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Development of a modified yeast display system for screening antigen-specific variable lymphocyte receptor B in hagfish (*Eptatretus burgeri*)

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ABSTRACT

The variable lymphocyte receptor B (VLRB) of jawless vertebrates has a similar function to the antibodies produced by jawed vertebrates, and has been considered as an alternative source to mammalian antibodies for use in biological research. We developed a modified yeast display vector system (pYD8) to display recombinant hagfish VLRB proteins on the extracellular surface of yeast for the isolation of antigen-specific VLRBs. After observing an up-regulation in the VLRB response in hagfish immunized with hemagglutinin 1 of avian influenza virus H9N2 subtype (H9N2-HA1), the antigen-specific VLRBs decorated on the yeast's surface were selected by quantitative library screening through magnetic-activated cell sorting (MACS) and fluorescent-activated cell sorting (FACS). We also demonstrated a strong specificity of the antigen-specific VLRBs, when expressed as a secreted protein using a mammalian expression system. Together, our findings suggest that the pYD8 vector system could be useful for screening antigen-specific hagfish VLRBs, and the specificity of secreted VLRB may have potential for a variety of biological applications.

1. Introduction

Yeast display (YD) systems are powerful tools for discovering recombinant antibodies as well as engineering antibodies with improved affinity, specificity and stability in a high-throughput manner. The YD involves the expression of antibody on the surface of the yeast, where it can interact with target antigens in solution. The antibodies are expressed fused to a mating protein Aga2 (mAga2), which is then linked by two disulfide bonds to the surface protein Aga1 (sAga1) attached covalently to the cell wall of the yeast (Boder and Wittrup, 1997). The YD system offers several advantages: i) a eukaryotic expression system capable of post-translational modifications such as disulfide bond formation, ii) relatively low technical and time requirements compared with mammalian display systems, and iii) compatibility with fluorescence-activated cell sorting (FACS) which allows quantitative discrimination of desired clones within the libraries through simultaneous data analysis (Siegel et al., 2004). Various antibodies have been successfully developed using a YD platform against a variety of specific molecules, such as HIV-1 gp120, tuberculosis Antigen 85, epidermal growth factor receptor and the tumor vascular marker Endosialin/TEM1 (Chao et al., 2004; Walker et al., 2009; Zhao et al., 2011; Ferrara et al., 2012).

Variable lymphocyte receptors (VLRs) are a unique form of immune receptors that mediate acquired immune systems in jawless vertebrates. Especially, VLRB is phylogenetically and functionally related to B-cell receptors of jawed vertebrates, thus constitutes a major component of the humoral response of the lamprey and hagfish in terms of recognizing and binding to foreign substances (Pancer et al., 2004). VLRBs are generated through rearrangement of germline genes, flanked by a large number of genomic cassettes that are matured through a

Abbreviations: YD, yeast display; mAga2, mating protein Aga2; sAga1, surface protein Aga1; FACS, fluorescence-activated cell sorting; VLRs, variable lymphocyte receptors; LRR, leucine-rich repeat; LRRNT, N-terminal leucine-rich repeat; LRRVs, variable leucine-rich repeat cassettes; CP, connecting peptide; LRRCT, C-terminal leucine-rich repeat; AIV, avian influenza virus; H9N2-HA1, hemagglutinin 1 of avian influenza virus H9N2 subtype; Cm^R, chloramphenicol resistance; HEK, Human embryonic kidney; HRP, horseradish peroxidase; MACS, magnetic-activated cell sorting; SEMs, standard error of the means

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unique gene assembly mechanism, wherein the non-coding intervening sequence in a germline VLRB gene is replaced with variable genomic cassettes (Alder et al., 2005; Rogozin et al., 2007). The mature VLRB has a series of leucine-rich repeat (LRR) protein segments, composed of an N-terminal LRR (LRRNT), LRR1, as many as eight variable LRR cassettes (LRRVs), a connecting peptide (CP), the C-terminal LRR (LRRCT) and invariant C-terminal cell surface-anchoring region, which is unlikely to be involved in antigen recognition, comprising of a Thr/Pro-rich stalk region and hydrophobic tail (Pancer et al., 2004; Herrin and Cooper, 2010). Recently, many studies have reported on the antibody-like function and use of lamprey VLRBs as an alternative source of antibodies, specific for various molecules such as murine B cell leukemia, malignant tumors, plant expressed proteins (Moot et al., 2016; Velásquez et al., 2017; Yun et al., 2017). Although hagfish VLRBs also have antibody-like activities and the potential to be used as an alternative antibody (Finstad and Good, 1964; Linthicum and Hildemann, 1970; Takaba et al., 2013; Im et al., 2016; Kim et al., 2018), there have only been few report relating to this in the literature.

In this study, we have developed a new YD vector system through the genetic fusion of hagfish VLRB gene with the Aga YD system for screening unidentified VLRB against target antigens. Using this chimeric VLRB-Aga system displayed on the surface of yeast cells, we successfully selected hagfish VLRB specific to hemagglutinin 1 (H9N2-HA1), which is a major surface protein of avian influenza virus (AIV) subtype H9N2. Furthermore, we proved that the VLRB selected by this YD system was also feasible in the mammalian expression system. Thus, in this study we have established an alternative high-throughput platform system that could enhance the screening and discovery of antigen-specific VLRBs.

2. Materials and Methods

2.1. Animals and immunization

Inshore hagfish (*Eptatretus burgeri*) 20–30 cm in length, recently captured by commercial fishermen (Bogyeong Hagfish Service, South Korea), were maintained in an aquarium at 14–15 °C. For immunization, the hagfish were first anesthetized by immersing them into a solution of ethyl 3-aminobenzoate methanesulfonic acid (0.1 g/l; Sigma) and subsequently injected intraperitoneally with 20 μg of H9N2-HA1 (A/Chicken/Hong Kong/G9/97; Sinobiological) in 100 μl of 0.67 × PBS, four times at 2 weeks intervals. One week after the final injection, peripheral blood was collected into 0.67 × PBS/10 mM EDTA, layered onto a 28% Percoll (GE Healthcare) gradient and centrifuged at 400 × *g* for 20 min at 4 °C. Hagfish leukocytes were then collected by centrifugation for 10 min at 500 × *g*. Total RNA was extracted from the blood leukocytes using a QIAamp RNA blood mini kit (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was treated with DNase I and reverse transcribed with a RevertAid First-strand cDNA Synthesis kit (Thermo Fisher Scientific). To remove surplus RNA from the original template, the reactants were incubated with RNase H (Thermo Fisher Scientific). All experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Gyeongsang National University.

2.2. Viruses

The AIVs used in this study [AIV H9N2 (A/Chicken/Korea/MS96/96), H6N2 (A/Chicken/Korea/ KBU0084), and H4N2 (A/Chicken/Korea/VI1410583)] were obtained from the Avian Disease Laboratory (Chung-buk National University, South Korea). The working stocks of virus were prepared by propagation in chicken embryonated eggs (allantoic fluid) (Brauer and Chen, 2015).

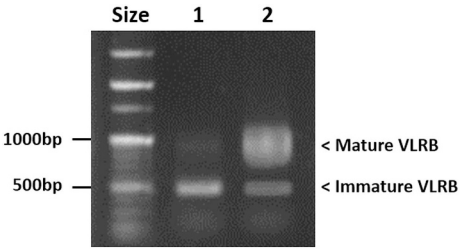

Fig. 1. Evaluation of the transcriptional expression of VLRB in hagfish challenged with H9N2-HA1. The cDNAs encoding immature and mature VLRBs were PCR amplified from peripheral blood leukocytes obtained before (1) or at 4 weeks after immunization (2), using VLRB-specific primers.

2.3. Plasmid and library construction

The pYD5, modified from commercial YD vector pYD1 (Invitrogen), is a parental vector with the surface expression cassette located at the N-terminal to the mAga2 (Wang et al., 2005). To construct the pYD8 vector, the chloramphenicol resistance (Cm^R)/ccdB gene, flanked by two different *Sfi* I sites, was amplified from pEF-DEST51 (Invitrogen) using primers *Nhe*I/*Sfi*I_ccdB_F (5′-AAGCTAGCGTTTTAGCAGAATTGGCCACCGGGGCCAAAAAAAGGCTTATGG AGAAAAAAATC-3′ and ccdB_Sfi/*Eco*RI_R (5′- AAGAATTCAAGGCCCCAGAGGCCTTATATTCCC CAGAACATCAG -3′), and inserted into the NheI and EcoRI sites of pYD5. To generate the VLRB library for selecting antigen-specific VLRBs, mature VLRB sequences spanning from the LRRNT to the LRRCT were amplified from the mRNA of blood leukocytes by extension PCR (SfiI_LRRNT_F, 5′- AAAGGCCACCGGGGCCTGTCCTTCACGGTGTTCCT GCA.

-3′ and SfiI_LRRCT_R, 5′-AAAAAAGGCCCCAGAGGCCAGGGCAGAT GATACTTCGGACGG-3′) and directionally cloned into the two SfiI sites of the pYD8 vector to replace Cm^R/ccdB. The ligated VLRBs/pYD8 were purified by ethanol precipitation and transformed into ElectroMAX DH10B cells (Invitrogen) by electroporation for plasmid DNA amplification. The electroporated cells were recovered in Super Optimal broth with Catabolite repression (Thermo Fisher Scientific) for 30 min, and 10-fold serially diluted from the cell suspension to determine library size from the colony counts. The amplified plasmid were purified using DNA Spin Maxiprep kits (iNtRON Biotechnology).

2.4. Yeast transformation

Before transformation, *Saccharomyces cerevisiae* strain EBY100 at an OD600 of 0.2, was grown in YPD medium (Sigma) in a shaking incubator (225 rpm) at 30 °C overnight. EBY100 was grown to an OD600 of 4–6, diluted to an OD600 of 0.3, and incubated again in a shaking incubator at 30 °C until an OD600 of approximately 1.6 was obtained, usually after 5 h. The cells were collected, washed twice with ice cold distilled-water and once with electroporation buffer (1 M sorbitol/1 mM $CaCl_2$), incubated in condition buffer (0.1 M LiAc/10 mM DTT) in a shaking incubator at 30 °C for 30 min, and then washed once by electroporation buffer. The conditioned cells were collected by centrifugation and re-suspended in electroporation buffer, and kept on ice. Approximately 1.6×10^9 cells/mL were sufficient for five electroporation reactions of 200 μl each. Ten μg/10 μl of the VLRB library in pYD8 were prepared by ethanol precipitation and mixed with 200 μl of the conditioned cells in pre-chilled GenePulser cuvette (0.2 cm electrode gap, BioRad). The cells were electroporated at 2.5 kV and 25 μF. Typical time for this ranged from 3.0 to 4.5 milliseconds. The electroporated cells were immediately transferred into recovery buffer (0.5 M sorbitol, 0.5% yeast extract, 1% peptone, and 1% dextrose) and incubated in a shaking incubator at 30 °C for 2 h. The recovered cells were serially diluted 10-fold from the cell suspension and grown in synthetic-defined agar plate containing 0.00072% CSM-TRP-URA (MP

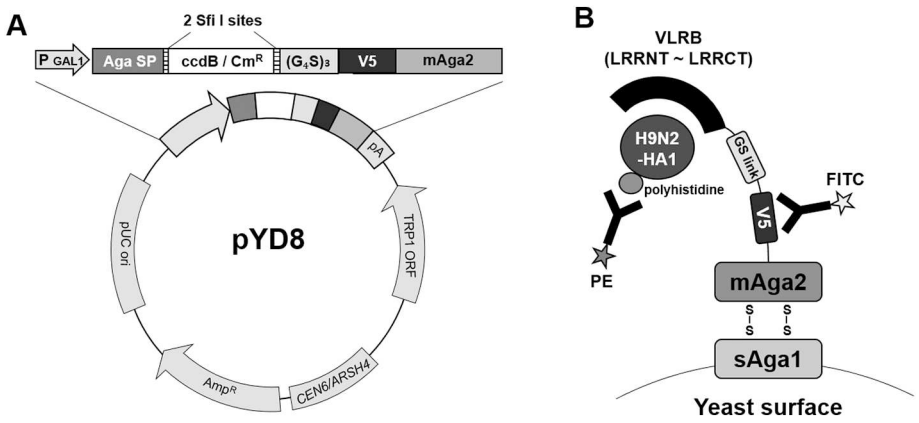

Fig. 2. The YD system based on pYD8. (A) Plasmid map of the pYD8. (B) Schematic representation of recombinant VLRB displayed on yeast surface. The recombinant VLRB composed of LRRNT to LRRCT were connected by the Aga anchor. The V5-tag served for VLRB detection via FITC-conjugated antibodies, and H9N2-HA1 were detected via PE-conjugated antibodies.

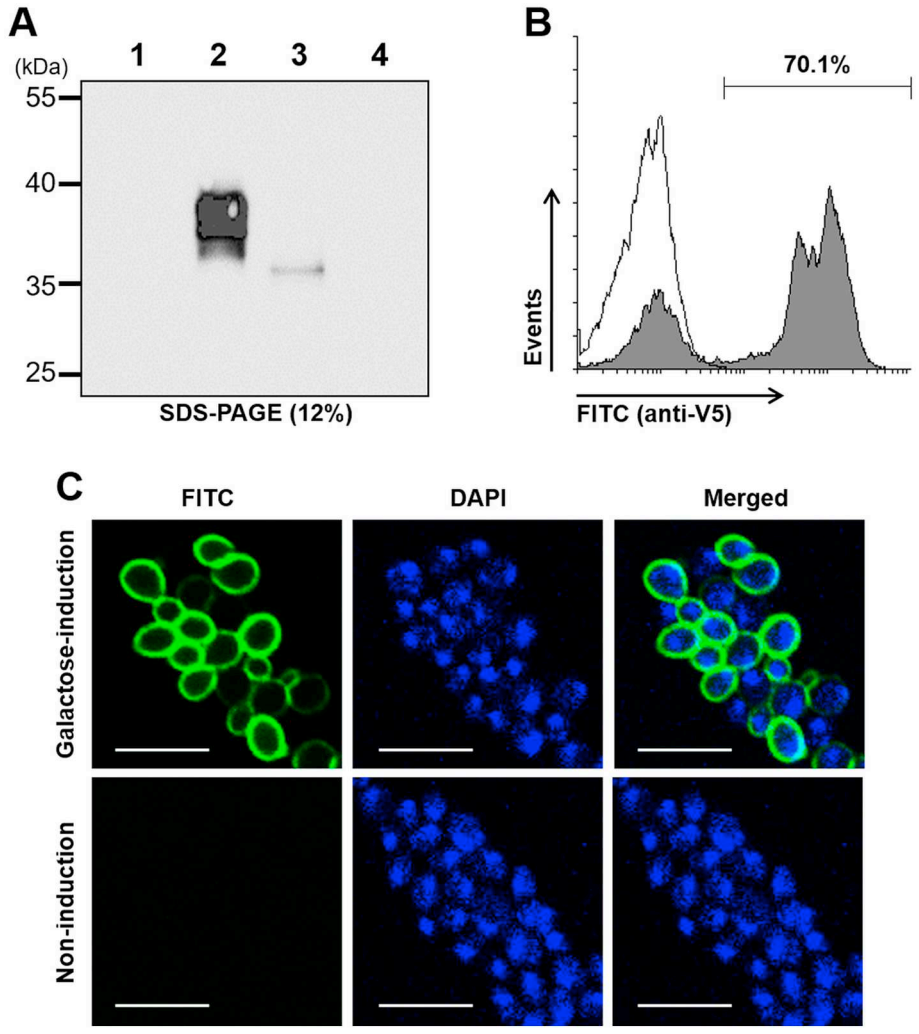

Fig. 3. Expression and display of monoclonal VLRB (VLRB.mV2) on the yeast surface based on pYD8 system. (A) Expression pattern of the VLRB.mV2 separated by 12% SDS-PAGE under reducing conditions and subjected to Western blot analysis with mouse anti-V5 IgG, 2D12, followed by goat anti-mouse IgG-HRP (lane1: yeast cell lysates of non-induced group, lane2: yeast cell lysates of gal-induced group, lane3: yeast cell supernatants of non-induced group, lane4: yeast cell supernatants of gal-induced group). (B) Flow cytometric analysis of the yeast cells displayed with the VLRB.mV2. The VLRB.mV2 was detected by antibody 2D12 followed by goat anti-mouse IgG-FITC in galactose-induced group (gray-shaded) or non-induction group as a negative control (black line). (C) Immunofluorescence staining of yeast cells decorating the VLRB.mV2 on their surfaces. The yeast cells were stained with antibody 2D12 followed by goat anti-mouse IgG-FITC in galactose-induced group or non-induction group as a negative control. The FITC-stained cells (green) were visualized by confocal imaging, and the images were merged with DAPI staining the cell nucleus (blue). Scale bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Biomedicals), 0.67% Yeast Nitrogen Base (Sigma), 2% dextrose, 1.5% agar (Sigma). Library size was determined from the colony counts after three days. The remaining cells were incubated in synthetic-defined medium containing 0.00072% CSM-TRP-URA, 0.67% Yeast Nitrogen Base, 2% dextrose, 30 °C for 30 h. For protein expression, the yeast cells, at OD600 = 0.2, were grown up to OD600 = 4–6 in the synthetic-defined medium containing 0.00072% CSM-TRP-URA, 0.67% Yeast Nitrogen Base, 2% galactose in a shaking incubator (225 rpm) at 20 °C for 48 h.

2.5. Transfection

Human embryonic kidney (HEK) 293-F cells (Thermo Fisher Scientific) were maintained in high-glucose Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum in a 37 °C incubator with 5% CO_2. The constructed plasmids (pkGHP/VLRB) were purified using DNA Spin miniprep kits (iNtRON Biotechnology) and quantified using a NanoDrop spectrophotometer (Im et al., 2018). For transfection, 293-F cells were seeded into 24-well plates, grown to 90% confluence, and transfected with the plasmids using Lipofectamine2000 (Thermo Fisher

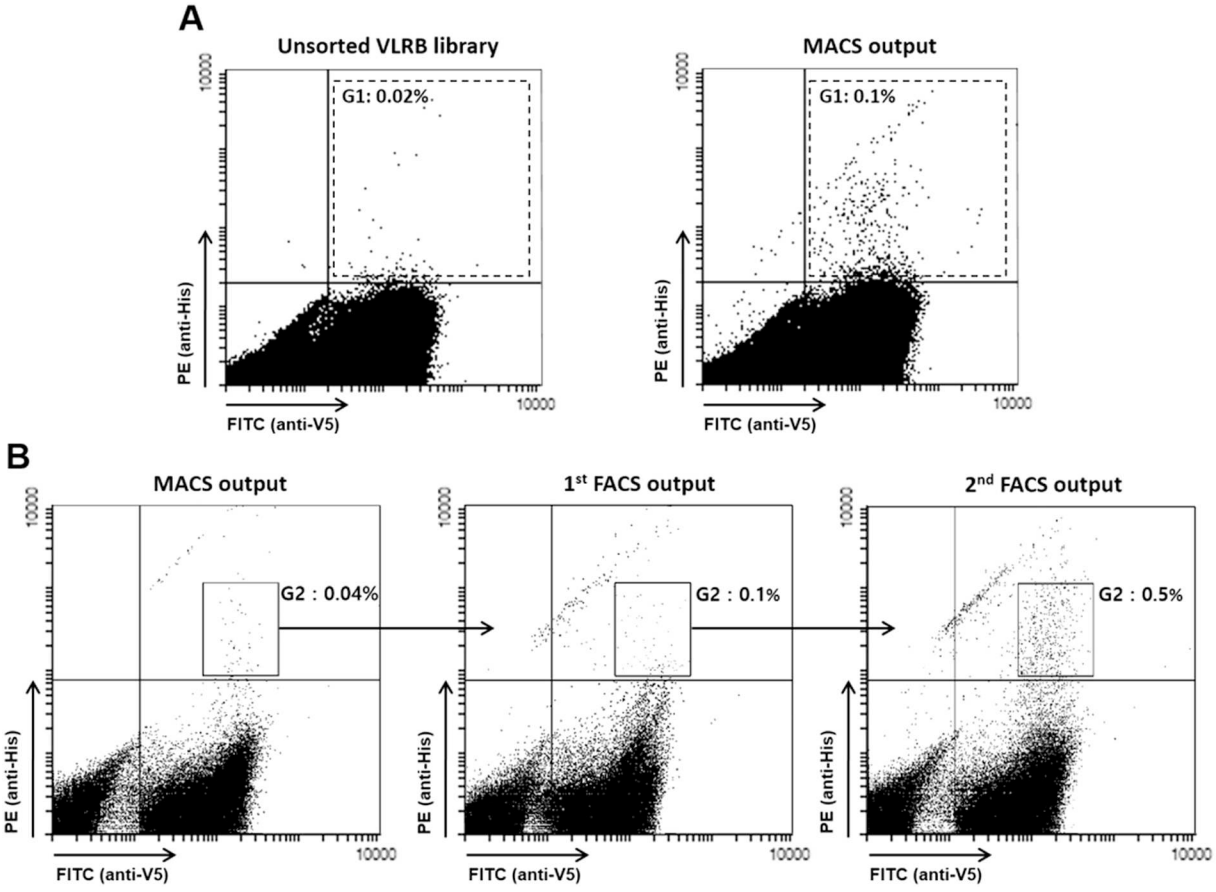

Fig. 4. Enrichment of H9N2-HA1-binding VLRB clones from the immunized hagfish library. (A) Enrichment of the antigen-binding clones by MACS with anti-His tag magnetic beads. G1 region of flow cytometric analysis was gated to calculate the double-positive yeast cells in the unsorted VLRB library and the output group of the MACS. (B) Two rounds of enrichment by FACS. G2 region of flow cytometric analysis was gated to sort and calculate the double-positive yeast cells in the output group of the MACS, output group of the first FACS and output group of the second FACS. The concentration of H9N2-HA1 used in this study was 500 nM.

Scientific) according to the manufacturer's protocol. After 4 h, the transfectants were transferred to expression medium (Thermo Fisher Scientific). After 48 or 72 h, each supernatant was harvested and centrifuged for removal of cells and debris.

2.6. Western blot and immunoblot analysis

The yeast cell lysates and culture supernatants were separated on a 12% SDS-PAGE gel under reducing conditions and transferred to methanol-activated PVDF membranes. The membranes were blocked with 5% skim milk in PBST (0.1% Tween 20 in PBS), and then incubated for 1 h with mouse anti-V5 IgG1 (2D12), which was previously reported to recognize the V5 epitope tag (Im et al., 2018). The blots were then incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Thermo Fisher Scientific). For immunoblotting, the viruses were mixed with 5 × SDS-loading buffer and dotted on a 100% methanol-activated PVDF membrane (1 μg/dot). The antigens were blocked and incubated with the culture supernatants from HEK 293-F cells containing secreted VLRB as a primary antibody for 1 h, incubated with antibody 2D12, and labeled with an HRP-conjugated antibody. The results were analyzed by a ChemiDoc™ XRS + System with Image Lab™ Software (BioRad) using a SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Fisher Scientific). After each incubation, the membranes were washed three times with PBST for 10 min during each washing step.

Confocal microscopy. The yeast cells were diluted to an OD600 of 0.4–0.6, and fixed with 4% formaldehyde in PBS, incubated on an 8-chamber slide treated with poly L-lysine for 1 h to allow attachment. The slides were blocked with 1% BSA in PBS for 30 min, incubated with antibody 2D12 for 1 h, and incubated with anti-mouse IgG-FITC (Jackson ImmunoResearch) for 30 min, then stained with DAPI for 15 min. After each incubation, the slides were washed three times with PBS for 5 min during each washing step. The slides were mounted with Vectashield (H-1000, Vector Labs), and images were viewed under a confocal microscope (Zeiss Axiovert).

2.7. Magnetic-activated cell sorting (MACS)

In 200 μl of MACS buffer (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, 0.5% BSA, and 0.0744% EDTA-Na_2), 3.0×10^7 induced yeast cells were reacted with 500 nM of H9N2-HA1 at 4 °C for 1 h. After washing with MACS buffer, the yeast cells were incubated with 20 μl of anti-His tag Microbeads (Miltenyi Biotec) at 4 °C for 30 min. After washing again, 3.0×10^7 cells in 500 μl of MACS buffer were loaded onto a LS column (Miltenyi Biotec) surrounded by a magnet and washed with 10 ml of MACS buffer. To increase the yield of MAC-sorted cells, three MACS were performed in parallel. The sorted cells were recovered in SD selective medium at 30 °C for 2 days.

2.8. Fluorescence-activated cell sorting (FACS)

The recovered and induced yeast cells (3.0×10^7) in 200 μl of FACS buffer (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, 0.1% BSA, and 0.0744% EDTA-Na_2) were incubated with 500 nM of H9N2-HA1 at RT for 1 h. Two-color labeling was performed with antibody 2D12 followed by goat anti-mouse IgG-FITC, and rabbit anti-

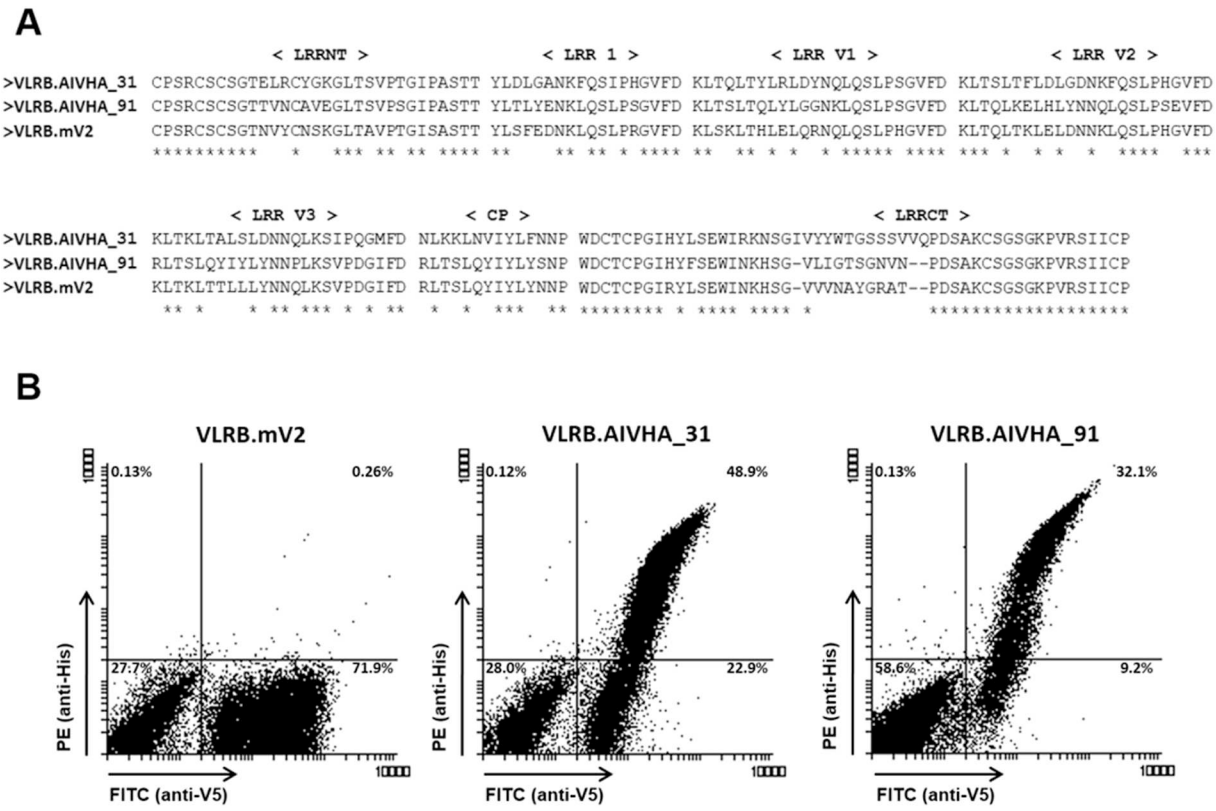

Fig. 5. Characterization of the antigen-specific VLRB (VLRB.AIVHA_31 and VLRB.AIVHA_91) sorted by a second round of FACS. (A) Structure-based sequence alignment of VLRB.AIVHA_31 and VLRB.AIVHA_91 compared with VLRB.mV2. Conserved residues are indicated below the sequences (*). (B) Flow cytometric analysis of individual yeast clones expressing VLRB.AIVHA_31 or VLRB.AIVHA_91 complexed with H9N2-HA1. The VLRB-antigen complexes were stained with antibody 2D12 followed by goat anti-mouse IgG-FITC, and phycoerythrin (PE)-conjugated antibody. Yeast clone of VLRB.mV2 served as a negative control.

polyhistidine-phycoerythrin (PE) (Abcam). The labeled cells in 500 µl of FACS buffer were run through FACS AriaIII (BD Bioscience). The sorted yeast cells were recovered in SD selective medium at 30 °C for 2 days.

2.9. ELISA

The viruses (e.g., AIV subtypes H9N2, H6N2 and H4N2) were coated to 96-well plates (500 ng/well). The antigens were blocked with 1% BSA in PBS and incubated for 1 h with the culture supernatants from HEK 293-F cells containing secreted VLRB as a primary antibody, after which incubation with antibody 2D12, and lastly, labeling with an HRP-conjugated antibody was performed. After each incubation step, the plates were washed three times with PBST.

2.10. Statistical analysis

All data were expressed as the means ± standard error of the means (SEMs). Paired samples *t*-test was used to compare the mean of all the sample groups and statistical comparisons were calculated using IBM SPSS Statistics software, version 19 (SPSS Inc). Statistically significant of the differences in mean were considered at $p < .05$.

3. Result

3.1. VLRB response of hagfish immunized with H9N2-HA1

To construct large VLRB libraries for the identification of antigen-specific hagfish VLRBs, we immunized hagfish larvae with H9N2-HA1. In order to observe transcriptional alterations of the VLRB, electrophoretic profiles of VLRB amplicons were obtained from mRNA extracted from hagfish leukocytes, before and after immunization with the antigen (Fig. 1). PCR products for incomplete germ-line VLRB gene (~500 bp) were amplified from the peripheral blood leukocytes of naïve hagfish (Fig. 1 Lane 1), whereas mature VLRB fragments (~1 kb) were obtained from the immunized hagfish (Fig. 1 Lane 2).

3.2. Yeast display vector, pYD8

For high-sensitivity screening of the large VLRB libraries, induced by the immunization with the H9N2-HA1, we developed a modified vector for the YD, pYD8 (Fig. 2A). For more efficient cloning and to exclude self-ligation, a Cm^R/ccdB gene was inserted downstream of the Aga signal peptide followed by glycine/serine residues, a V5 epitope tag and the mAga2, under the control of the GAL1 promoter. The VLRB library encoding LRRNT to LRRCT were directionally cloned into two different *Sfi*I sites in place of Cm^R/ccdB. The pYD8 system displayed the VLRBs on the surface of the yeast via a disulfide bond between the C-terminally fused mAga2 and yeast membrane anchored sAga1, with a free N-termini (Fig. 2B). The glycine/serine residues function as flexible linkers, and the V5 tag served to quantify the level of surface VLRB via FITC-conjugated antibodies, using a FACS. The H9N2-HA1 was tagged with polyhistidine, and those bound to antigen-specific VLRB decorated on yeast were detected by PE-conjugated antibody specific to polyhistidine.

3.3. Surface expression of monoclonal VLRB

The expression and surface display of a randomly selected VLRB clone (VLRB.mV2) fused to V5/mAga2 on the yeast using the pYD8 system can be observed in Fig. 3, under galactose-inducible conditions compared with the non-induction group (negative controls). Lysates of the yeast exhibited high expression of the VLRB, whereas such

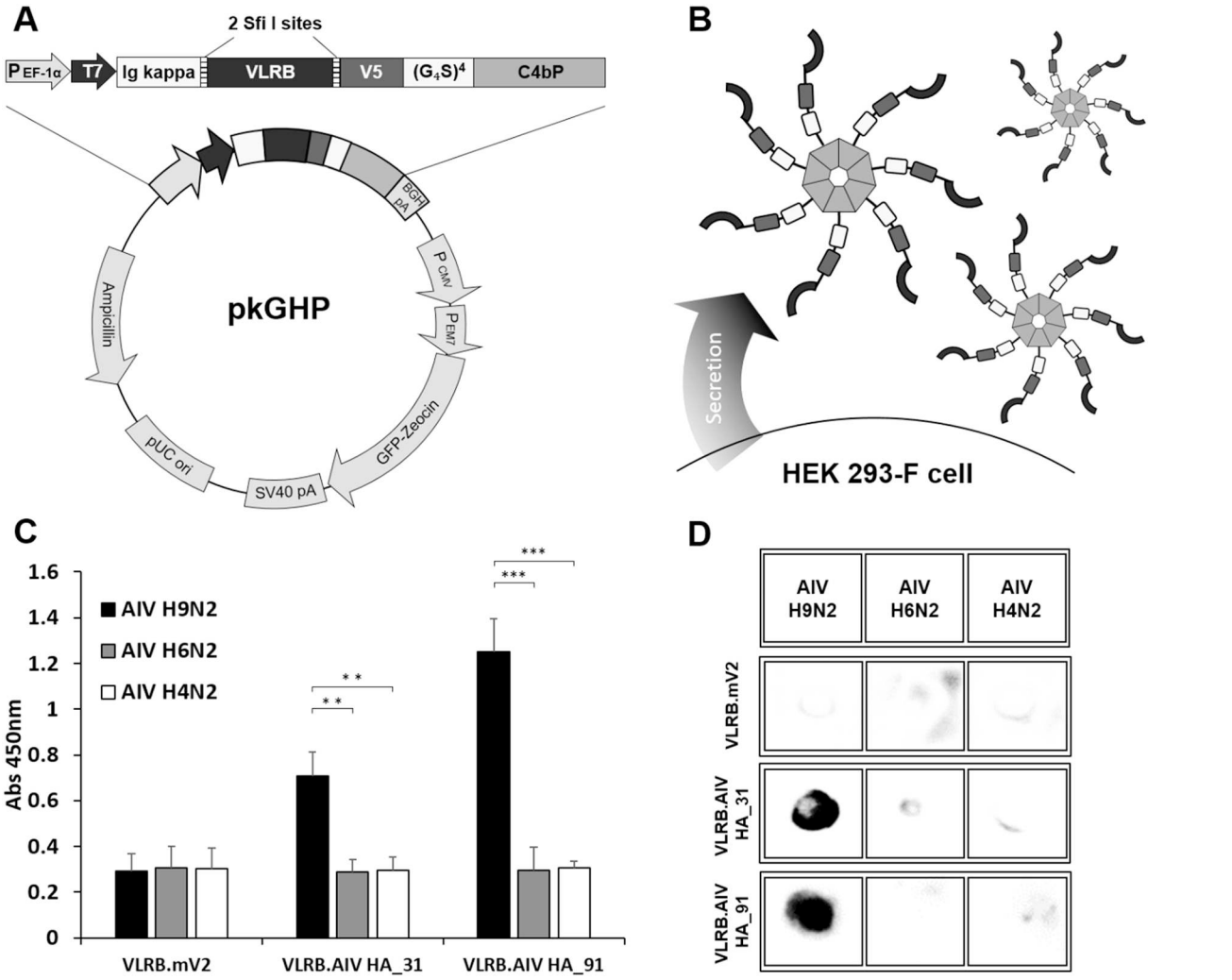

Fig. 6. Characterization of antigen-specific VLRB (VLRB.AIVHA_31 and VLRB.AIVHA_91) as a secreted protein from mammalian expression system. (A) Plasmid map of the pkGHP. The two *Sfi* I sites share the same restriction site with pYD8. (B) Schematic representation of recombinant VLRB secreted from HEK 293-F cell. The recombinant VLRB consisted of LRRNT to LRRCT were heptamerized by the C4bp domain, which featured seven identical antigen-binding subunits. The V5-tag served for VLRB detection, and the glycine/serine residues function as flexible linkers. (C) ELISA-based comparison showing the specificity of the VLRB.AIVHA_31 and VLRB.AIVHA_91 as secreted proteins from HEK 293-F cell to AIV H9N2 (black bar), H6N2 (gray bar) and H4N2 (white bar). The secreted VLRB.mV2 was used as a negative control. 'Abs 450 nm' refers to absorbance at 450 nm. Error bars indicate SEMs for $n = 3$ experiments. Statistical significances were assessed by the paired samples *t*-test (*$p < .05$, **$p < .01$, ***$p < .001$). (D) Immunoblotting analysis of the VLRB.AIVHA_31 and VLRB.AIVHA_91 as secreted proteins to various subtypes of AIV (H6N2, H4N2) compared with the AIV H9N2 subtype. The secreted VLRB.mV2 served as a negative control.

expression was not seen in the supernatants of the yeast cells, as shown from the Western blot analysis (Fig. 3A). Flow cytometric analysis revealed proper surface expression of VLRB on the yeast, wherein 70.1% of the yeast cells were positively stained with FITC (Fig. 3B). Furthermore, cellular surface localization of the VLRB on the yeast was confirmed using confocal imaging analysis (Fig. 3C).

3.4. Screening of H9N2-HA1-specific VLRBs with MACS and FACS

We constructed pYD8/VLRB cDNA libraries, consisting of mature VLRBs from the immunized hagfish (Fig. 1) conjugated in the pYD8 vector. Two-color labeling was performed with FITC as an indicator of VLRB displayed on the yeast library and with PE for the H9N2-HA1, thereby the VLRB-antigen complexes appeared as double-positive cells in the upper-right quadrants of the dot plots in FACS analysis. The initial populations in gated G1 region of the unsorted VLRB library was 0.02% of the yeast cells (Fig. 4A). After enrichment by MACS with the antigen and anti-histidine magnetic beads, 0.1% of the yeast population was observed in gated G1 region, indicating a 5-fold enrichment of the double-positive population. Then the gated region was optimized from G1 to G2 region for more specific selection of double-positive populations in two successive rounds of FACS enrichments, and 0.04% of the yeast population was calculated in the gated G2 region of MACS output (Fig. 4B). Each round of FACS output showed 0.1% and 0.5% of the yeast cells in the gating G2 region, indicating 2.5-fold and 5-fold enrichment of the double-positive populations, respectively. After the second round of FACS, the gated yeast cells in the G2 region were collected and analyzed by DNA sequencing. These gated cells were cultured on the plate and then fifty clones, randomly selected from the culture, were classified into two groups of VLRB sequences, VLRB.AIVHA_31 and VLRB.AIVHA_91 that had a completely different sequence in their variable region (Fig. 5A). These populations of individual yeast clones, which exist as monoclonal VLRBs, were double stained, and contained a higher number of double-positive populations (VLRB.AIVHA_31: 48.9%, VLRB.AIVHA_91: 32.1%) as determined from the FACS analysis, indicating high reactivity for the antigen (Fig. 5B). The VLRB.mV2 clone that contained the PE-negative cell population served as a negative control.

3.5. Specificity of secreted VLRB to AIV H9N2 obtained from mammalian expression system

The pkGHP VLRB vector used in the mammalian expression system has been shown previously to be as a useful vector for the production of secreted VLRBs (Im et al., 2018). To prove the specificity of the antigen-specific clones (VLRB.AIVHA_31 and VLRB.AIVHA_91) as secreted proteins, the VLRB genes of these clones were transferred directly from the pYD8 plasmid into the pkGHP plasmid via two shared *Sfi* I sites (Fig. 6A). The VLRBs secreted from the pkGHP VLRB system have multivalency derived from the complete heptamerization of seven identical antigen-binding subunits, as previously shown by Lee et al., (2018) and Im et al., (2018) (Fig. 6B). The VLRB.AIVHA_31 and VLRB.AIVHA_91 secreted into the supernatant of transfected HEK 293-F cells were tested for their reactivity to several AIV subtypes by ELISA (Fig. 6C). ELISA results indicate that VLRB.AIVHA_31 and VLRB.AIVHA_91 showed significant binding avidity to AIV H9N2 with an O.D value of 0.71 ± 0.11 and 1.25 ± 0.15, respectively, while there was no significant response to other AIV subtypes including H6N2 and H4N2. Furthermore, similar results were obtained with immunoblotting (Fig. 6D). These antigen-specific VLRBs exhibited a strong signal to only AIV H9N2 without any response to the other AIV subtypes. The VLRB.mV2 served as negative control in both ELISA and immunoblotting.

4. Discussion

The properties of VLRB as an antigen receptor are multifarious. One specific property of VLRBs is that they show a unique antigen recognition distinct to that of conventional antibodies. In response to foreign material, the VLRB genes in the germline configuration are processed into mature VLRB genes by a gene assembly-like process with variable LRRV cassettes (Alder et al., 2005; Rogozin et al., 2007). During immunization, a vast number of mature VLRBs are generated, as evident in the immunized hagfish in the present study (Fig. 1). Furthermore, structural analyses of monoclonal VLRB specific for hen egg lysozyme revealed that a flexible loop-structure protruding from the LRRCT of the VLRB, was capable of interacting with an epitope located in the active site of the hen egg lysozyme, which was not easily recognized by Ig-based antibodies (Velikovsky et al., 2009). In addition to the above property, VLRB has simple and single polypeptide structure, and this allow various protein engineering techniques to be performed on VLRB production. Previous studies have demonstrated the use of advanced screening methods and expression systems for production of antigen-specific VLRBs (Tasumi et al., 2009; Lee et al., 2012; Yu et al., 2012; Im et al., 2018; Lee et al., 2018a; Lee et al., 2018b)

In this study, we developed and optimized the pYD8 vector for the efficient expression of hagfish VLRB on the surface of the yeast, as confirmed by Western blot, flow cytometry and confocal imaging analysis (Figs. 2 and 3). In addition, high-throughput screening of antigen-specific hagfish VLRB was also accomplished through MACS and FACS (Fig. 4). Although a previous study reported on the use of the YD system using flocculation proteins of α-agglutination (Flo1p) in the selection of antigen-specific lamprey VLRB (Tasumi et al., 2009), the Aga-based pYD8 system used here has many advantages. The surface density of VLRB on the yeast was 1–10 × 10^4 copies per cell in Aga YD system, which is approximately 4 to 10 times higher than that of Flo1p YD system, with only 2.5–10 × 10^3 copies per cell expressed (Chao et al., 2006; Tasumi et al., 2009). Therefore, the antigen-specific hagfish VLRB on the yeast surface could be more efficiently screened as multivalent binders, and consequently possessed high reactivity to the antigen in FACS analysis (Fig. 5). Furthermore, the antigen-specific VLRBs as secreted protein in culture supernatants of HEK 293-F cell have significant specificity to the target AIV virus (H9N2), when compared to the other AIV subtypes tested in ELISA and immunoblotting (Fig. 6). This indicates that the binding abilities of the antigen-specific VLRBs could be maintained between the Aga-based yeast expression system and mammalian expression system.

Taken together, our results demonstrate that the newly developed pYD8 vector system could be useful for screening antigen-specific hagfish VLRB and also appear to be compatible with mammalian expression systems. Furthermore, the specificity of the generated VLRB to the virus suggests that it may have potential in various bio-applications.

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