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Generation and characterization of hagfish variable lymphocyte receptor B against glycoprotein of viral hemorrhagic septicemia virus (VHSV)

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ABSTRACT

Variable lymphocyte receptors B (VLRBs) are non-immunoglobulin components of the humoral immune system in jawless vertebrates including hagfish (*Eptatretus burgeri*) and lamprey (*Petromyzon marinus*). Hagfish VLRBs consist of leucine rich repeat (LRR) modules with a superhydrophobic C-terminal tail, the latter of which leads to extremely low expression levels in recombinant protein technology. Here, we present an artificially oligomerized VLRB (arVLRB) that conjugates *via* the C4bp oligomerization domain derived from human C4b-binding protein (hC4bp) rather than the superhydrophobic tail. The resulting arVLRB had a tightly multimerized form with seven monomeric VLRB arms and showed high expression and secretion levels in a mammalian expression system. To isolate antigen-specific arVLRB, we constructed large VLRB libraries from hagfish immunized with the fish pathogen, viral hemorrhagic septicemia virus (VHSV). The selected arVLRBs were found to recognize various types of antigens, including the recombinant target protein, purified viruses, and progeny viruses, with high antigen binding abilities and specificities. We also performed *in vitro* affinity maturation of the arVLRBs through LRRCT mutagenesis, and found that this enhanced their antigen-binding properties by at least 125-fold. Our epitope mapping analysis revealed that ^{37}DWDTPL^{42}, which is located in a region conserved among the glycoproteins of all VHSV isolates, is the recognition epitope of the arVLRBs. Thus, our newly developed arVLRB could prove useful in the development of universal diagnostic tools and/or therapeutic agents for the virus. Together, our novel findings provide valuable insights into hagfish VLRB and its potential use as a novel alternative to conventional antibodies for biotechnological applications.

1. Introduction

In jawless vertebrates (agnathans), variable lymphocyte receptors (VLRs) play the central role in antigen recognition for adaptive immunity, and are equivalent to the conventional immunoglobulin (Ig) domain-based antigen receptors of jawed vertebrates (gnathostomes). VLRs generate a vast diversity of antigen-binding receptors ($> 10^{14}$) *via* somatic rearrangement of the leucine-rich repeats (LRRs) found within the intervening sequence of an incomplete germline VLR gene, and are expressed clonally on lymphocyte-like cells (LLCs) (Alder et al., 2005; Pancer et al., 2004). VLRs contain the following domains: an N-terminal LRR domain (LRRNT), the first LRR (LRR1), a diverse number of variable LRRs (LRRV), a connecting peptide (CP), a C-terminal LRR domain (LRRCT), an invariant threonine/proline-rich region (Stalk), and a transmembrane or GPI (glycosylphosphatidylinositol)-anchor region. Crystal structure analyses of VLR-antigen complexes revealed that: i) the repeated LRR β-strands of the LRRVs and the CP modules form an inner concave surface that contributes a complementary binding site for their specific antigens; and ii) a flexible, highly variable loop at LRRCT constructs a distinctive tip that is inserted toward the cleft side of the VLR and acts to improve the antigen-binding affinity (Kim et al., 2007; Velikovsky et al., 2009; Han et al., 2008).

Three different types of receptors, VLRA, VLRB, and VLRC, have been classified in both hagfish and lamprey. VLRA and VLRC, which exist on the surfaces of LLCs as membrane-anchored receptors, are phylogenetically and functionally similar to the αβ T- and γδ T-cells, respectively, of jawed vertebrates (Alder et al., 2008; Guo et al., 2009; Im et al., 2016; Kasamatsu et al., 2010; Li et al., 2013). Similarly to Igs,

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lamprey VLRBs are expressed on the surface of LLCs *via* the GPI-anchor domain and are also secreted in serum as humoral agglutinins (Herrin et al., 2008). VLRBs are secreted as disulfide-linked pentamers or tetramers that conjugate through their C-terminal cysteine (Cys)-rich tails, and thus bear some structural resemblance to IgM. Since the discovery of antigen-specific VLRBs in lamprey, many researchers have successfully produced antigen-specific monoclonal lamprey VLRBs against bacteria, cell surface antigens, and other kinds of soluble proteins (Hong et al., 2012; Tasumi et al., 2009; Wezner-Ptasinska and Otlewski, 2015; Yu et al., 2012). However, only a few studies have sought to characterize hagfish VLRB beyond confirming its genetic and structural similarities with lamprey VLRB. Interestingly, hagfish VLRB has a cluster of hydrophobic amino acids in the C-terminus (superhydrophobic tail) rather than a Cys-rich tail, as seen in lamprey VLRB (Li et al., 2013). Given hydrophobicity of the target protein is a key factor in recombinant protein technology, the high hydrophobicity in C-terminus of hagfish VLRB might lead to low production levels and complicate the experimental production of hagfish VLRB (Goh et al., 2004).

Many naturally occurring proteins use oligomerization to increase their valency, binding affinity and structural stability (Deyev and Lebedenko, 2008; Jain et al., 2007). Numerous researchers have attempted to produce artificially multimerized antibody fragments with improved binding avidity and serum half-life by fusion of diverse multimerization domains, such as those of streptavidin (Dübel et al., 1995) and p53 (Rheinnecker et al., 1996), and with leucine zippers (Wild et al., 1994). Here, in an effort to develop an antigen-specific VLRB antibody screening system from a vast repertoire of hagfish VLRBs, superhydrophobic tail of hagfish VLRB was replaced with the C4-binding protein (C4bp) oligomerization domain derived from human C4bp (hC4bp, as a fusion partner) (Hofmeyer et al., 2013). This domain plays an important role in the oligomerization of seven alpha chains to form a heptameric complex. We hypothesized that this C-terminal conversion of hagfish VLRB could help solve the issues with the limited production and multimeric structure of recombinant VLRBs (rVLRBs). In present study, we generated artificially oligomerized rVLRBs (arVLRBs) of hagfish immunized with the fish pathogen, viral hemorrhagic septicemia virus (VHSV), which is associated with severe economic losses in the aquaculture industry, and apply it as a model to investigate the specificity and binding ability of hagfish VLRB. We also examined the *in vitro* affinity maturation of VLRB by modular engineering of LRRCT, and characterized the linear antigenic site on VHSV by precise epitope mapping of VLRB.

2. Methods

2.1. Cell lines and viruses

Hirame natural embryo (HINAE) cells derived from flounder embryo were maintained at 25 °C in Leibovitz's L-15 medium (Gibco Life Technologies, USA) containing 10% fetal bovine serum (FBS; Life Technologies), 100 units/mL penicillin, 100 μg/mL streptomycin, and 250 ng/ml amphotericin B. The virus stock was prepared per a previous report (Lazarte et al., 2017). Epithelioma papulosum cyprinid (EPC) cells were infected with W-VHSV 150402-P2 at a multiplicity of infection (MOI) of 1. After 1 h, the inoculum was replaced with L-15 medium containing 2% FBS, and the cells were incubated at 14 °C until a cytopathic effect (CPE) was apparent. When an extensive CPE was observed, the medium was collected, the cellular debris was removed by low-speed centrifugation, and the concentration of the virus in the collected supernatant was determined by calculating the 50% Tissue Culture Infective Dose ($TCID_{50}$).

2.2. Hagfish immunization

Juvenile inshore hagfish (*Eptatretus burgeri*) of 20–30 cm in length were purchased from commercial fishermen (Bogyeong Hagfish Services, South Korea) and maintained in aquaria at a constant water temperature (14–15 °C). As per a previous report (Im et al., 2016), the fish were sedated by immersion in ethyl 3-aminobenzoate methanesulfonic acid (0.1 g/L; Sigma-Aldrich, USA) and then intraperitoneally (i.p.) injected with 30 μg of VHSV in 100 μl of 0.67 × PBS (91 mM NaCl, 1.8 mM KCl, 2.8 mM Na2HPO4, and 0.9 mM KH2PO4). This injection was repeated four times at 2-week intervals. For further experiments, peripheral blood was collected in 0.67 × PBS with 10 mM EDTA, layered onto a 28% Percoll (GE Healthcare, USA) gradient, and centrifuged at 400*g* for 20 min at 4 °C. Leukocytes were collected and saved for RNA extraction.

2.3. Construction of vectors and VLRB cDNA library cloning

The pTracer-EF/V5-His mammalian expression vector (Invitrogen Life Technologies, USA) was modified to introduce a murine Ig κ-chain leader sequence (Ig κ) and two Sfi I sites. The DNA fragment flanked by Kpn I (bold) and two Sfi I (underlined)/Not I (italicized) sites was PCR amplified (forward primer: 5′-CA**GGTACC**ATGGAGACAGACACACTC CTG-3′; reverse primer: 5′-T*GCGGCCGC*GGGCCCCAGAGGCCTTTTTTG GCCCCGGTGGCCGAGTCACCAGTGGAACCTGGAAC-3′) from the template, pSecTac2A (Invitrogen Life Technologies). The PCR product was digested with Kpn I/Not I and ligated into the Kpn I/Not I sites of pTracer-EF/V5-His to generate pKINGeo. For more efficient cloning and to exclude self-ligation, a PCR product harboring the chloramphenicol resistance/ccdB gene flanked with Sfi I sites was amplified from pEF-DEST51 (Invitrogen Life Technologies) (forward primer: 5′-AGGCCAC CGGGGCCAAAAAAGGCTTATGGAGAAAAAAATC-3′; reverse primer: 5′-AGGCCCCAGAGGCCTTATATTCCCCAGAACATCAG-3′). The obtained product was digested with Sfi I and inserted into the two Sfi I sites of pKINGeo to construct pKINGeo/ccdB.

To construct the pKepta/ccdB vector, the C4bp oligomerization domain (aa 540 ~ 597 of human C4bp α; GenBank accession no. NM_000715) were introduced next to the second SfiI site of the template. Polymerase chain reaction (PCR) with complementary primer sets was used to amplify fragments spanning from the Ig κ sequence to the second SfiI site (KpnI Ig κ Fwd, 5′- GCTTGGTACCATGGAGACAGACA CACTCCT and ccdB SfiI C4bp Rev, 5′- GCCTTCGGGGGTCTCCCACGG GCCCCAGAGGCCTTATATTCCC) or the C4bp oligomerization domain (C4bp con Fwd, 5′- TGGGAGACCCCCGAAGGCTGTGAACAAGTGCTCA CAGGCAAAAGACTCATGCAGTGTCTCCCAAACCCAGAGGATGTGAAAA TGGCCCTGGAGGTATATAAGCTG and C4bp con Rev PmeI, 5′- GCGG GTTTAAACTTATAGTTCTTTATCCAAAGTGGATTGTCTTGCGCTGTCTC TCTGTAGTTCCAGTTGTTCAATTTCCAGAGACAGCTTATATACCTC CAG). The amplified fragments were joined by PCR using the KpnI Ig κ Fwd and C4bp con Rev PmeI primers, and then cloned into the KpnI/PmeI sites of the template.

To obtain natively occurring hagfish VLRB cDNAs, total RNA was extracted from hagfish blood leukocytes using a QIAamp RNA blood mini kit (Qiagen, Germany) according to the manufacturer's instructions and reverse transcribed with a RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, USA). Intact form of VLRB library (LRRNT ~ hydrophobic tail) were amplified from total RNA of non-immunized hagfish leukocyte and cloned into the SfiI sites of pKINGeo/ccdB using appropriate primers (LRRNT SfiI Fwd: 5′- AGGCCACCGGG GCCTGTCCTTCACGGTGTTCCTG-3′; hydrophobic tail SfiI Rev: 5′-TGGCCCCAGAGGCCTCAGAGCAGCTGCGAGGCGT-3′). A partial VLRB fragment library (LRRNT ~ stalk) was amplified from total RNA of VHSV-immunized hagfish leukocytes and cloned into the SfiI sites of pKepta/ccdB using appropriate primers (LRRNT SfiI Fwd, 5′-AGGCCA CCGGGGCCTGTCCTTCACGGTGTTCCTG-3′ and Stalk SfiI Rev, 5′-TGG CCCCAGAGGCCCGCGTTCATGACACGGCCGA-3′). To construct the plasmid encoding monomeric K43 ($K43^{mono}$), a stop codon was inserted before the C4bp oligomerization domain of the template, K43, using appropriate primers (LRRNT SfiI Fwd and Stalk stop SfiI Rev, 5′-TGG