

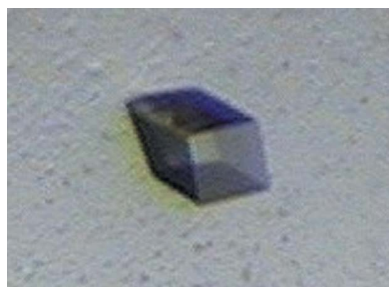
**Ji Yeon Lee,^a Hyoun Sook Kim,^a
 In Wha Baek,^a Jang Mi Back,^a
 Mi Ra Han,^a Sun-Young Kong,^b
 Ji Hyeon Kim,^b Robert N.
 Kirchdoerfer,^c Jae-Ouk Kim,^d
 Max D. Cooper,^e Ian A. Wilson,^c
 Hyun-Jung Kim^{b*} and
 Byung Woo Han^{a*}**

^aResearch Institute of Pharmaceutical Sciences,
 College of Pharmacy, Seoul National University,
 Seoul 151-742, Republic of Korea,

^bCollege of Pharmacy, Chung-Ang University,
 Seoul 156-756, Republic of Korea, ^cDepartment
 of Molecular Biology and The Skaggs Institute of
 Chemical Biology, The Scripps Research
 Institute, La Jolla, CA 92037, USA, ^dLaboratory
 Science Division, International Vaccine Institute,
 Seoul 151-919, Republic of Korea, and
^eDepartment of Pathology and Laboratory
 Medicine, Emory University School of Medicine,
 Atlanta, GA 30322, USA

Correspondence e-mail:
 hyunjungkim@cau.ac.kr, bwihan@snu.ac.kr

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Overexpression, crystallization and preliminary X-ray crystallographic analysis of the variable lymphocyte receptor 2913 ectodomain fused with internalin B

In jawless vertebrates, variable lymphocyte receptors (VLRs) play a crucial role in the recognition of antigens as part of the adaptive immune system. Leucine-rich repeat (LRR) modules and the highly variable insert (HVI) of VLRs contribute to the specificity and diversity of antigen recognition. VLR2913, the antigen of which is not known, contains the same HVI amino-acid sequence as that of VLR RBC36, which recognizes the H-trisaccharide from human blood type O erythrocytes. Since the HVI sequence is rarely identical among all known VLRs, identification of the antigen for VLR2913 and the main contributing factors for antigen recognition based on a comparison of VLR2913 and VLR RBC36 has been attempted. To initiate and facilitate this structural approach, the ectodomain of VLR2913 was fused with the N-terminal domain of internalin B (InlB-VLR2913-ECD). Three amino-acid residues on the concave surface of the LRR modules of InlB-VLR2913-ECD were mutated, considering important residues for hydrogen bonds in the recognition of H-trisaccharide by VLR RBC36. InlB-VLR2913-ECD was overexpressed in *Escherichia coli* and was crystallized at 295 K using the sitting-drop vapour-diffusion method. X-ray diffraction data were collected to 2.04 Å resolution and could be indexed in the tetragonal space group $P4_12_12$ (or $P4_32_12$), with unit-cell parameters $a = 91.12$, $b = 91.12$, $c = 62.87$ Å. Assuming that one monomer molecule was present in the crystallographic asymmetric unit, the calculated Matthews coefficient (V_M) was $2.75 \text{ \AA}^3 \text{ Da}^{-1}$ and the solvent content was 55.2%. Structural determination of InlB-VLR2913-ECD by molecular replacement is in progress.

1. Introduction

Variable lymphocyte receptors (VLRs) recognize foreign antigens to achieve adaptive immunity in jawless vertebrates, in contrast to the immunoglobulin (Ig) type antigen receptors of jawed vertebrates (Pancer *et al.*, 2004; Alder *et al.*, 2005). In lamprey and hagfish, the only two surviving jawless vertebrates, VLRs have been isolated by repeated injections with a cocktail of particulate antigens and mitogens (Pancer *et al.*, 2004, 2005). VLRs recognize antigens using leucine-rich repeat (LRR) modules and a highly variable insert (HVI) composing the ectodomain of the functional receptor of VLRs (Han *et al.*, 2008; Velikovskiy *et al.*, 2009; Kim *et al.*, 2007). For diverse antigen recognition by VLRs, various numbers of LRR modules are generated by recombinatorial DNA assembly into the *vlr* loci, which differs from the analogous process for antibody V, D and J gene segments (Alder *et al.*, 2005; Pancer *et al.*, 2004; Tasumi *et al.*, 2009).

VLRs further enhance antigen-binding specificity through an HVI in the C-terminal leucine-rich repeat (LRRCT), which adopts various secondary structures and conformations (Han *et al.*, 2008; Velikovskiy *et al.*, 2009). The HVI amino-acid sequence and length are important for antigen-specific interaction by VLRs (Han *et al.*, 2008; Deng *et al.*, 2010; Velikovskiy *et al.*, 2009). The amino-acid sequence of the HVI is highly diverse among all known VLRs. It is therefore a rare occurrence for the HVI of VLR2913 to be identical to that of VLR RBC36, which recognizes the H-trisaccharide from human blood type O erythrocytes (Han *et al.*, 2008). Owing to the rare identity of the HVIs between VLR2913 and VLR RBC36, H-trisaccharide or a similar glycan are potential antigen candidates for VLR2913, the specific antigen of which remains undetermined.

We are currently attempting to discover the antigen for VLR2913 and characterize the modes of VLR–antigen recognition through hypothetical interactions between VLR2913 and H-trisaccharide. To elucidate the crucial factor for antigen recognition by VLRS and to obtain a high level of soluble VLR2913, we designed key-residue mutant forms of VLR2913 fused with internalin B (InIB-VLR2913-ECD), which displays high thermodynamic and pH stabilities (Han *et al.*, 2008; Lee *et al.*, 2012). VLR RBC36 recognizes H-trisaccharide *via* hydrogen bonds to three key residues and van der Waals interactions with several residues on the concave surface (Han *et al.*, 2008). Since hydrogen bonds would contribute to antigen recognition more than van der Waals interactions, we mutated the three key residues on the concave surface of the LRR modules of InIB-VLR2913-ECD to mimic hydrogen bonds based on the sequence alignment and the crystal structure of VLR RBC36 in complex with H-trisaccharide (Han *et al.*, 2008). We overexpressed the soluble InIB-VLR2913-ECD with three mutations (A70D, N118D and D119Q) in the LRR modules using an *Escherichia coli* expression system. In this report, we describe the cloning, overexpression, purification, crystallization and preliminary X-ray crystallographic analysis of InIB-VLR2913-ECD.

2. Materials and methods

2.1. Cloning and expression

The VLR2913 ectodomain was cloned into the expression vector pET-21a(+) (Novagen, Darmstadt, Germany), adding a hexahistidine-containing eight-residue tag to the C-terminus. LRRV1 to LRRCT (119 amino acids) of VLR2913 was inserted into the vector, and the N-terminal domain (81 amino acids) of internalin B was fused to the N-terminus of VLR2913 (Lee *et al.*, 2012). The mutation of three amino-acid residues (A70D, N118D and D119Q) in the LRR modules of InIB-VLR2913-ECD was performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, USA).

The recombinant protein was overexpressed in *E. coli* BL21 (DE3) RIPL cells using Luria broth culture medium. Protein expression was induced using 0.5 mM isopropyl β -D-1-thiogalactopyranoside and the cells were incubated for 16 h at 293 K following growth to mid-log phase at 310 K.

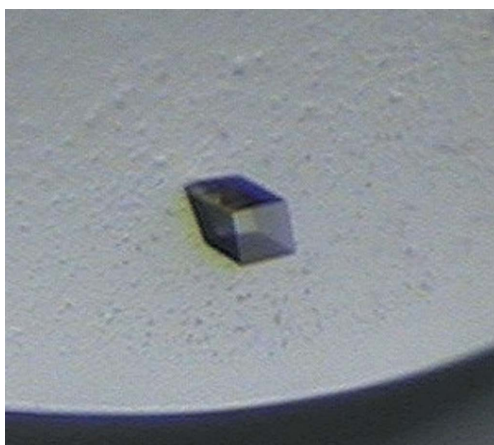


Figure 1
Crystal of InIB-VLR2913-ECD. The dimensions of the crystal are approximately $100 \times 100 \times 150 \mu\text{m}$.

Table 1

Data-collection statistics for InIB-VLR2913-ECD.

Values in parentheses are for the highest resolution shell.

X-ray wavelength (Å)	1.54178
Temperature (K)	100
Space group	$P4_12_12$ or $P4_32_12$
Unit-cell parameters (Å)	$a = 91.12, b = 91.12, c = 62.87$
Resolution range (Å)	50–2.04 (2.08–2.04)
No. of total/unique reflections	132286/17451
R_{merge}^\dagger (%)	5.2 (32.1)
Data completeness (%)	99.9 (100)
Multiplicity	7.6 (7.3)
Average $I/\sigma(I)$	44.6 (8.2)

$^\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the intensity of the i th measurement of reflection hkl and $\langle I(hkl) \rangle$ is the average intensity of reflection hkl .

2.2. Purification

The cells were lysed by sonication in lysis buffer (20 mM Tris–HCl pH 7.5, 500 mM NaCl, 35 mM imidazole, 1 mM phenylmethylsulfonyl fluoride). The supernatant was applied onto a HiTrap Chelating HP column (GE Healthcare, Little Chalfont, England) which had previously been equilibrated with 20 mM Tris–HCl pH 7.5, 500 mM NaCl, 35 mM imidazole. The InIB-VLR2913-ECD protein was eluted with a linear gradient of 0.035–1.0 M imidazole in the same buffer. The eluted sample was further purified by gel filtration on a HiLoad 16/600 Superdex 200 prep-grade column (GE Healthcare) which was equilibrated with 20 mM Tris–HCl pH 8.5, 100 mM NaCl. The sample was applied onto a HiTrap Q ion-exchange column (GE Healthcare) and eluted with a linear gradient of 0.1–1.0 M NaCl in 20 mM Tris–HCl pH 8.5. The protein purity was confirmed by SDS–PAGE, which indicated that the protein was 99% pure. Purified InIB-VLR2913-ECD was concentrated to a final concentration of 20.0 mg ml^{-1} using an Amicon Ultra 3K centrifugal filter device (Millipore, Billerica, Massachusetts, USA).

2.3. Crystallization and X-ray data collection

InIB-VLR2913-ECD was crystallized at 295 K using the sitting-drop vapour-diffusion method by mixing equal volumes (1.5 μl each) of protein solution and reservoir solution. Crystals with approximate dimensions of $100 \times 100 \times 150 \mu\text{m}$ (Fig. 1) appeared after one week using a reservoir solution consisting of 0.17 M ammonium sulfate, 25.5% (w/v) PEG 4000, 15% (v/v) glycerol (Wizard III condition No. 30; Emerald BioSystems, Bainbridge Island, Washington, USA). No further cryoprotectant was required for cryo-cooling in liquid nitrogen. X-ray diffraction data were collected at 100 K using a MicroMax-007 HF microfocuss X-ray generator and an R-Axis IV⁺⁺ imaging-plate area detector (Rigaku, Tokyo, Japan) at the Korea Basic Science Institute (KBSI), Republic of Korea. The statistics of data collection are summarized in Table 1. For each image, the crystal was rotated by 1° and the raw data were processed using the HKL-2000 program suite (Otwinowski & Minor, 1997).

3. Results and discussion

VLR2913-ECD was constructed as a fusion protein with internalin B (InIB-VLR2913-ECD) to facilitate its structural characterization. The protein was successfully overexpressed using an *E. coli* system and was purified to 99% homogeneity by affinity chromatography, size-exclusion chromatography and anion-exchange chromatography. InIB-VLR2913-ECD crystals were obtained using the sitting-drop vapour-diffusion method at 295 K with reservoir solution consisting of 0.17 M ammonium sulfate, 25.5% (w/v) PEG 4000, 15% (v/v)

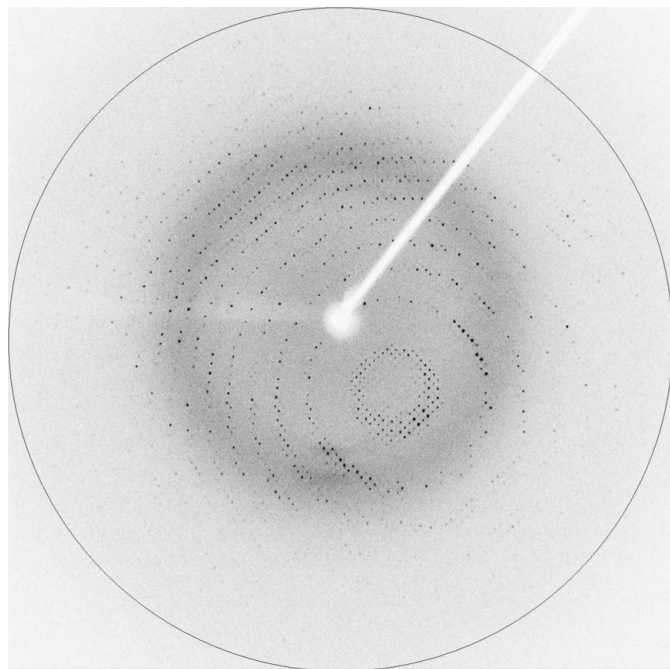


Figure 2

An X-ray diffraction image from an InlB-VLR2913-ECD crystal. The edge of the detector corresponds to a resolution of 2.04 Å and is represented as a circle. The crystal-to-detector distance was 150 mm and the exposure time for data collection was 3 min per image.

glycerol (Fig. 1). X-ray diffraction data were collected to 2.04 Å resolution (Fig. 2) and indexed in a tetragonal space group. A total of 132 286 measured reflections were merged into 17 451 unique reflections, giving an R_{merge} of 5.2% and a completeness of 99.9%. The space group was determined to be $P4_12_12$ (or $P4_32_12$) on the basis of systematic absences and symmetry. The unit-cell parameters were $a = 91.12$, $b = 91.12$, $c = 62.87$ Å (Table 1). If one monomer molecule

is assumed to be present in the crystallographic asymmetric unit, the calculated Matthews coefficient (V_M) is $2.75 \text{ \AA}^3 \text{ Da}^{-1}$ and the solvent content is 55.2%. We are currently attempting to solve the structure of InlB-VLR2913-ECD using molecular replacement. The structural study of InlB-VLR2913-ECD is expected to provide structural insight to elucidate the different modes of antigen recognition by VLRs.

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