Phosvitin plays a critical role in the immunity of zebrafish embryos via acting as a pattern recognition receptor and an antimicrobial effector

Shaohui Wang\textsuperscript{a,*}, Yuan Wang\textsuperscript{a,*}, Jie Ma\textsuperscript{a}, Yunchao Ding\textsuperscript{b} and Shicui Zhang\textsuperscript{a,b,**}

\textsuperscript{a}Institute of Evolution & Marine Biodiversity and \textsuperscript{b}Laboratory of Evolution and Development, Department of Marine Biology, Ocean University of China

*These authors contributed equally to this work.

** To whom correspondence and reprint request should be addressed.

Shicui Zhang, PhD

Professor of Marine Biology

Ocean University of China

Room 205, Ke Xue Guan

5 Yushan Road, Qingdao 266003

PR China

E-mail: sczhang@ouc.edu.cn

Running title: Immune role of phosvitin in zebrafish embryo
Abstract

How fish embryos that develop externally survive microbial attacks is poorly understood. Here we clearly demonstrated that the embryo extract of zebrafish and its early embryo both displayed antimicrobial activity against microbes including pathogenic *Aeromonas hydrophila*, and phosvitin (Pv), a nutritional protein abundant in eggs, was related to this antimicrobial activity. We also showed that recombinant Pv (rPv) acted as a pattern recognition receptor capable of recognizing the microbial signature molecules LPS, lipoteichoic acid and peptidoglycan as well as binding Gram-negative and positive microbes *Escherichia coli*, *A. hydrophila* and *Staphylococcus aureus*, and functioned as an antimicrobial agent capable of killing the microbes. Furthermore, we revealed that its C-terminal 55 residues (Pt5) with the functional sites Arg242 and Ala290/Ile293 were indispensable for Pv antimicrobial activity. Importantly, microinjection of rPv or Pt5 into early embryos significantly enhanced their resistance to *A. hydrophila* challenge, and this enhanced bacterial resistance was markedly reduced by co-injection of anti-Pv antibody plus rPv (or Pt5), but not by injection of anti-actin antibody plus rPv. Moreover, the generated mutants with *in vitro* antimicrobial activity, when injected into the embryos, could also promote their resistance to *A. hydrophila*, but those without *in vitro* antimicrobial activity could not. It is thus proposed that Pv participates in the protection of early embryos against pathogenic attacks via binding and disrupting potential pathogens. This work also opens a new way for the study of the immunological roles of yolk proteins in oviparous animals which rely on yolk proteins for embryonic development.

Keywords: Zebrafish; Embryogenesis; Vitellogenin; Phosvitin; Antimicrobial peptide

Introduction

Eggs of most fish are released and fertilized externally, and the resulting embryos and larvae are therefore exposed to an aquatic environment which is full of potential pathogens capable of causing various types of diseases. During the early stages of development, fish embryos have little or only limited ability to synthesize immune-relevant molecules endogenously and their lymphoid organs are not yet fully formed (1, 2). Moreover, embryogenesis of fish is one of the most vulnerable stages in life history (3). How they survive the pathogenic attacks in such a hostile environment is one of the key issues for reproductive and developmental immunology, but study as such is rather limited to date.

Fish eggs are in most cases cleidoic, i.e. closed free-living system following fertilization; they are therefore supposed to depend upon the maternal provision of immune-relevant molecules for protection against invading pathogens before full maturation of immunological systems. Previous studies on several fish species have shown that maternal IgM is able to be transferred from mother to offspring (4-13). Likewise, maternal transfer of the innate immune factors including the complement component C3 (14-19), lectins (20-22), protease inhibitors (23, 24) and lysozymes (25, 26) to offspring has also been reported in different teleost species. Moreover, immunization of parents results in a significant increase in IgM levels (7, 12) and anti-protease and lysozyme activities (7)
in the eggs compared to control. These transferred maternal molecules have been proposed to be involved in the immune defense against pathogens in developing fish embryos and larvae. For example, Wang et al. (19, 27) have recently demonstrated that the maternal complement components operating via the alternative pathway are attributable to the protection of early embryos of zebrafish *Danio rerio* against microbial attacks. Besides above substances, yolk proteins consisting of phosvitin (Pv) and lipovitellin (Lv) are also maternally-transferred molecules stored in fish eggs. Both Pv and Lv are the proteolytically cleaved products of a high molecular mass lipoglycophosphoprotein, vitellogenin (Vg), and are traditionally regarded as the yolk reserves of nutrients essential for growth and development (28). Interestingly, as a major component of yolk proteins, chicken Pv has been shown to be able to inhibit the growth of the Gram-negative bacterium *Escherichia coli* (29) via chelating ions through its numerous phosphorylated serine residues (30, 31). On the other hand, Vg, the precursor of Pv, has also been revealed to be an antimicrobial agent involved in immune defense in fish (32-36). Similarly, mosquito Vg has recently been shown to be able to interfere with the anti-*Plasmodium* response in the malaria mosquito *Anopheles gambiae* (37). From these it may be prudent to hypothesize that Pv, in addition to being a simple nutritional reserve, is also involved in the immune defense of developing embryos in fish. The aim of this study is thus to verify this hypothesis, using the model fish *D. rerio*. Clearly, such a study will definitely shed light on the mechanisms how early embryos of fish survive the pathogenic attacks.

**Materials and Methods**

**Preparation of embryo extracts**

The embryos of *D. rerio* were collected at about 10 h post fertilization, washed three times with sterilized PBS (pH7.4) and homogenized on ice. After centrifugation at 5000 g at 4°C for 5 min, and the embryo extracts were pooled, aliquoted and stored at -70°C until used.

**Preparation of bacteria**

The Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Staphylococcus aureus* were incubated at 37°C in LB medium for 16 h, and the Gram-negative bacterium *Aeromonas hydrophila* LSA 20 (pathogenic to *D. rerio*) was grown at 28°C in tryptic soy broth (TSB) medium for 16 h. They were harvested by centrifugation at 3000 g at 4°C for 15 min. The bacterial pellets were re-suspended in 10 mM PBS (pH7.4), giving a concentration of 5×10^7 cells/ml (for both *E. coli* and *S. aureus*) or a concentration of 2.5×10^8 cells/ml (for *A. hydrophila*), and used for the following experiments.

**Assays for antimicrobial activity of Pv in embryo extracts**

To inactivate the complement activity, the embryo extracts were heated at 45°C for 30 min (27). Heating at this temperature had little effect on Pv because Pv was heat-resistant (38). To precipitate Pv in embryo extract, an aliquot of 1 ml heated extract (with ~3.2 μM Pv) was mixed with ~1 μg anti-fish Pv antibody (polyclonal antibody; E91679Fi, Usenk), incubated at 4°C for 2 h and then 20 μl of protein A/G PLUS-Agarose (Santa Cruz Biotechnology) was added to the mixture. After incubation overnight at 4°C, the mixture was centrifuged at 3000 g for 10 min, and the supernatant was pooled and used for
antimicrobial activity assay. For control, 1 ml of embryo extract was mixed with ~1 µg anti-β-actin antibody (polyclonal antibody; AA128-1; Beyotime, Nantong, China) and processed similarly.

The antimicrobial activity of embryo extracts against E. coli, A. hydrophila and S. aureus was assayed as described by Wang et al. (27). PBS solution instead of embryo extracts was used as control. The percent of bacterial growth inhibition was inferred from the difference between the numbers of colonies in the test and control.

**Assays for antimicrobial activity of Pv in developing embryos**

To test if Pv has any ability to protect developing embryos, 30 dechorionated embryos were microinjected at 8-cell stage in the yolk sac with ~6 nl of sterilized PBS (blank control) or anti-Pv antibody solution (~0.33 ng) or anti-β-actin antibody solution (~0.33 ng) or purified recombinant Pv (rPv; see below) solution (~0.6 ng) or BSA solution (~0.6 ng), respectively, and challenged 1 h later by injection of ~6 nl (~500 cells) of live A. hydrophila suspension. The mortality was recorded and cumulative mortality calculated at 24 h after bacterial injection. To confirm the specificity of the antimicrobial activity of rPv in vivo, anti-Pv antibody was injected together with rPv into the embryos, which were then challenged by injection of live A. hydrophila. For control, the embryos were injected with anti-actin antibody plus rPv, and treated similarly.

To verify the killing of A. hydrophila by developing embryos, 8-cell stage embryos were dechorionated and microinjected with live A. hydrophila as above. Five embryos were collected each time at 0, 6 and 12 h post bacterial injection. The normal embryos were also collected as control. Total DNAs were isolated from each embryo according to the method of Wang et al. (27) and used for PCR. The PCR was carried out to amplify a specific region of A. hydrophila 16S rRNA gene using the sense primer 5’-AATACCGCATACGCCCTAC-3’ and anti-sense primer 5’-AACCCACATCTCAGACAC-3’, which were designed on the basis of A. hydrophila 16S rRNA sequence (GenBank accession no. DQ207728).

**Titration of Pv content in eggs/embryos**

Totally, 60 fertilized eggs and 120 embryos collected at 12 and 24 h post fertilization were washed three times with sterilized 0.9% saline, homogenized and centrifuged at 5000 g at 4°C for 5 min. The supernatants were pooled and used to measure the contents of Pv in the extracts using fish Pv ELISA kit (E91679Fi, Uscnk) according to manufacturer’s protocol. The mean diameter of D. rerio eggs measured was ~600 µm and therefore the egg/embryo volume was approximately ~1.1×10^5 cm³. Accordingly, the content of Pv in each egg/embryo was inferred as described by Liang et al. (16).

**Expression and purification of recombinant Pv (rPv)**

The cDNA region encoding zebrafish Pv was amplified by PCR with the upstream primer 5’-GCCATATGGTCAGAAACATTGAAG-3’ (NdeI site is underlined), and the downstream primer 5’-CGGAATTCTTTAGAAATACATTTC-3’ (EcoRI site is underlined). The PCR product was digested with NdeI and EcoRI and sub-cloned into the plasmid expression vector pET28a (Novagen) previously cut with the same restriction enzymes. The identity of the insert was verified by
sequencing and the plasmid was designated pET28a/Pv.

The cells of *E. coli* BL21 were transformed with the plasmid pET28a/Pv and cultured overnight in LB broth containing 50 μg/ml kanamycin. The culture was diluted 1:1000 with LB broth and subjected to further incubation at 37°C for 2 h, and the expression of rPv was induced by adding isopropyl β-D-thiogalactoside (IPTG) to the culture at a final concentration of 0.1 mM. The rPv was purified and electrophoresed on 12% SDS-PAGE. MALDI/TOF MS analysis was performed on Bruker Ultraflex MALDI/TOF MS mass spectrometer (Bremen, Germany).

Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard.

**Antimicrobial activity assay of rPv**

The antimicrobial activity of rPv against the Gram-negative bacteria *E. coli* and *A. hydrophila* and the Gram-positive bacterium *S. aureus* was measured by the colony forming unit assay as described by Fan et al. (39). The percent of bacterial growth inhibition by rPv was calculated as follows: [(number of colonies (control-test)/number of colonies (control))]×100 (n=3).

**Scanning electron microscopy (SEM)**

Aliquots of 150 μl of *E. coli* (a representative Gram-negative bacterium) and *S. aureus* (a representative Gram-positive bacterium) suspensions containing 5×10⁷ cells/ml were mixed with 150 μl of 3.2 μM rPv in 50 mM Tris-HCl buffer (TB; pH 8.0) or with 150 μl of TB alone as control. The mixtures were incubated at 25°C for 1 h, fixed in 2.5% glutaraldehyde in 100 mM PBS (pH7.4) and dropped onto 24 mm × 24 mm cover glasses. The samples were post-fixed in 1% osmium tetroxide, dehydrated with graded ethanol, dried by the critical point method and coated with gold. Observation was made under a JEOL JSM-840 scanning electron microscope.

**Iron-binding activity assay**

Chrome azurols (CAS) assay buffer was prepared as described by Schwny and Neilands (40). The iron-binding activity of rPv was assayed spectrophotometrically as described by Liu et al. (41). The iron-binding activity of rPv was expressed as OD₆₃₀ ratio, which indicates the relative absorbance of the samples in comparison with control.

**Labeling of rPv with fluorescein isothiocyanate (FITC)**

Purified rPv (~32 μM) and BSA were labeled as described by Li et al. (33). The purity of the conjugate, FITC-labeled rPv, was confirmed by SDS-PAGE and Coomassie blue staining, and its F/P ratio (F/P ratio is defined as the ratio of moles of FITC to moles of protein in the conjugate) was calculated by the equation F/P = (2.77 × A₄₉₅)/(A₃₈₀-0.35 × A₄₉₅) from the absorbance readings of the conjugate samples (42). To assess if rPv activity is affected by labeling with FITC, colony forming unit assay was conducted to compare the growth inhibition rates of *E. coli* by FITC-labeled rPv and non-labeled rPv.

**Assay for binding of FITC-labeled rPv to microbial cells**

Binding of rPv to the microbes *E. coli, A. hydrophila* and *S. aureus* was assayed by the method of Li et al. (33). The binding of
FITC-labeled rPv to microbial cells was observed under an Olympus BX51 fluorescence microscope. The microbes treated with FITC-labeled BSA under the same conditions were used as control. To test if non-labeled rPv could competitively inhibit the binding of FITC-labeled rPv to the microbes, non-labeled rPv was mixed with labeled rPv at a ratio of 1:1. The mixture was then incubated with the microbes and the binding observed as above.

**Assay for binding of rPv to various ligands**

LPS from *E. coli*, and lipoteichoic acid (LTA) and peptidoglycan (PGN) from *S. aureus* (all from Sigma-Aldrich) were individually dissolved in re-distilled water, all giving a concentration of 40 μg/ml. A volume of 50 μl (2 μg) of each solution was applied to each well of a 96-well microplate and air-dried at room temperature overnight. The plates were incubated at 60°C for 30 min to fix rPv, and the wells were each blocked with 200 μl of 1 mg/ml BSA in 10 mM PBS (pH 7.4) at 37°C for 2 h. After washing four times with 200 μl of 10 mM PBS supplemented with 1% Tween-20, 50 μl of DIG-labeled rPv solutions with different concentrations of DIG-labeled rPv (0, 0.02, 0.04, 0.08, 0.16, 0.32 and 0.5 μM) was added into the wells. After incubation at room temperature for 3 h, the wells were each rinsed four times with 200 μl of 10 mM PBS supplemented with 1% Tween-20, 100 μl of anti-DIG-Fabs (Roche) diluted at 1:1,000 with 10 mM PBS (pH 7.4) containing 1% dry milk powder was added into the wells. The wells were incubated at 37°C for 2 h, washed and reacted with 75 μl of 0.4 mg/ml O-phenylenediamine (OPD; Amresco) in 51.4 mM Na₂HPO₄, 24.3 mM citric acid and 0.045% H₂O₂ (pH 5.0) at 37°C for 20 min. Subsequently, 25 μl of 2 mM H₂SO₄ was added into each well to terminate the reactions, and absorbance at 492 nm was monitored by a microplate reader (GENios Plus, Tecan). For control, BSA at the same concentrations was treated similarly.

For Scatchard analysis, LPS, LTA and PGN were labeled with biotin hydrazide (Sigma-Aldrich) by the method of Gotoh et al. (43). rPv was dissolved in re-distilled water, giving a concentration of 40 μg/ml. A volume of 50 μl (2 μg) of rPv solution was applied to each well of a 96-well microplate and air-dried at room temperature overnight. The plates were incubated at 60°C for 30 min to fix rPv, and the wells were each blocked with 200 μl of 20 mg/ml BSA in 10 mM PBS (pH 7.4) at 37°C for 2 h. After washing four times with 200 μl of 10 mM PBS supplemented with 1% Tween-20, a total of 50 μl solution with different concentrations (0, 0.02, 0.04, 0.08, 0.16, 0.32 and 0.5 μg/ml) of biotin-labeled LPS, LTA or PGN was added each well and then processed as above. For control, BSA instead of rPv at the same concentrations was treated similarly. The equilibrium association constant (Kd) and apparent maximum number of binding (Bmax) are determined according to the Scatchard plot using the software of GraphPad Prism 5.01.

**Expression of truncated Pv and bioactivity assay**

To determine the structure-activity relationship, the N-terminal 55, 102, 134, 174 and 194 residues of Pv were deleted, respectively. The upstream primers used were:

- 5′-GCCTATGGTCCCATACATTGAA-3′; 55d,
- 5′-GCCATAGAGCTGGAGTTGAAGT
-3’; 134d, 5’-GCCATATGTTCTTGTGAAAAGTGA-3’; 174d, 5’-GCCATATGAGCTCAAGCTTAAAGG-3’; 194d, 5’-GCCATATGCTCTCTGTATGCTTAAAGA-3’ (NdeI site is underlined); and the downstream primer used was all the same, 5’-CGGAATTCTTATGGAATATCATTTCC-3’ (EcoRI site is underlined). The C-terminal 55 residues of Pv were also deleted using the primer pair 5’-GCCATATGGTCAGAAACATTGAAG-3’ (NdeI site is underlined) and 5’-CGGAATTCACTGTCTCCTTGGA-3’ (EcoRI site is underlined).

Construction of the expression vector plasmid, transforming into E. coli BL21 as well as expression and purification of recombinant proteins were all performed as above. The plasmids constructed were verified by sequencing and designated Pt1 (N-terminal 55 residues deleted), Pt2 (N-terminal 102 residues deleted), Pt3 (N-terminal 134 residues deleted), Pt4 (N-terminal 174 residues deleted), Pt5 (N-terminal 194 residues deleted) and Pt6 (C-terminal 55 residues deleted).

Antimicrobial activity and ligand binding assays were performed as above. Pt5 and Pt6 were also microinjected into the early embryos of D. rerio, respectively, to test their antimicrobial activity in vivo. To verify the specificity of the antimicrobial activity of Pt5 in vivo, the embryos were co-injected with Pt5 plus anti-Pv antibody, and then challenged by injection of live A. hydrophila.

Site-directed mutation of Pv and bioactivity assay

The plasmid Pt5 was used for mutational analyses. Mutants were generated using TaKaRa MutanBEST Kit (TaKaRa, Dalian, China) according to the kit’s protocol. The specific primers used were listed in Table 1. The mutations were confirmed by DNA sequencing. Expression and purification of the mutated recombinant proteins, antimicrobial activity and ligand binding assays were carried out as above. Pt5 and Pt6 were used as positive and negative controls, respectively. To test their antimicrobial activity in vivo, the mutants m1, m5 and m9 with in vitro antimicrobial activity and the mutants m12 and m13 with no in vitro activity (see below) were selected and microinjected into the early embryos of D. rerio, respectively, followed by the challenge with A. hydrophila.

Statistical Analysis

All the experiments were conducted at least three times. Statistical analyses were performed using the computer program SPSS 13.0. The statistical significance of difference between mean values was calculated by ANOVA and difference at p<0.05 was considered significant. All the data were expressed as mean±SD.

Results

Antimicrobial activity of Pv in embryo extracts

We first sought to examine if Pv in the embryo extract of D. rerio has any antimicrobial activity. The protein concentration of the extract prepared from the embryos collected at approximately 10 h post fertilization was ~20 mg/ml. As shown in Fig. 1, the embryo extract exhibited conspicuous antimicrobial activities against the Gram-negative bacteria E. coli and A. hydrophila as well as the Gram-positive bacterium S. aureus. When the complement activities, which had been demonstrated to
be present in the egg cytosol and heat-sensitive (19), were inactivated by heating, the antimicrobial activities of the embryo extracts against both 
*E. coli* and *A. hydrophila* as well as *S. aureus* were remarkably reduced. However, some antimicrobial activities remained in the heated extracts. As Pv was heat-resistant (38), we therefore thought that it might be related to the remaining antimicrobial activities. As expected, the remaining antimicrobial activities of the heated extracts against *E. coli*, *A. hydrophila* and *S. aureus* were significantly reduced by the pre-incubation with anti-fish Pv antibody, but not by the pre-incubation with anti-actin antibody (Fig. 1A, B and C). These data suggested that in addition to complement, Pv was also a factor responsible for the antimicrobial activity in the embryo extract.

**Involvement of Pv in antimicrobial activity in early embryos**

To test if Pv plays any protective role in early development, 8-cell stage embryos were each microinjected with the antibody against fish Pv to block Pv action, followed by injection with live *A. hydrophila* (pathogenic to *D. rerio*). The majority of the non-bacterial challenge control embryos (~95%) developed normally (Fig. 1D). Interestingly, the challenge with live *A. hydrophila* resulted in a significant increase in the mortality of the embryos microinjected with anti-Pv antibody, with the 24 h cumulative mortality of ~80%, while the same challenge caused only ~52% cumulative mortality at 24 h in the embryos injected with either anti-actin antibody or bovine serum albumin (BSA) or PBS alone (Fig. 1D). Moreover, anti-Pv antibody-induced embryonic death rate was remarkably reduced by the co-injection of the antibody plus purified rPv (see below), with the 24 h cumulative mortality of ~65%, but not by the injection of anti-actin antibody plus rPv (Fig. 1D). All these data suggested that Pv was involved in the antimicrobial activity of developing embryos.

To prove the killing of live *A. hydrophila* by the embryos, PCR analysis was performed to amplify a specific region of *A. hydrophila* 16S rRNA gene using the total DNAs isolated from each embryo as template (27). As shown in Fig. 2A, no band was observed in the control sample (from an embryo without injected *A. hydrophila*), but intense bands were found in the embryos collected soon after bacterial injection (0 h), and the band intensities apparently decreased with time (at 6 h and 12 h), suggesting the lysis of the bacterium by the embryos. Besides, the microinjection of anti-Pv antibody into the embryos blocked decrease in the band intensity during the initial 12 h (Fig. 2B), that is, little *A. hydrophila* lysis took place in the embryos, whereas the injection of anti-actin antibody into the embryos failed to block reduction of the band intensity at 6 and 12 h, that is, *A. hydrophila* lysis continued in the embryos (Fig. 2C). These data implicated the presence of a link between Pv and lysis of the bacterium in the early embryos.

**Microbicidal activity of rPv**

To verify that Pv is indeed involved in antimicrobial activity in developing embryos, we expressed zebrafish Pv in *E. coli* and examined its microbicidal activity directly *in vitro*. An expression vector including the cDNA encoding Pv of *D. rerio* and 5' additional tag of PET28a was constructed and transformed into *E. coli* cells. The recombinant peptide was induced by IPTG and purified by affinity
chromatography on a Ni-NTA resin column. The purified rPv with the TRX-His-tag yielded a single band of about 29 kDa on SDS-PAGE gel after Coomassie blue staining, corresponding to the expected size. MALDI-TOF MS analysis showed that the protein purified was zebrafish Pv (Supplementary S1). The purified rPv was then used to detect its antimicrobial activity against the Gram-negative bacteria *E. coli* and *A. hydrophila* and the Gram-positive bacterium *S. aureus* by colony forming unit assay. As expected, the growth of both *E. coli* and *A. hydrophila* as well as *S. aureus* was all inhibited by rPv in a dose-dependent manner (Table 2). rPv was able to significantly inhibit the growth of *E. coli, A. hydrophila* and *S. aureus*, with the half inhibitory concentrations (IC₅₀) of ~3.1 μM, ~3.0 μM and ~3.0 μM, respectively. In contrast, the peptide TRX-His-tag expressed did not suppress these bacterial growth (data not shown; also see An et al. (44)), implicating that the TRX-His-tag extra part in rPv had little effect on its antimicrobial activity. Besides, scanning electron microscopy examination revealed that rPv caused damage to the cells of both *E. coli* and *S. aureus*. The cells of *E. coli* and *S. aureus* incubated with rPv were severely damaged, with the appearance of collapsed architecture (Fig. 3B and D), but the bacterial cells incubated with TB alone remained intact and their cell surfaces were smooth (Fig. 3A and C). All these suggested that consistent with its involvement in antimicrobial activity *in vivo*, Pv was also a microbicidal factor *in vitro*, being capable of killing the Gram-negative and positive bacteria such as *E. coli* and *S. aureus*.

**Contents of Pv in eggs/embryos**

To evaluate if early embryos possess sufficient Pv *in vivo* to fulfill antimicrobial activity, we measured the endogenous content of Pv. An ELISA analysis revealed that the concentrations of Pv in each of the newly fertilized eggs as well as 12 h and 24 h embryos were ~36.0 μM, ~9.0 μM and ~1.3 μM, respectively. This indicated that at least at the initial 12 h post fertilization, the concentration of Pv in each embryo was significantly higher than IC₅₀, and therefore sufficient enough to kill invading pathogens *in vivo*.

**Binding of rPv to bacteria**

Next we explored the mechanisms by which Pv inhibits bacterial growth. As Pv isolated from chicken eggs was able to chelate ions through its phosphorylated serine residues (30, 31), we therefore determined if rPv has any capacity to bind iron which is necessary for bacterial growth and proliferation (45). When chrome azurols (CAS) assay solution was mixed with rPv, no color change was found in the mixture media and the OD₅₃₀ values did not change with rPv concentrations (Supplementary S2A). Moreover, the growth of both *E. coli* and *S. aureus* in the presence or absence of excess iron showed little difference (Supplementary S2B and C). These suggested that rPv did not bind Fe³⁺. As the phosphorylated serine residues of chicken Pv were shown to contribute to its chelating effect including binding to Fe³⁺ (46), the inability of zebrafish rPv to bind to Fe³⁺ might be due to the lack of phosphorylation of the serine residues in the rPv expressed in *E. coli*.

We then tried to test if rPv is able to bind to microbes. To do this, we labeled rPv and BSA with FITC. The F/P ratios of FITC-labeled rPv and BSA were 4.9 and 5.4, respectively, showing that they had similar F/P ratios. Antimicrobial activity
examination by colony forming unit assay revealed that the growth of *E. coli* pre-incubated with 1.6 µM of FITC-labeled rPv and non-labeled rPv was both significantly inhibited, with the similar inhibitory rates of ~21% and ~22%, respectively. In addition, FITC-labeled rPv appeared as a single band on a SDS-PAGE, suggesting that FITC-labeled rPv remained as monomer (Supplementary S3). These indicated that rPv activity was not affected by the labeling with FITC. Pre-incubation of FITC-labeled rPv with the Gram-negative bacteria *E. coli* and *A. hydrophila* and the Gram-positive bacterium *S. aureus* showed that rPv was able to bind to *E. coli, A. hydrophila* and *S. aureus* (Fig. 4A, D and G). Similarly, FITC-labeled chicken Pv also bound to the microbes (data not shown), suggesting that the phosphorylation of serine residues in Pv was not necessary for its binding to the microbes. Notably, the binding of FITC-labeled rPv to the microbes was found to be competitively inhibited by non-labeled rPv (Fig. 4B, E and H). In contrast, FITC-labeled BSA employed as control did not bind to the microbial cells tested (Fig. 4C, F and I). All these suggested that both zebrafish rPv and chicken Pv had the capacity to bind to microbes.

**Binding of rPv to various ligands**

To better understand the mechanisms by which rPv binds microbes, an ELISA was carried out to investigate what molecules on the microbial surfaces are recognized by rPv. It was found that rPv was able to bind to all the immobilized ligands tested, including LPS, LTA and PGN, in a dose-dependent manner, while BSA did not (Fig. 5A, B and C). To directly characterize the affinities of rPv to the ligands, we further performed the Scatchard analysis. The Bmax (ng/pmol protein) and the *Kd* (µg/ml) of the affinity of rPv to LPS, LTA and PGN were approximately 7.7 and 1.0, 7.1 and 1.3 as well as 8.7 and 1.5 (Fig. 5D, E and F), respectively. It was clear that the binding of rPv to LPS, LTA and PGN was specific and saturable. No specific binding of BSA to LPS, LTA and PGN was detected. These denoted that rPv was capable of binding to the microbial signature molecules LPS, LTA and PGN, thereby establishing it is a multivalent pattern recognition molecule.

**Effects of N- and C-terminal deletion on antimicrobial activity of rPv**

To determine the structure-activity relationship, various truncated phosvitins (Pt1-Pt6), with N-terminus or C-terminus deleted, were expressed in *E. coli* (Supplementary S4) and subjected to functional analyses. As shown in Fig. 6, deletion of the N-terminal 55, 102, 134, 174 and 194 residues had little effect on antimicrobial activity against *E. coli, A. hydrophila* and *S. aureus*, but deletion of the C-terminal 55 residues resulted in a remarkable decrease in antimicrobial activity (Fig. 6B, C and D). To detect if the reduction of antimicrobial activity of truncated peptides is related to any alteration in ligand binding, their affinity to LPS, LTA and PGN were assayed. Consistent with the fact that Pt6 displayed little antimicrobial activity, its affinity to LPS, LTA and PGN was significantly reduced (Fig. 6F). In contrast, Pt5 retaining the large antimicrobial activity still possessed the affinity to the ligands similar to that of full rPv (Fig. 6E). These suggested that the antimicrobial activity of Pv and its truncated products were correlated with their capacity to bind the ligands. Scatchard analysis revealed that the Bmax and *Kd* values of the binding of Pt5 to LPS, LTA and PGN were about 6.0 and 1.1, 6.1 and 1.3 as well as 6.5
and 1.6 (Fig. 6E), respectively, while the Bmax and Kd values of the binding of Pt6 to LPS, LTA and PGN were 2.3 and 3.2, 2.0 and 3.2 as well as 2.0 and 3.3 (Fig. 6F). Compared to Pt5, both the Bmax and Kd values of Pt6 to bind LPS, LTA and PGN were markedly decreased. All these indicated that the C-terminal 55 residues of rPv were indispensable for both antimicrobial activity and binding to the ligands.

To investigate if Pt5 and Pt6 function similarly in vivo as in vitro, the embryos were each microinjected with Pt5 or Pt6, followed by the challenge with injection of live A. hydrophila. About 95% of the embryos microinjected with Pt5 and Pt6 developed normally. Notably, the challenge with live A. hydrophila caused a significant increase in the mortality of the embryos injected with Pt6 or PBS, with the 24 h cumulative mortality of ~52%, but the same challenge induced only ~42% cumulative mortality at 24 h in the embryos injected with Pt5 (Fig. 1D). Moreover, the embryo-protecting role of Pt5 was counteracted by the co-injection of Pt5 plus anti-Pv antibody (Fig. 1D). These showed that like rPv, Pt5 with in vitro antimicrobial activity, was also involved in the antimicrobial activity of developing embryos.

**Effects of amino acid replacement on antimicrobial activity**

Antimicrobial peptides are generally accepted to exert antimicrobial activities via their positively charged surface and amphipathic (47, 48). Therefore inverse PCR was used to generate mutants using the plasmid Pt5 harboring the C-terminal 55 residues as template to assay the effects of charged and hydrophobic residue replacement on antimicrobial activity (Fig. 7A). All the mutants had a closely similar molecular mass (Fig. 7B), we thus compared the antimicrobial activity of each mutant with that of control under a same concentration. Compared with control, the single mutation R242G (m12; positively charged R to neutral G) and the double mutation A201G/I203M (m13; hydrophobic A and I to hydrophilic G and M) resulted in a significant decrease in their antimicrobial activity against *E. coli, A. hydrophila* and *S. aureus* (Fig. 7C, D and E) as well as their affinity to LPS, LTA and PGN (Supplementary S5). For all the other mutants including mutations I203M (m1), K209E (m2), H211L (m3), K212E (m4), R214G (m5), T225F (m6), F234S (m7), F234L (m8), E235K (m9), K239E (m10) and Q240H (m11), no marked decrease in antimicrobial activity against *E. coli, A. hydrophila* and *S. aureus* was observed (Fig. 7C, D and E). Consistently, the Scatchard analysis showed that the binding affinities of m12 and m13 to LPS, LTA and PGN were significantly reduced, while the affinities of the mutants such as m1, m3, m5, m7 and m9 to LPS, LTA and PGN remained comparable to that of Pt5 (Table 3). These indicated that the positively charged residue R242 and the hydrophobic residues A201/I203 were the functional determinants contributing to the antimicrobial activity of rPv.

To test if the mutants play similar roles in vivo, the embryos were each microinjected with m12 and m13, both of which had little antimicrobial activity in vitro, as well as with m1, m5 and m9, which all remained antimicrobial activity comparable to Pt5 in vitro, followed by the challenge with injection of live *A. hydrophila*. The majority of the embryos (~95%) microinjected with all the mutants above developed normally.
Interestingly, the challenge with live *A. hydrophila* induced a significant increase in the mortality of the the embryos injected with m12 or m13, with the 24 h cumulative mortality of more than 50%, while the same challenge caused ~42% cumulative mortality at 24 h in the embryos injected with m1 or m5 or m9 (Fig. 7F). It was obvious that the mutants with *in vitro* antimicrobial activity were able to protect the developing embryos, while the mutants without *in vitro* antimicrobial activity could not.

**Discussion**

Pv, a major component of yolk proteins, is a maternally-transferred molecule abundant in fish eggs. The physiological role known for Pv so far is to provide necessary nutrients for early developing embryos and larvae. Here we clearly demonstrated that in *D. rerio*, both the embryo extract and the developing embryo displayed antimicrobial activity against microbes including *A. hydrophila* (pathogenic to *D. rerio*), and all the findings point to Pv being one of the most important factors involved in the antimicrobial activity. First, the antimicrobial activity of embryo extract was significantly decreased by the pre-incubation with anti-fish Pv antibody, a process that causes the precipitation of Pv. Second, the microinjection of anti-Pv antibody into the early developing embryos (which were then challenged by injection of live *A. hydrophila*) resulted in a significantly increased mortality, while the antibody-induced embryonic death rate was markedly reduced by the co-injection of purified rPv plus anti-Pv antibody. Third, rPv was capable of directly inhibiting the growth of *E. coli*, *A. hydrophila* and *S. aureus*. Fourth, injection of exogenous rPv or its truncated product Pt5 into the embryos markedly promoted their resistance to *A. hydrophila*, and this promoted bacterial resistance was significantly reduced by the co-injection of anti-Pv antibody plus rPv (or Pt5), but not by the injection of anti-actin antibody plus rPv. Fifth, like rPv and Pt5, the generated mutants with *in vitro* antimicrobial activity, when injected into the embryos, were able to protect the developing embryos against *A. hydrophila* challenge, but those without *in vitro* antimicrobial activity could not. Finally, the early embryos contained sufficient Pv, which is high enough to defend against potential pathogens *in vivo*. Taken together, these indicate that Pv, in addition to being a simple nutritional reserve, also has an antimicrobial activity, a novel function assigned to Pv. This nature of Pv possibly has an important physiological significance. Fish embryos develop externally in most cases and are exposed to an aquatic environment full of potential pathogens, but they have little or only limited ability to mount an efficient and protective response. How they survive the pathogenic attacks in such a hostile environment is poorly understood. Our results suggest it is highly likely that Pv may be physiologically involved in the antimicrobial defense of zebrafish early embryos.

Non-self recognition is a pivotal process in innate immune response. Recognition of non-self recognition in innate immunity is mediated by a set of host’s molecules known as pattern recognition receptors (PRRs) that recognize the microbial cell wall constituents called pathogen-associated molecular patterns (PAMPs) present on the surface of microbes but absent in the host, such as LPS, LTA and PGN. We showed that zebrafish rPv had a strong affinity to LPS from Gram-negative bacteria, LTA from Gram-positive bacteria and PGN from both
Gram-positive and negative bacteria. In agreement, rPv also bound to Gram-negative bacteria *E. coli* and *A. hydrophila* as well as Gram-positive bacterium *S. aureus*. Recently, significant advances have been made in identifying non-self recognition molecules, and few of them have been tested for the existence of multiple specificities recognizing PAMPs (50). It is clear that Pv is a pattern recognition receptor with a wide spectrum of specificity capable of identifying the microbial signature molecules LPS, LTA and PGN.

An important part of innate immunity is that a group of proteins have microbicidal activity in addition to their immune recognition function. These proteins all play essential roles in the host non-specific defenses by preventing or limiting infections via their ability to recognize potential pathogens; most of these proteins also exert their antimicrobial effects by interacting with and destabilizing either the microbial plasma membrane or cell wall, eventually leading to cell death (51). In this study, we found that rPv was able to cause direct damage to *E. coli* and *S. aureus*, as evidenced by SEM, suggesting that rPv can kill both the Gram-negative bacterium *E. coli* and the Gram-positive bacterium *S. aureus*. It is obvious that in addition to being a pattern recognition receptor, Pv is also an effector (microbicidal) molecule capable of killing microbes. Since microinjection of exogenous rPv into early embryos promoted their resistance to pathogenic *A. hydrophila* challenge, and this promoted bacterial resistant activity was markedly reduced by the co-injection of anti-Pv antibody plus rPv, we thus propose that Pv may also defend early developing embryos *in vivo* against pathogenic attacks by the same mechanisms via binding to and disrupting invading pathogens. However, this demands further study at host-pathogen interaction level.

Most antimicrobial proteins (AMPs) are cationic and amphipathic molecules with a net positive charge ranging from +2 to +9 and high percentage of hydrophobic residues (47). Zebrafish Pv had a net positive charge +2.2 (pH7.0) and 78 hydrophobic residues (~32%), conforming to the characteristics of AMPs. To gain insight into the structure-activity relationship, N-terminal and C-terminal deletion was carried out. Deletion of the N-terminal 194 residues did not impair the antimicrobial activity of the resultant peptide (Pt5), but truncation of the C-terminal 55 residues resulted in almost complete loss of the antimicrobial activity of the resultant peptide (Pt6). In addition, the microinjection of Pt5 into the embryos can enhance their resistance to pathogenic *A. hydrophila*, but the injection of Pt6 cannot. These suggest that the C-terminal 55 residues are the “core” structure contributing to antimicrobial activity of rPv. This is also corroborated by the facts that Pt5 with antimicrobial activity had a net positive charge +4.4 (pH7.0), while Pt6 without antimicrobial activity possessed a net negative charge -2.3 (pH7.0).

Site-directed mutagenesis of Pt5 showed that the single mutation R242G (m12) and the double mutation A201G/I203M (m13) resulted in a marked decrease in its antimicrobial activity against *E. coli*, *A. hydrophila* and *S. aureus* as well as its affinity to LPS, LTA and PGN, but the replacement of the other positively charged and hydrophobic residues such as I203M (m1; hydrophobic I to hydrophilic M), R214G (m5; positively charged R to negatively charged G) and E235K (m9;
negatively charged E to positively charged K) caused no changes in their antimicrobial activity. These were also supported by in vivo studies showing that a markedly greater number of the embryos injected with m1, m5 or m9 survived the challenge with live A. hydrophila than the embryos injected with m12 or m13. It is clear that the positively charged residue R242 and the hydrophobic residues A201 and I203 are the functional determinants critical for Pt5, but the other positively charged and hydrophobic residues mutated are not. This suggests that the distribution of positively charged and hydrophobic residues rather than net positive charges and hydrophobicity could be the major factors of Pt5 (also rPv) to mediate specific electrostatic surface and amphipathicity, consistent with the mechanism for specific high-affinity binding between proteins proposed by Sinha and Smith-Gill (52). Of note, the antimicrobial activity of Pt5 (also rPv) against E. coli, A. hydrophila and S. aureus was correlated with its affinity to LPS, LTA and PGN; and consistent with the loss of antimicrobial activity of the mutants m12 and m13, their affinity to LPS, LTA and PGN was also lost. The correlation between antimicrobial activity and ligand binding suggests that the functional sites R248 and A201/I203 may be simultaneously involved in multiple activities including binding to microbial signature molecule LPS, LTA and PGN and destabilizing/disrupting microbial cell membranes.

In summary, this study first demonstrates that Pv is associated with the immune defense of zebrafish developing embryos and then elucidates that Pv is a multivalent pattern recognition receptor capable of identifying LPS, LTA and PGN as well as an antimicrobial effector capable of damaging Gram-negative and positive microbes. It also reveals that the C-terminal 55 residues with the functional sites R242 and A201/I203 are critical for Pv antimicrobial activity. As the deposit of Pv in eggs is widespread in oviparous animals, the Pvm-mediated immune defense may widely present in in the early embryos of different species. This work also opens a new avenue for the study of the immunological roles of yolk proteins in animals that rely on yolk proteins for embryonic development.

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**Figure Legends**

**Fig. 1** Antimicrobial activities of Pv in vitro and in vivo. (A) Antimicrobial activity of the embryo extract against E. coli; (B) Antimicrobial activity of the embryo extract against A. hydrophila; (C) Antimicrobial activity of the embryo extract against S. aureus; (D) Effects of microinjected recombinant Pv, Pt5 and Pt6 as well as anti-fish Pv antibody on the antimicrobial activity of the embryos. The early (8-cell stage) embryos were first microinjected, and then challenged by injection of live A. hydrophila 1 h later. The mortality was recorded and the cumulative mortality calculated at 24 h following the bacterial injection. About 95% of the non-bacterial challenge control embryos developed normally. The challenge with live A. hydrophila resulted in a significant increase in the mortality of the embryos microinjected with anti-Pv antibody, but not the embryos injected with anti-actin antibody or BSA or PBS. Microinjection of rPv or Pt5 into the embryos was able to increase their resistance to A. hydrophila, while the injection of Pt6 into the embryos was not. Co-injection of anti-Pv antibody together with rPv or Pt5 was able to counetact their activity to enhance the embryonic resistance to A. hydrophila. All data were expressed as mean values ± SEM (n=3). The bars represent the standard error of mean values. The symbol * means a significant difference (p<0.05). NE, embryo extract; HE, heated embryo extract; PvAb, anti-fish Pv antibody; AcAb, anti-actin antibody; rPv, recombinant phosvitin; BSA, bovine serum albumin; Pt1, Pt2, Pt3, Pt4, Pt5 and Pt6 are the 6 truncated products of Pv (see Fig. 6).

**Fig. 2** PCR analysis of A. hydrophila 16S rRNA gene. The 8-cell stage embryos were microinjected with live A. hydrophila, and 5 embryos were collected each time at 0, 6 and 12 h post the bacterial injection. Total DNAs were isolated from each embryo and used as template to amplify the specific region of A. hydrophila 16S rRNA gene. All the PCR products were electrophoresed in 1% agarose and the bands were recorded using the gel imaging system. (A) Embryos injected with A. hydrophila only. (B) Embryos injected with anti-fish Pv antibody, followed by injection with A. hydrophila. (C) Embryos injected with anti-actin antibody, followed by injection with A. hydrophila. M: Marker; C: Control.

**Fig. 3** Micrographs showing E. coli and S. aureus incubated with TB (A and C) or with recombinant Pv (B and D) at 25°C for 1 h. Scale bars represent 1 μm.

**Fig. 4** Binding of FITC-labeled recombinant Pv to microbial cells. (A, D and G) Binding of FITC-labeled recombinant Pv to E. coli, A. hydrophila and S. aureus. (B, E and H) Binding of FITC-labeled rPv to E. coli, A. hydrophila and S. aureus was weakened in the presence of non-labeled rPv. (C, F and I) No binding of FITC-labeled BSA to E. coli, A. hydrophila and S. aureus. Inserts show the corresponding bright-field images. Scale bars represent 20 μm.

**Fig. 5** Interaction of recombinant Pv with various ligands. (A, B and C) Binding of recombinant Pv to various ligands. LPS, LTA and PGN dissolved in re-distilled water was applied to wells of a 96-well microplate and air-dried overnight at room temperature, followed by ELISA. BSA instead of recombinant Pv was used as control. (D, E and F) Saturation curve of the binding of rPv to LPS, LTA and PGN. rPv dissolved in re-distilled water was applied to wells of a 96-well microplate and air-dried overnight at room
temperature, followed by Scatchard analysis. BSA was treated at the same concentrations as control. Data were expressed as mean values ± SEM (n=3).

**Fig. 6** Antimicrobial activity of truncated Pv peptides and their affinities to various ligands. (A) A diagram showing Pv truncation. (B) Antimicrobial activity against *E. coli*. (C) Antimicrobial activity against *A. hydrophila*. (D) Antimicrobial activity against *S. aureus*. (E) Saturation curve of the binding of P5 to LPS, LTA and PGN. (F) Saturation curve of the binding of P6 to LPS, LTA and PGN. BSA instead of recombinant Pv was used as control. The numbers in (A) indicated the positions at which Pv was truncated. Pt1 (55-249), Pt2 (102-249), Pt3 (134-249), Pt4 (174-249), Pt5 (194-249) and Pt6 (1-194) represented the truncated peptides. Data were expressed as mean values ± SEM (n=3). The bars represent the standard error of mean values. The symbol * means a significant difference (p<0.05).

**Fig. 7** Antimicrobial activity of Pt5 mutants. (A) A diagram showing the site-directed mutagenesis. (B) SDS-PAGE of the mutants, showing that they all have a similar molecular mass. (C) Antimicrobial activity against *E. coli*. (D) Antimicrobial activity against *A. hydrophila*. (E) Antimicrobial activity against *S. aureus*. BSA was as control. (F) Effects of microinjected m1, m5, m9, m12 or m13 as well as anti-fish Pv antibody on the antimicrobial activity of the embryos. The early (8-cell stage) embryos were first microinjected, and then challenged by injection of live *A. hydrophila* 1 h later. The mortality was recorded and the cumulative mortality calculated at 24 h following the bacterial injection. About 95% of the non-bacterial challenge control embryos developed normally. The challenge with live *A. hydrophila* resulted in a significant increase in the mortality of the embryos microinjected with anti-Pv antibody, but not the embryos injected with PBS. Microinjection of m1, m5 or m9 into the embryos was able to increase their resistance to *A. hydrophila*, while the injection of m12 or m13 into the embryos was not. Co-injection of anti-Pv antibody with m1, m5 or m9 was able to counteract their activity to enhance the embryonic resistance to *A. hydrophila*. Data were expressed as mean values ± SEM (n=3). The bars represent the standard error of mean values. The symbol * means a significant difference (p<0.05). PvAb, anti-fish Pv antibody; AcAb, anti-actin antibody.
Table 1. Primers used for site-directed mutagenesis.

| Mutation | Sequence (5' to 3') |
|----------|---------------------|
| I203M | sense: GACTGCCACCAGTAGAGCCTT anti-sense: TTAGACATAGGAGCCTGGACATAG |
| K309E | sense: CAGGAAATCCACAAAGATCGGT anti-sense: AAAGGCTCAATGTGTTGCGACATG |
| H211L | sense: CTCAAAGATCGGTACTTGGGCACAC anti-sense: GAATTTCCTGAAAGGCTCAATGATGG |
| K212E | sense: CACGAGATCGGTACTTGGGCACAC anti-sense: GAATTTCCTGAAAGGCTCAATGATGG |
| R214G | sense: CACAAAGATGGTACTTGGGCACAC anti-sense: GAATTTCCTGAAAGGCTCAATGATGG |
| Y225F | sense: GATCGGTACTTGGGCACACCTAG anti-sense: TTTGTGGAATTTCCCTGAAAGGCT |
| F234S | sense: GCAGCTAGCTCTGAACAAATGCA anti-sense: AGCAGCTTCCACTGCTAGTATCCT |
| F234L | sense: GCAGCTAGCTGGAAACAAATGCA anti-sense: AGCAGCTTCCACTGCTAGTATCCT |
| E235K | sense: GCAGCTAGCTTTAAACCAATGCA anti-sense: AGCAGCTTCCACTGCTAGTATCCT |
| K239E | sense: GAACAAATGCAGAACAGAATAGA anti-sense: AAAGCTAGCTGAGCAGCTTCCACT |
| Q240H | sense: AACATTAGATTCTTGGAAATG anti-sense: TCTGCATTGTTCAAGGCTAGCTG |
| R242G | sense: AACAGAATGGATCTTGGAAATG anti-sense: TCTGCATTGTTCAAGGCTAGCTG |
| A201G, I203M | sense: GACTGCCACCAGTAGAGCCTT anti-sense: TTAGACATAGGAGCCTGGACATAG |

The mutations are marked in bold and underlined.
### Table 2 Antimicrobial activity of recombinant Pv against *E. coli*, *S. aureus* and *A. hydrophila*

| Bacteria     | rPv (µM) | Colony counts (cfu) | Inhibition (%) | IC₅₀ (µM) |
|--------------|----------|---------------------|----------------|----------|
| *E. coli*    | 0 (control) | 307.3±21.1          | —              | 3.1      |
|              | 1.6      | 244.0±13.3          | 20±3           |          |
|              | 3.2      | 125.3±31.3          | 59±6           |          |
|              | 8.0      | 6.3±1.8             | 98±2           |          |
| *A. hydrophila* | 0 (control) | 298.7±16.6          | —              | 3.0      |
|              | 1.6      | 236.7±9.7           | 21±2           |          |
|              | 3.2      | 119.3±13.3          | 60±3           |          |
|              | 8.0      | 12.0±1.6            | 96±3           |          |
| *S. aureus*  | 0 (control) | 313.3±35.3          | —              | 3.0      |
|              | 1.6      | 260.0±26.6          | 18±5           |          |
|              | 3.2      | 132.7±20.6          | 57±4           |          |
|              | 8.0      | 9.0±1.0             | 97±3           |          |
Table 3 The $B_{\text{max}}$ and $K_d$ values of the binding of m1, m3, m5, m7, m9, m12 and m13 to LPS, LTA and PGN, respectively.

| Mutants | Binding to LPS | Binding to LTA | Binding to PGN |
|---------|----------------|----------------|---------------|
|         | $K_d$ (\(\mu g/ml\)) | $B_{\text{max}}$ (ng/pmol) | $K_d$ (\(\mu g/ml\)) | $B_{\text{max}}$ (ng/pmol) | $K_d$ (\(\mu g/ml\)) | $B_{\text{max}}$ (ng/pmol) |
| m1      | 1.1            | 5.9            | 1.3            | 6.0            | 1.5            | 6.3            |
| m3      | 1.1            | 6.0            | 1.3            | 6.1            | 1.6            | 6.3            |
| m5      | 1.1            | 5.8            | 1.3            | 5.9            | 1.6            | 6.1            |
| m7      | 1.2            | 5.8            | 1.2            | 5.8            | 1.6            | 6.1            |
| m9      | 1.2            | 5.7            | 1.3            | 5.9            | 1.7            | 6.1            |
| m12     | 2.3            | 2.4            | 2.2            | 2.5            | 2.7            | 2.4            |
| m13     | 2.8            | 2.1            | 2.6            | 2.1            | 3.0            | 2.2            |
Figure 1

A Antimicrobial activity against *E. coli*
B Antimicrobial activity against *A. hydrophila*
C Antimicrobial activity against *S. aureus*

D

Mortality (%)

No bacterial challenge  Challenged with *A. hydrophila*
Figure 2

Figure 3
Figure 4

| FITC-labeled rPv | FITC-labeled rPv + non-labeled rPv | FITC-labeled BSA |
|-------------------|-----------------------------------|-----------------|
| ![Image A]        | ![Image B]                        | ![Image C]      |
| ![Image D]        | ![Image E]                        | ![Image F]      |
| ![Image G]        | ![Image H]                        | ![Image I]      |

**Legend**
- **A**, **D**, **G**: FITC-labeled rPv
- **B**, **E**, **H**: FITC-labeled rPv + non-labeled rPv
- **C**, **F**, **I**: FITC-labeled BSA

**Bacteria**
- **E. coli**
- **A. hydrophila**
- **S. aureus**
Figure 5

(A) Binding to LPS

(B) Binding to LTA

(C) Binding to PGN

(D) Specific binding (ng/μmol) to LPS

(E) Specific binding (ng/μmol) to LTA

(F) Specific binding (ng/μmol) to PGN

Scatchard analysis

- rPV
- BSA

Bmax and Kd values:

- LPS: Bmax=7.7, Kd=1.0
- LTA: Bmax=7.1, Kd=1.3
- PGN: Bmax=8.7, Kd=1.5
Phosvitin plays a critical role in the immunity of zebrafish embryos via acting as a pattern recognition receptor and an antimicrobial effector

Shaohui Wang, Yuan Wang, Jie Ma, Yunchao Ding and Shicui Zhang

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