Crystal structure of an anti-idiotypic variable lymphocyte receptor

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Variable lymphocyte receptors (VLRs), the leucine-rich repeat (LRR)-based antigen receptors of jawless fish, have great utility in a wide variety of biochemical and biological applications, similar to classical Ig-based antibodies. VLR-based reagents may be particularly useful when traditional antibodies are not available. An anti-idiotypic lamprey VLR, VLR39, has previously been identified that recognizes the heavy-chain CDR3 of the B-cell receptor (BCR) of a leukemic clone from a patient with chronic lymphocytic leukemia (CLL). VLR39 was used successfully to track the re-emergence of this clone in the patient following chemotherapy. Here, the crystal structure of VLR39 is presented at 1.5 Å resolution and compared with those of other protein-specific VLRs. VLR39 adopts a curved solenoid fold and exhibits substantial structural similarity to other protein-binding VLRs. VLR39 has a short LRRCT loop that protrudes outwards away from the concave face and is similar to those of its protein-specific VLR counterparts. Analysis of the VLR39–BCR interaction by size-exclusion chromatography and biolayer interferometry using the scFv version of the BCR confirms that VLR39 recognizes the BCR Fv region. Such VLR-based reagents may be useful for identifying and monitoring leukemia in CLL patients and in other clinical diagnostic assays.

1. Introduction

The adaptive immune system of jawless fish, including the sea lamprey (*Petromyzon marinus*), is similar in function to that of other vertebrates (Boehm *et al.*, 2012). However, lamprey antigen receptors, known as variable lymphocyte receptors (VLRs), are assembled using gene segments encoding leucine-rich repeats (LRRs; Pancer *et al.*, 2004; Alder *et al.*, 2005), and not immunoglobulin (Ig) domains as in other vertebrates. Mature VLRs are assembled from one of three genetic loci, *VLRA*, *VLRB* or *VLRC*, depending on the type of lymphocyte (Boehm *et al.*, 2012). In lamprey B-like cells, which express membrane-bound and secreted VLR antibodies, the antigen receptor is derived from the *VLRB* locus.

Several VLR-based antibodies derived from immunized lampreys have been isolated and characterized, most of which are VLRB-type antibodies (Deng *et al.*, 2013). These antibodies exhibit high affinity and selectivity for glycan (Han *et al.*, 2008; Alder *et al.*, 2008; Tasumi *et al.*, 2009; Hong *et al.*, 2013; Collins *et al.*, 2017) and protein (Herrin *et al.*, 2008; Alder *et al.*, 2008; Yu *et al.*, 2016; Altman *et al.*, 2015; Kirchdoerfer *et al.*, 2012; Velikovskiy *et al.*, 2009; Nakahara *et al.*, 2013) antigens. Compared with classical Ig antibodies, relatively few VLR antibodies and VLR–antigen complexes have been structurally characterized. A handful of VLRB–glycan structures have been determined (Han *et al.*, 2008; Luo *et al.*,

2013; Collins *et al.*, 2017), but only two VLRB–protein complexes: the BclA-specific VLR4 (Kirchdoerfer *et al.*, 2012) and the hen egg lysozyme (HEL)-specific VLRB.2D (Velikovsky *et al.*, 2009). From these studies, it is apparent that both glycan- and protein-specific VLRBs share the same curved solenoid fold and interact with their cognate antigen *via* both the concave face and a C-terminal third complementarity-determining region (CDR3)-like loop termed the ‘LRRCT loop’.

The protein-binding VLR4 and VLRB.2D have one feature in common that distinguishes them from the glycan-binding VLRs. The LRRCT loops of VLR4 and VLRB.2D are short (seven amino acids) and point upwards away from the concave face (Kirchdoerfer *et al.*, 2012; Velikovsky *et al.*, 2009). In contrast, all of the glycan-binding VLRs that have been structurally characterized have longer LRRCT loops (ten amino acids) that extend out across the concave face and form a ligand-binding cleft (Han *et al.*, 2008; Luo *et al.*, 2013; Collins *et al.*, 2017). Notably, the LRRCT loops of the protein-specific

VLRs lack the Trp that directly contacts the antigen in glycan-binding VLRs (Han *et al.*, 2008; Luo *et al.*, 2013).

Our previous studies suggested that VLR39 recognizes the HCDR3 (heavy-chain CDR3) of the patient’s BCR, indicating that VLR39 is a protein-specific and anti-idiotype antibody (Nakahara *et al.*, 2013), which is supported by several other observations. Firstly, the patient’s own mononuclear cells (MNCs), containing the leukemic B-cell clone, were used as the immunogen for the lamprey vaccination. Thus, some VLRs isolated from responding cells should be B-cell-specific and perhaps even tumor-specific. Accordingly, VLR39 stains the B cells but not the T cells in the patient’s blood sample, suggesting that VLR39 recognizes an epitope that is expressed only on B cells. VLR39 does not stain B cells from other CLL patients, who express different BCR idiotypes, indicating that VLR39 is specific to the BCR of the patient’s B-cell clone. Similar results were observed in an ELISA using purified Fab fragments. Importantly, VLR39 stains 293 cells transfected with the scFv version of the patient’s BCR and does so in a dose-dependent manner. Cell-staining experiments with amino-acid variants and chimeric BCRs map the epitope to HCDR3 of the patient’s BCR. These data strongly suggest that the Fv portion of the BCR is recognized by VLR39 and that HCDR3 is sufficient for VLR39 binding.

To examine the VLR39–HCDR3 interaction in more detail, we determined the crystal structure of VLR39 and studied the interaction using purified components. Our data provide a detailed structural view of VLR39 and support the notion that VLR39 is an anti-idiotype antibody. Together with our previously published data (Nakahara *et al.*, 2013), these data suggest that tumor-specific VLRs can be isolated from lampreys and that they are useful for tracking the recurrence of leukemia in CLL patients. VLRs may also be useful in other clinical diagnostic assays.

2. Materials and methods

2.1. Protein expression, purification and characterization

The identification of the VLR39 clone has been described elsewhere (Nakahara *et al.*, 2013). For these studies, the N-terminally His₆-tagged ectodomain of VLR39 and the heavy-chain and light-chain V regions of the BCR connected by a (Gly₄-Ser)₃ linker (with V_H followed by V_L) were synthesized and cloned into a modified pHCMV3 vector. VLR39 and scFv proteins were expressed in Freestyle HEK293S cells using the 293Fectin reagent and standard transient transfection methods. Secreted proteins were harvested from the medium and purified using an Ni-NTA column (Qiagen) followed by size-exclusion chromatography (SEC; Superdex 75, GE Healthcare) in 25 mM HEPES pH 7.5, 200 mM NaCl. For formation of the VLR39–scFv complex, purified scFv was co-concentrated with an excess of VLR39 and analyzed on a Superdex 75 column. The elution profiles were monitored by absorbance at 280 nm. For crystallization of VLR39, the protein was concentrated to an A₂₈₀ of 12.1 and used immediately or stored at –80°C before use. For biolayer

Figure 1
Crystal structure of VLR39. (a) Schematic showing the domain organization of VLR39. SP, signal peptide; CP, connecting peptide. Not drawn to scale. (b) Structure of VLR39, shown as a cartoon, in two orthogonal orientations looking onto and perpendicular to the concave binding face.

interferometry (BLI) studies, recombinant proteins were diluted into $0.5\times$ kinetics buffer containing phosphate-buffered saline (PBS), bovine serum albumin (BSA) and Tween 20. The association of scFv with immobilized His₆-VLR39 was measured on an Octet Red instrument (ForteBio) at 28°C using Ni-NTA sensors.

2.2. Crystallization and data collection

As no crystals could initially be obtained for VLR39, 5 mM β -mercaptoethanol (BME) was added to 12.3 mg ml⁻¹ VLR39 protein solution prior to crystallization, an approach that we have successfully employed in other LRR protein crystallizations (unpublished observations) and also based on the observation that CD14 proteins form intermolecular disulfide bonds during crystallization (Kelley *et al.*, 2013). Crystals were grown in a sitting-drop format on our robotic Rigaku CrystalMation system at The Scripps Research Institute (as 100 nl + 100 nl drops) using the vapor-diffusion method, with 100 mM Tris pH 8.5, 200 mM MgCl₂, 24% PEG 4000 as the precipitant at 4°C. The crystals were flash-cooled in liquid nitrogen without additional cryoprotectant. Diffraction data

from single crystals were collected on beamline BL12-2 at the Stanford Synchrotron Radiation Lightsource (SSRL). Data were processed using *XDS* (Kabsch, 2010). Data-collection and processing statistics are reported in Table 1 and Supplementary Table S1.

2.3. Structure solution and refinement

Molecular-replacement (MR) solutions for the initial VLR39 structure were found using *Phaser* in the *PHENIX* suite (Adams *et al.*, 2010) with a single copy of VLR4 (PDB entry 3twi; Kirchdoerfer *et al.*, 2012) as the search model. Refinement was carried out using *PHENIX* and iterative building of the models into $2F_o - F_c$ and $F_o - F_c$ electron-density maps was carried out with *Coot* (Emsley *et al.*, 2010). The progress of the refinement process was judged by monitoring R_{cryst} and R_{free} . Structure validation was performed using the PDB Validation Server (<https://validate.wwpdb.org>). Refinement statistics are reported in Table 1 and Supplementary Table S1. The structure factors and atomic coordinates of VLR39 have been deposited in the RCSB Protein Data Bank (<http://www.rcsb.org/>) and have PDB code 5wk4.

Figure 2
Concave face of VLR39. (a) Upward-facing side chains of the concave face of VLR39 with the potential to interact with the BCR are shown as sticks. (b) Side chains comprising the three 'ridges' (R1, R2 and R3) on the concave face, as described in Deng *et al.* (2013), are shown as a surface representation. (c) Surface representation of the VLR39 concave face, with the two glycine residues forming the base of the apparent binding pocket colored gray. (d) Electrostatic potential of the concave face shown as a surface representation contoured at $\pm 7 kT e^{-1}$, as calculated using APBS (Baker *et al.*, 2001) and PyMOL. Electronegative regions are colored red; electropositive regions are colored blue.

Table 1

X-ray data-collection and refinement statistics for VLR39.

Values in parentheses are for the highest resolution shell.

Data collection	
Beamline	12-2, SSRL
Wavelength (Å)	0.9795
Space group	<i>P</i> 1
Unit-cell parameters (Å, °)	<i>a</i> = 60.4, <i>b</i> = 61.4, <i>c</i> = 80.6, <i>α</i> = 110.1, <i>β</i> = 99.8, <i>γ</i> = 93.5
Resolution (Å)	34.72–1.50 (1.55–1.50)
No. of observations	536547 (33560)
No. of unique reflections	145338 (9013)
Multiplicity	3.7 (3.7)
Completeness (%)	85.1 (52.7)
<i>I</i> / <i>σ</i> (<i>I</i>)	11.1 (1.5)
<i>R</i> _{merge} †	0.06 (0.74)
<i>R</i> _{p.i.m.} ‡	0.04 (0.44)
CC _{1/2} §	1.00 (0.73)
Refinement	
Resolution (Å)	34.72–1.50 (1.55–1.50)
No. of reflections, work set	145247 (8998)
No. of reflections, test set	7053 (443)
<i>R</i> _{cryst} ¶ (%)	19.3 (28.8)
<i>R</i> _{free} †† (%)	22.3 (34.9)
Average <i>B</i> factor (Å ²)	
Protein	22.8
Solvent	30.0
Wilson <i>B</i> factor (Å ²)	19.3
No. of residues	1148
No. of ions	4
No. of waters	943
R.m.s.d. from ideal geometry	
Bonds (Å)	0.006
Angles (°)	0.88
Ramachandran plot‡‡ (%)	
Favored	95.6
Allowed	4.4
Outliers	0

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the scaled intensity of the *i*th measurement of reflection *hkl* and $\langle I(hkl) \rangle$ is the average intensity for that reflection. ‡ $R_{\text{p.i.m.}}$ is a redundancy-independent measure of the quality of intensity measurements. $R_{\text{p.i.m.}} = \sum_{hkl} \{1/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the scaled intensity of the *i*th measurement of reflection *hkl*, $\langle I(hkl) \rangle$ is the average intensity for that reflection and *N* is the redundancy. § CC_{1/2} is the Pearson correlation coefficient between two random half data sets. ¶ $R_{\text{cryst}} = \sum_{hkl} |F_{\text{obs}}| - |F_{\text{calc}}| / \sum_{hkl} |F_{\text{obs}}| \times 100$. †† R_{free} was calculated as for R_{cryst} , but using a test set comprising 5% of the data which were excluded from refinement. ‡‡ Calculated using PHENIX.

Figures were generated and structural superpositions were performed with PyMOL.

3. Results

3.1. Overall structure

To perform structural studies, the ectodomain of VLR39 (Fig. 1*a*) was expressed as a soluble monomer in HEK293S cells. We determined the crystal structure of VLR39 by molecular replacement using VLR4 (PDB entry 3twi) as the initial search model, followed by iterative refinement and model building (Table 1, Supplementary Table S1). VLR39 is a curved solenoid (Fig. 1*b*), similar to other VLR proteins (Boehm *et al.*, 2012). Solvent-exposed residues on the concave face (Fig. 2*a*, top), which is the antigen-binding interface of VLR proteins, include all residue types. A small extended pocket is formed on the concave face in the middle of the R2 and R3 'ridges' (as described in Deng *et al.*, 2013; Fig. 2*b*),

which results in part from two glycine residues at the base on adjacent LRRs (Fig. 2*c*). Interestingly, the center of the pocket is positively charged owing to Arg101 as well as backbone amide groups (Fig. 2*d*).

3.2. Structural comparison to other VLRLs

The domain organization of VLR39 is similar to that of the other two protein-binding VLRLs that have been structurally characterized, VLR4 and VLRB.2D (Fig. 3*a*, left), with the exception that VLR39 has one additional LRR (LRRV2). The LRRCT loops of all three VLRLs are relatively short, comprised of only seven residues (Fig. 3*a*, right). The overall structure of VLR39 is very similar to those of VLR4 and VLRB.2D, with r.m.s.d.s for all atoms of only 0.36 and 0.50 Å, respectively (Fig. 3*b*). The LRRCT loops of all three VLRLs adopt similar conformations and protrude upwards, away from

Figure 3

Comparison of protein-binding VLR structures. (a) Schematic showing the domain organization of the three known protein-specific VLRLs (left). VLR39 has one extra LRR compared with VLR4 and VLRB.2D. Right: the amino-acid sequences of the LRRCT loops. (b) Superposition of VLR39 (green), VLR4 (cyan) and VLRB.2D (magenta) structures. VLR4, PDB entry 3twi; VLRB.2D, PDB entry 3g3b (Velikovskiy *et al.*, 2009). The r.m.s.d.s for all atoms for the superposition of VLR39 with VLR4 and VLRB.2D is shown.

Table 2
LRRCT loop sequences of glycan-specific VLRs.

VLR	LRRCT loop sequence
RBC36	MRWDGKAVND
O13	MRWDGKAVND
Tn4-22	MRWDGKAVND
aGPA.23	GEGWPWRHS

the concave surface. Notably, similar to the extended pocket observed in the VLR39 structure (Fig. 2), VLR4 and VLRB.2D also have pocket-like features in the center of their concave faces (Velikovsky *et al.*, 2009; Kirchdoerfer *et al.*, 2012; Supplementary Fig. S1).

3.3. Recognition of the BCR

To determine whether VLR39 interacts directly with the BCR, we analyzed complex formation by size-exclusion chromatography using purified recombinant VLR39 and the scFv version of the patient's BCR. The proteins co-eluted from the SEC column in a peak corresponding to the size of the complex, eluting prior to the peak containing excess VLR39 monomer alone (Fig. 4a). VLR39 binds to the scFv with an affinity of 0.44 μM (Fig. 4b), which is similar to the affinity of VLR4 and VLRB.2D monomers for their cognate

antigens (Kirchdoerfer *et al.*, 2012; Velikovsky *et al.*, 2009). These data confirm that VLR39 interacts directly with the BCR and that VLR39 recognizes an epitope in the Fv region, consistent with our previous data suggesting that VLR39 recognizes the HCDR3 loop of the BCR (Nakahara *et al.*, 2013).

4. Discussion

Together with our previous work (Nakahara *et al.*, 2013), these data suggest that VLR39 recognizes an epitope in the Fv region of a BCR derived from a leukemic patient. A structural comparison of VLR39 with anti-glycan VLRs (Han *et al.*, 2008; Luo *et al.*, 2013; Collins *et al.*, 2017) would seem to rule out the possibility that VLR39 recognizes a glycan epitope on the BCR. The LRRCT loops of glycan-binding VLRs are longer, extend out over the concave face, and contain Trp residues. The LRRCT loop of VLR39 is similar to that of protein-binding VLRs (Kirchdoerfer *et al.*, 2012; Velikovsky *et al.*, 2009). The LRRCT loop is short and points outwards, away from the concave face (Fig. 1b). Also, the LRRCT loops of VLR39 and the other two protein-binding VLRs (Fig. 3a) lack the conserved Trp of glycan-binding VLR LRRCT loops (Table 2). Since the scFv lacks N-glycosylation sites, our SEC

Figure 4
VLR39 forms a complex with the scFv of the BCR. (a) Size-exclusion chromatography profile of the VLR39–scFv complex. Fractions from the center of each peak were run on SDS–PAGE gels and stained with Coomassie Blue (inset). (b) Measurement of the affinity of VLR39 for the scFv by bioluminescence resonance energy transfer. Binding of the scFv at concentrations of 0.3–1.35 μM to His₆–VLR39 at 0.025 mg ml^{−1} immobilized on an Ni–NTA sensor was measured on an Octet Red system (ForteBio). The binding curve was fitted with a 1:1 ligand-binding model.

and BLI data (Fig. 4) also support the notion that VLR39 recognizes a protein epitope, and that the epitope resides in the V region of the BCR. Unfortunately, the binding mode cannot be easily inferred from the apo structure. The concave face and LRRCT loop of VLR39 and the HCDR3 of the BCR (VTVKYYDFWGYFDY) contain a wide variety of residue types. What role, if any, the positively charged pocket on the VLR39 concave face (Fig. 2) plays in antigen binding is unclear. It is possible that this pocket accommodates one of the negatively charged Asp residues on HCDR3. The concave faces of VLR4 and VLRB.2D also have small pockets at the center of their binding interfaces (Supplementary Fig. S1; Kirchdoerfer *et al.*, 2012; Velikovsky *et al.*, 2009). A high-resolution structure of the VLR39–scFv complex will reveal whether the epitope recognized by VLR39 is exclusively HCDR3 or whether the epitope is comprised of additional determinants. In this regard, it will also be informative to analyze complex formation between VLR39 and the scFv containing a mutated HCDR3. These studies and crystallization trials of the VLR39–scFv complex are under way. Notably, as with other protein-binding VLRBs, the affinity of VLR39 for the scFv (0.44 μ M) is significantly lower than the affinities of most IgGs for their cognate antigens (which are in the nanomolar range). However, *in vivo*, the multimeric (decameric and dodecameric) nature of VLRBs likely overcomes this problem *via* avidity effects, similar to IgM antibodies. VLRBs can also be affinity-matured *in vitro* to achieve near-single-digit nanomolar affinities (Velikovsky *et al.*, 2009). Together with our previous data (Nakahara *et al.*, 2013), the findings here suggest that VLR39 recognizes an epitope in the V region of the BCR. Such VLR-based reagents may be useful for tracking the re-emergence of leukemic clones in CLL patients and other clinical diagnostic applications.

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