Diffraction source	Rigaku MicroMax-007
Wavelength (Å)	1.54178
Temperature (K)	100
Detector	R-Axis IV ⁺⁺
Crystal-to-detector distance (mm)	140
Rotation range per image (°)	1
Total rotation range (°)	202
Exposure time per image (s)	180
Space group	<i>P</i> 3 ₂ 21
Unit-cell parameters (Å, °)	<i>a</i> = 53.9, <i>b</i> = 53.9, <i>c</i> = 135.5, <i>α</i> = 90, <i>β</i> = 90, <i>γ</i> = 120
Mosaicity (°)	0.555
Resolution range (Å)	50–1.95 (2.02–1.95)
Total No. of reflections	201260
No. of unique reflections	17254
Completeness (%)	99.1 (98.1)
Multiplicity	11.7 (11.8)
<i>I</i> / <i>σ</i> (<i>I</i>)	50.147 (18.417)
<i>R</i> _{merge} [†] (%)	5.3 (14.6)
Overall <i>B</i> factor from Wilson plot (Å ²)	22.1

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

bodies. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

The purified *Amphi-IgSF-V* protein was dialysed against crystallization buffer (20 mM Tris–HCl pH 8.0, 50 mM NaCl) and concentrated to 3 and 8 mg ml^{−1} for crystallization. A crystal of *Amphi-IgSF-V* was obtained using screening kits from Hampton Research at 291 K using the sitting-drop vapour-diffusion method. Two drops comprising equal volumes of protein solution and reservoir crystallization buffer (1 µl each) were mixed and were equilibrated against 150 µl of the same reservoir solution in a VDX plate (Hampton Research). After 7 d, an ideal crystal of *Amphi-IgSF-V* appeared using a protein concentration of 8 mg ml^{−1} and a reservoir consisting of 0.2 M sodium chloride, 0.1 M sodium acetate trihydrate pH 4.6, 30% (v/v) 2-methyl-2,4-pentanediol. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

Data collection from the crystal was carried out in-house using a Rigaku MicroMax-007 rotating-anode X-ray generator operated at 40 kV and 30 mA (Cu *Kα*; λ = 1.54178 Å) equipped with an R-Axis IV⁺⁺ image-plate detector. Before diffraction, the crystal was soaked in reservoir solution supplemented with 15% (v/v) glycerol as a cryoprotectant for a few seconds and then flash-cooled in a stream of gaseous nitrogen at 100 K. The collected intensities were indexed, integrated, corrected for absorption, scaled and merged using *HKL-2000* (Otwinowski & Minor, 1997).

3. Results and discussion

The refolding efficiency of the *Amphi-IgSF-V* inclusion bodies was 10–13%. The *Amphi-IgSF-V* protein was first purified by Superdex 200 16/60 HiLoad size-exclusion chromatography (Fig. 1*a*). Considering the molecular mass of the *Amphi-IgSF-V* monomer (~11 kDa), peak 1 (~22 kDa) corresponded to an *Amphi-IgSF-V* dimer. The collected protein was then purified by Resource-Q anion-exchange chromatography and the specific peak appeared at an NaCl concentration of 20–24% (Fig. 1*b*). SDS–PAGE analysis showed a single band corresponding to the expressed *Amphi-IgSF-V* (~11 kDa; Fig. 1, inset). The purified *Amphi-IgSF-V* protein was pooled and concentrated for crystallization.

An ideal crystal of *Amphi-IgSF-V* (Fig. 2) appeared in a drop consisting of 0.2 M sodium chloride, 0.1 M sodium acetate trihydrate pH 4.6, 30% (v/v) 2-methyl-2,4-pentanediol using a protein concentration of 8 mg ml^{−1}. The *Amphi-IgSF-V* crystal belonged to space group *P*3₂21, with unit-cell parameters *a* = *b* = 53.9, *c* = 135.5 Å, and diffracted to 1.95 Å resolution (Fig. 3). The Matthews coefficient was calculated to be 2.58 Å³ Da^{−1}, corresponding to 52.38% solvent content for two molecules in the asymmetric unit (Matthews, 1968). Selected data statistics are listed in Table 3.

As shown in the sequence alignment (Supplementary Fig. S1[†]), *Amphi-IgSF-V* shares eight highly conserved residues (Gly7, Cys14, Trp27, Arg54, Leu63, Asp72, Tyr76 and Cys78) which were found in

[†] Supporting information has been deposited in the IUCr electronic archive (Reference: PU5424).

almost all IgSF V domains. In addition, *Amphi*-IgSF-V has 27, 20, 31 and 31% identity to the V domains of amphioxus VCBP, catfish novel immune-type receptor (NITR), opossum T-cell receptor delta chain and human Ig light chain, respectively. The low homology between *Amphi*-IgSF-V and the other domains indicates that the *Amphi*-IgSF-V might have a different three-dimensional structure.

The basal chordate amphioxus has an important status in the evolution of immunity (Han *et al.*, 2010). Genome sequencing suggests the existence of a species-specific immune system in Florida amphioxus (Putnam *et al.*, 2008). Crystallization and preliminary X-ray diffraction analysis of *Amphi*-IgSF-V will facilitate study of the evolution of IgSF and provide a clue for the study of the immune system in amphioxus. Structure determination and refinement are currently under way using the molecular-replacement method.

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