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Zebrafish Peptidoglycan Recognition Proteins Are Bactericidal Amidases Essential for Defense against Bacterial Infections

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SUMMARY

Peptidoglycan recognition proteins (PGRPs) are structurally conserved through evolution, but their functions in innate immunity are different in invertebrates and vertebrates. We asked what the functions of PGRPs in fish are and whether they are indispensable for defense against infection because fish are the first vertebrates that developed adaptive immunity, but they still rely solely on innate immunity during early development of embryos. We identified and cloned three zebrafish PGRPs and showed that they are highly expressed in eggs, developing embryos, and adult tissues that contact external environment. Zebrafish PGRPs have both peptidoglycan-lytic amidase activity and broad-spectrum bactericidal activity, which is a unique feature. Furthermore, we demonstrated that in the developing zebrafish embryo, one of these PGRPs is essential for defense and survival during bacterial infections. These data demonstrate an absolute requirement for innate immunity in defense against infections in fish embryos and for a PGRP protein for survival in vertebrates.

INTRODUCTION

Innate immunity arose in the early multicellular organisms and has remained an essential component of defense mechanisms in all metazoans. Although all vertebrates developed acquired immunity, early vertebrates' eggs and embryos, which are laid and develop in water, must solely rely on innate immunity in defense against infections until their acquired immune system develops.

Peptidoglycan recognition proteins (PGRPs or PGLYRPs) is a family of innate immunity molecules that were first identified in insects (Yoshida et al., 1996; Kang et al., 1998) and then in mammals (Kang et al., 1998; Liu et al., 2001), other vertebrates, mollusks, and echinoderms (Dziarski and Gupta, 2006b). The invertebrate and vertebrate PGRP proteins are highly conserved in their structure and recognize bacteria through their cell wall component, peptidoglycan.

Peptidoglycan is a major component of bacterial cell wall and is a polymer of β(1-4)-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). MurNAc has short peptides, typically containing four alternating L and D amino acids. The third amino acid in Gram-negative bacteria and Gram-positive bacilli is *m*-diaminopimelic acid (DAP), and in most other Gram-positive bacteria (including Gram-positive cocci), it is L-lysine (Lys) (Dziarski and Gupta, 2006a; Dziarski and Gupta, 2006b).

Despite the structural similarities, invertebrate and vertebrate PGRPs have acquired different functions. Insects have many PGRPs that function in cell activation, phagocytosis, or hydrolysis of peptidoglycan (Werner et al., 2000). The cell activating PGRPs stimulate either the Toll (*Drosophila* PGRP-SA, PGRP-SD, and PGRP-SC1) (Michel et al., 2001; Bischoff et al., 2004; Garver et al., 2006) or the Imd (*Drosophila* PGRP-LC) (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002) signal transduction pathways, which induce transcription of antimicrobial peptides active against Gram-positive bacteria and fungi (Lemaitre et al., 1996) or Gram-negative bacteria (Lemaitre et al., 1995), respectively. The Toll and Imd pathways are preferentially triggered by either Lys-type or DAP-type peptidoglycan, respectively (Leulier et al., 2003; Kaneko and Silverman, 2005). Other PGRPs, such as silkworm PGRP-S, activate the prophenol-oxidase cascade, which also generates antimicrobial products (Yoshida et al., 1996; Park et al., 2006). Finally, some PGRPs, such as *Drosophila* PGRP-SC1, PGRP-LB, and PGRP-SB1, are *N*-acetylmuramoyl-L-alanine amidases (Kim et al., 2003; Mellroth et al., 2003; Mellroth and Steiner, 2006), which hydrolyze the lactyl-amide bond between MurNAc and L-Ala. The function of these insect amidases is to prevent excessive activation of the immune system by bacteria (Mellroth et al., 2003; Zaidman-Remy et al., 2006; Bischoff et al., 2006).

Mammals have a family of four PGRPs: PGLYRP-1, PGLYRP-2, PGLYRP-3, and PGLYRP-4 (Liu et al., 2001;

Dziarski and Gupta, 2006b). These proteins were initially hypothesized to function as pattern-recognition receptors similar to insect PGRPs (Liu et al., 2001). However, we have demonstrated that human PGRPs have two major functions: amidase and antibacterial activities. PGLYRP-2, is an *N*-acetylmuramoyl-L-alanine amidase and is constitutively expressed in the liver and secreted into the bloodstream (Gelius et al., 2003; Wang et al., 2003; Zhang et al., 2005). Its expression is also induced in keratinocytes upon bacterial stimulation (Wang et al., 2005; Li et al., 2006). PGLYRP-1 is present in the granules of polymorphonuclear leukocytes (Tydell et al., 2002; Dziarski et al., 2003). PGLYRP-3 and PGLYRP-4 are expressed in tissues that come in contact with the environment (Lu et al., 2006). PGLYRP-1, PGLYRP-3, and PGLYRP-4 are bactericidal and kill a wide range of Gram-positive and Gram-negative bacteria (Lu et al., 2006; Wang et al. 2007). These PGLYRPs do not have amidase activity, but their bactericidal activity relies on targeting bacterial cell wall and does not involve peptidoglycan hydrolysis or permeabilization of the cytoplasmic membrane (Lu et al., 2006; Wang et al., 2007).

Insect PGRPs are not known to be bactericidal, except for *Drosophila* PGRP-SB1, which has both *N*-acetylmuramoyl-L-alanine amidase and bactericidal activities (Mellroth and Steiner, 2006). However, PGRP-SB1 is bactericidal for only one bacterial species, *Bacillus megaterium*, which is likely due to its amidase activity (Mellroth and Steiner, 2006). By contrast, mammalian PGLYRPs have broad-spectrum bactericidal activity that does not rely on amidase activity (Tydell et al., 2002; Lu et al., 2006; Wang et al., 2007).

To gain insight into the evolution of the function of PGLYRPs in innate immunity, we have identified a family of PGLYRPs in an earlier vertebrate, the zebrafish, and we studied their functions and role in defense against bacterial infections. Zebrafish and other teleosts have a well-developed immune system that is similar to the mammalian immune system and that, unlike invertebrates, includes both innate and adaptive immunity (Yoder et al., 2002; Lam et al., 2004; Trede et al., 2004). However, fish were the first vertebrates to develop adaptive immunity, and it is not known whether their innate immune system has greater similarities to the invertebrate or to the higher vertebrate systems. Zebrafish, thus, serves as an important model for the evolutionary analysis of immune mechanisms and the role of innate immunity in lower vertebrates (Trede et al., 2001; Yoder et al., 2002; Lam et al., 2004; DeVries et al., 2006; Trede et al., 2004). We show here that innate immunity and PGLYRPs in particular are required for defense against bacterial infections in developing fish embryos.

RESULTS

Cloning and Sequence Analysis of Zebrafish PGLYRPs

In order to identify *pglyrp* genes in the zebrafish, we searched GenBank databases for genes homologous to human PGLYRPs. We identified four *pglyrp* genes in the zebrafish genome (approximately 70% of the genome is sequenced). We have cloned three of these genes and have named them *pglyrp-2*, *pglyrp-5*, and *pglyrp-6*. Zebrafish PGLYRP-2 was designated "2" because it had the highest homology to mammalian PGLYRP-2 compared to other zebrafish PGLYRPs. The other two zebrafish PGLYRPs were not considered orthologs of the remaining three mammalian PGLYRPs because unlike these mammalian PGLYRPs, they are predicted amidases, they are larger than PGLYRP-1, and they do not have two PGRP domains, which are present in mammalian PGLYRP-3 and PGLYRP-4. Thus, we named them PGLYRP-5 and PGLYRP-6. We were unable to clone the full-length cDNA for the fourth zebrafish PGLYRP that was identified from an EST clone. The genome organization (Figure S1 in the Supplemental Data available online) and sequence analysis (Figure S2) of zebrafish PGLYRPs is presented in the Supplemental Results.

Zebrafish PGLYRP proteins have no predicted signal peptides. However, our immunohistochemistry data demonstrate that all three proteins were present in the lumen of blood vessels; this indicates that they are secreted. Zebrafish PGLYRPs were not glycosylated (data not shown), unlike human PGLYRPs. Zebrafish PGLYRPs, similar to most vertebrate and invertebrate PGRPs, all have one C-terminal PGRP domain, which have 43%, 36%, and 42% conserved identities and 59%, 54%, and 57% conserved similarities, respectively, with PGRP domains in human PGLYRPs (Figure S2). All three zebrafish PGLYRPs are predicted to have amidase activity because they all have the conserved four Zn^{2+}-binding amino acids, corresponding to His411, Tyr447, His522, and Cys530 in human PGLYRP-2, which is an amidase (Wang et al., 2003). These four amino acids are conserved in all amidase-active PGRPs and in bacteriophage and bacterial type-2 amidases, such as bacteriophage T7 lysozyme. By contrast, human PGLYRP-1, PGLYRP-3, and PGLYRP-4, which are not amidases, do not have the last Cys conserved (corresponding to Cys530 in human PGLYRP-2).

Thus, we have identified four *pglyrp* genes in zebrafish, and we have cloned full-length cDNA for three of these genes. Zebrafish PGLYRP-2, PGLYRP-5, and PGLYRP-6 each have one C-terminal PGRP domain that is conserved through evolution, and these proteins are predicted to be amidases.

Differential Expression of Zebrafish PGLYR-2, PGLYR-5, and PGLYR-6

We performed both RNA and protein analysis to identify tissues that express the different PGLYRP proteins. Total RNA isolated from different tissues from adult zebrafish was analyzed by RNA blots. Both PGLYRP-2 and PGLYRP-5 mRNA were strongly expressed in the egg and had lower expression in the liver (Figure 1). PGLYRP-5 mRNA was also expressed in the intestine and gill (Figure 1). In contrast, PGLYRP-6 was strongly expressed in the intestine and weakly expressed in the liver (Figure 1).

In situ hybridization of whole fish sections with RNA probes for the three PGLYRPs demonstrated a similar

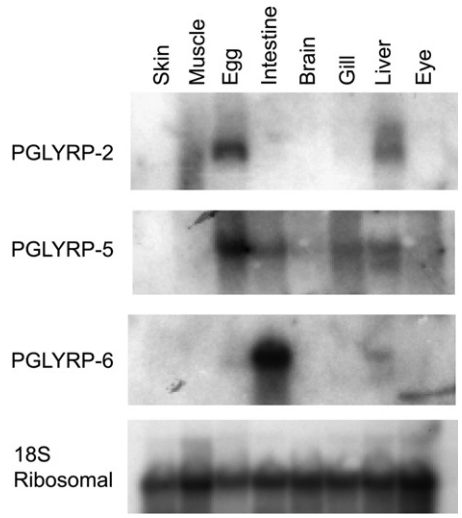

Figure 1. Zebrafish PGLYRP-2, PGLYRP-5, and PGLYRP-6 mRNAs Are Differentially Expressed in Various Tissues

RNA blots with RNA isolated from different adult zebrafish tissues were hybridized with the indicated probes and exposed to X-ray film. Hybridization with the 18S ribosomal probe demonstrates equal loading of the RNA samples. The results are from one of three similar experiments.

pattern of differential expression. PGLYRP-2 and PGLYRP-5 transcripts were strongly expressed in the developing oocytes (stages II, III, and IV shown for PGLYRP-2, Figure 2A; stage V shown for PGLYRP-5, Figure 2E). PGLYRP-2 and PGLYRP-5 mRNAs were also expressed in hepatocytes in the liver (Figures 2C and 2G). PGLYRP-6 was strongly expressed in the absorptive cells of the intestine with no expression in the goblet cells (Figure 2I). The staining was specific for all PGLYRPs as only the antisense probes (Figures 2A, 2C, 2E, 2G, and 2I) and not the sense probes (Figures 2B, 2D, 2F, 2H, and 2J) hybridized.

We next identified tissues that express PGLYRP proteins in sections of whole adult fish by using antibodies specific to each of the zebrafish proteins. PGLYRP-2, PGLYRP-5, and PGLYRP-6 proteins were expressed in hepatocytes and in the lumen of sinusoids in the liver (Figures 3A–3C). PGLYRP-2 and PGLYRP-5 proteins were highly expressed in the exocrine acinar cells of the pancreas, whereas PGLYRP-6 had lower expression (Figures 3E–3G). In contrast, PGLYRP-6 was highly expressed in the absorptive cells of the intestine, whereas PGLYRP-2 and PGLYRP-5 had lower expression (Figures 3I–3K). There was no expression of either PGLYRP proteins in the goblet cells. PGLYRP-2 was strongly expressed in stages III to V of the developing oocyte (Figure 3M), whereas PGLYRP-5 had somewhat lower expression (Figure 3N) and PGLYRP-6 had the lowest expression (Figure 3O). The three PGLYRPs were detected in the gills and were present in a population of epithelial cells lining the gill lamella and in the vascular lumen of the gills (Figures 3Q–3S). PGLYRP-2, PGLYRP-5, and PGLYRP-6 were expressed in the alarm cells but not in the mucous cells and not in the taste buds of the integumentary

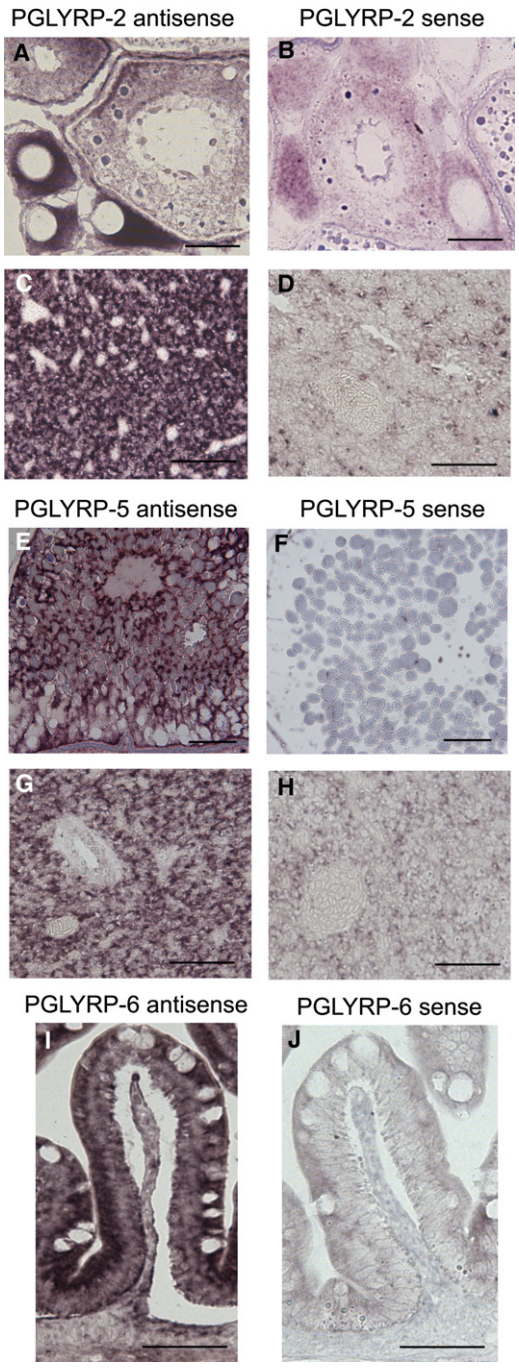

Figure 2. PGLYRP-2, PGLYRP-5, and PGLYRP-6 Transcripts Are Differentially Expressed in Developing Oocytes, Liver, and Intestine of Adult Zebrafish

Sections of zebrafish developing oocytes (A and B) and liver (C and D) were hybridized with PGLYRP-2 antisense (A and C) or sense (B and D) RNA probes. Sections of developing oocytes (E and F) and liver (G and H) were hybridized with PGLYRP-5 antisense (E and G) or sense (F and H) probes. Sections of intestine (I and J) were hybridized with PGLYRP-6 antisense (I) or sense (J) probes. Scale bars represent 50 μm. The results are from one of three similar experiments.

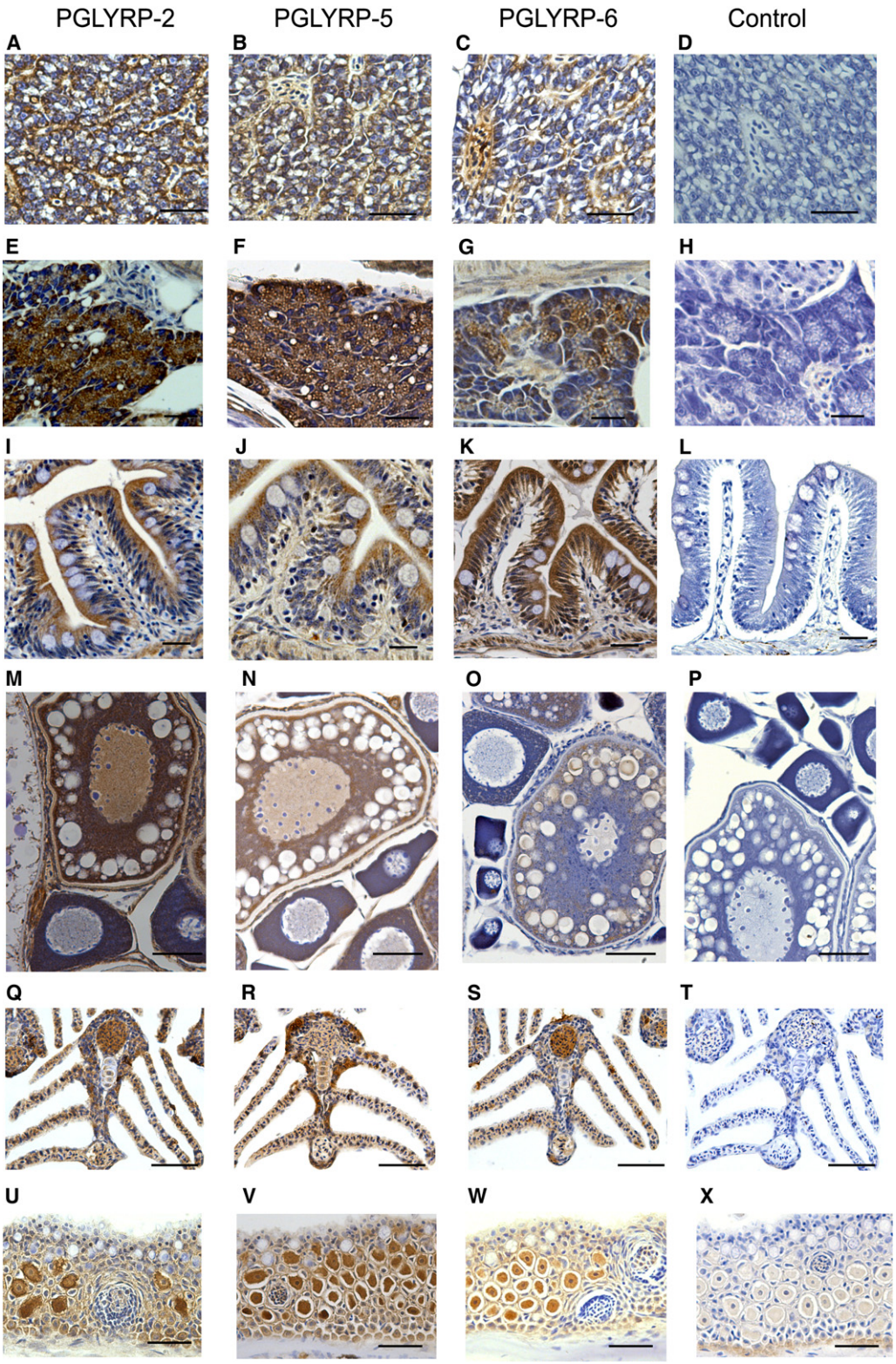

Figure 3. Zebrafish PGLYRP-2, PGLYRP-5, and PGLYRP-6 Proteins Are Expressed in the Liver, Pancreas, Intestine, Developing Oocytes, Gills, and Skin of Adult Zebrafish

Sections of zebrafish liver (A–D), pancreas (E–H), intestine (I–L), developing oocytes (M–P), gills (Q–T), and skin (U–X) were reacted with PGLYRP-2 (A, E, I, M, Q, and U), PGLYRP-5 (B, F, J, N, R, and V), PGLYRP-6 (C, G, K, O, S, and W), or control (D, H, L, P, T, and X) antibody, stained with peroxidase, and counterstained with hematoxylin. Scale bars for (A)–(L) represent 25 μm, and scale bars for (M)–(X) represent 50 μm. The results are from one of three similar experiments.

system (Figures 3U–3W). PGLYRP-2, PGLYRP-5, and PGLYRP-6 were also expressed in the epithelial cells of the kidney tubules (data not shown). There was no staining with the control antibody (Figures 3D, 3H, 3L, 3P, 3T, and 3X), indicating specific detection of PGLYRP proteins in these tissues.

These data demonstrate that zebrafish PGLYRPs are selectively expressed in a wide range of tissues including those, such as gills, skin, and eggs, that are exposed to bacteria in the surrounding water and are also expressed in tissues, such as liver, that would be exposed to bacteria during a systemic infection.

Amidase Activity of Zebrafish PGLYRP-2, PGLYRP-5, and PGLYRP-6

On the basis of the presence of all four conserved amino acids needed for the amidase activity of insect and mammalian PGLYRPs and bacteriophage type-2 amidases (Figure S2), we hypothesized that zebrafish PGLYRPs also have amidase activity for bacterial peptidoglycan. To test this hypothesis, we determined whether purified zebrafish PGLYRP proteins hydrolyze peptidoglycan.

We first used soluble polymeric *Staphylococcus aureus* peptidoglycan labeled with biotin on the N-terminal glycine in its peptide as a substrate. All three zebrafish PGLYRPs hydrolyzed *S. aureus* peptidoglycan (Figure 4A). Amidase-active PGRPs and other type-2 amidases hydrolyze the lactyl-amide bond between the MurNAc and L-Ala, which is the first amino acid in the stem peptide. To determine which bond in peptidoglycan is hydrolyzed by zebrafish PGLYRPs, we used synthetic peptidoglycan fragments (MurNAc-pentapeptides) and identified the digestion products by using mass spectrometry. Furthermore, on the basis of our sequence analysis, zebrafish PGLYRP-2 and PGLYRP-6 were predicted to be DAP specific (Figure S2). We tested whether zebrafish PGLYRPs hydrolyze both DAP- and Lys-containing muramyl peptides (Figure 4B). We also used a muramyl-peptide with N-acetylated Lys, MurNAc-L-Ala-D-isoGln-L-Lys(NHAc)-D-Ala-D-Ala (which mimics Gly that is often bound to the γ-amino group of Lys in Gram-positive peptidoglycans), to determine whether zebrafish PGLYRPs have a preference for hydrolyzing such a substituted peptide.

The results demonstrated that all three zebrafish PGLYRPs are *N*-acetylmuramoyl-L-alanine amidases (Figure 4C). PGLYRP-6 hydrolyzed both Lys- (nonacetylated and N-acetylated) and DAP-containing muropeptides equally well. PGLYRP-2 hydrolyzed DAP-containing and N-acetylated-Lys-containing muropeptides equally well, but it was less effective on nonacetylated Lys-containing muropeptide. PGLYRP-5 had lower activity than PGLYRP-2 and PGLYRP-6 and showed preference for DAP-containing muropeptide (Figure 4).

These data show that all three zebrafish PGRPs are type 2 amidases. The amidase activity is consistent with the earlier discussed prediction of amidase activity of all three zebrafish PGLYRPs on the basis of the presence of all four conserved Zn^{2+}-binding amino acids. Our results are consistent with the idea that peptidoglycan-binding groove specific for DAP-containing peptide can also accommodate Lys-containing peptide, which is less bulky, i.e., contains H found in Lys instead of COOH found in DAP (the only difference between Lys and DAP).

Bactericidal Activity of Zebrafish PGLYRP-2, PGLYRP-5, and PGLYRP-6

We have recently demonstrated that three human PGLYRPs (PGLYRP-1, PGLYRP-3, and PGLYRP-4) are bactericidal (Lu et al., 2006; Wang et al., 2007). Therefore, here we tested the hypothesis that zebrafish PGLYRPs may be also bactericidal. Moreover, because the antibacterial activity of amidase-active *Drosophila* PGRP-SB1 is limited to *B. megaterium* (Mellroth and Steiner, 2006), we tested whether zebrafish PGLYRPs have a broad or a narrow spectrum bactericidal activity.

All three zebrafish PGLYRPs were strongly bactericidal for Gram-positive bacteria: They reduced the numbers of *Bacillus subtilis*, *S. aureus*, and *Listeria monocytogenes* by 4–5 logs in 1, 2, or 4 hr, respectively (Figure 5A). These PGLYRPs also had similar bactericidal effect on Gram-negative bacteria, although the sensitivity of Gram-negative bacteria to various proteins somewhat varied: *Salmonella enterica* was highly sensitive to all three proteins, whereas *Escherichia coli*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* were less sensitive to PGLYRP-6, and *P. vulgaris* and *P. aeruginosa* were also less sensitive to PGLYRP-5 or PGLYRP-2, respectively (Figure 5A).

The LD_{99} (the concentration that kills 99% of bacteria) of PGLYRP-2, PGLYRP-5, and PGLYRP-6 for *B. subtilis* were 13 μg/ml (0.2 μM), 6 μg/ml (0.2 μM), and 1.3 μg/ml (0.02 μM), respectively; and for *S. enterica*, the LD_{99} were 15 μg/ml (0.2 μM), 33 μg/ml (1.0 μM), and 33 μg/ml (0.6 μM), respectively (Figure 5B). Thus, Gram-positive *B. subtilis* is most sensitive to PGLYRP-6, and Gram-negative *S. enterica* is most sensitive to PGLYRP-2.

Because the bactericidal activity of human PGLYRPs required Zn^{2+}, we then tested whether bactericidal activity of zebrafish PGLYRPs also required Zn^{2+}. All three zebrafish PGLYRPs, similar to human PGLYRPs, required 5 μM Zn^{2+} for their bactericidal activity. They were not bactericidal when purified and assayed without Zn^{2+}, and removal of Zn^{2+} with EGTA, which has a high affinity for Zn^{2+} (the log stability constant for Zn^{2+} is 12.9, compared to 11.0 for Ca^{2+}), abolished the bactericidal activities of all three PGLYRPs (Figure 5C).

These results demonstrate that zebrafish PGLYRPs, in addition to their amidase activity and unlike other vertebrate amidases, are also bactericidal. Zebrafish PGLYRPs, similar to human bactericidal PGLYRPs, have broad-spectrum Zn^{2+}-dependent bactericidal activity, and at least one zebrafish PGLYRP (PGLYRP-6) has greater bactericidal activity than human PGLYRPs.

PGLYRP-2 and PGLYRP-5 Are Expressed in the Developing Embryos, and PGLYRP-5 Is Required for In Vivo Protection against Infection

Lower vertebrates such as fish depend on innate immunity, particularly during the early stages of development

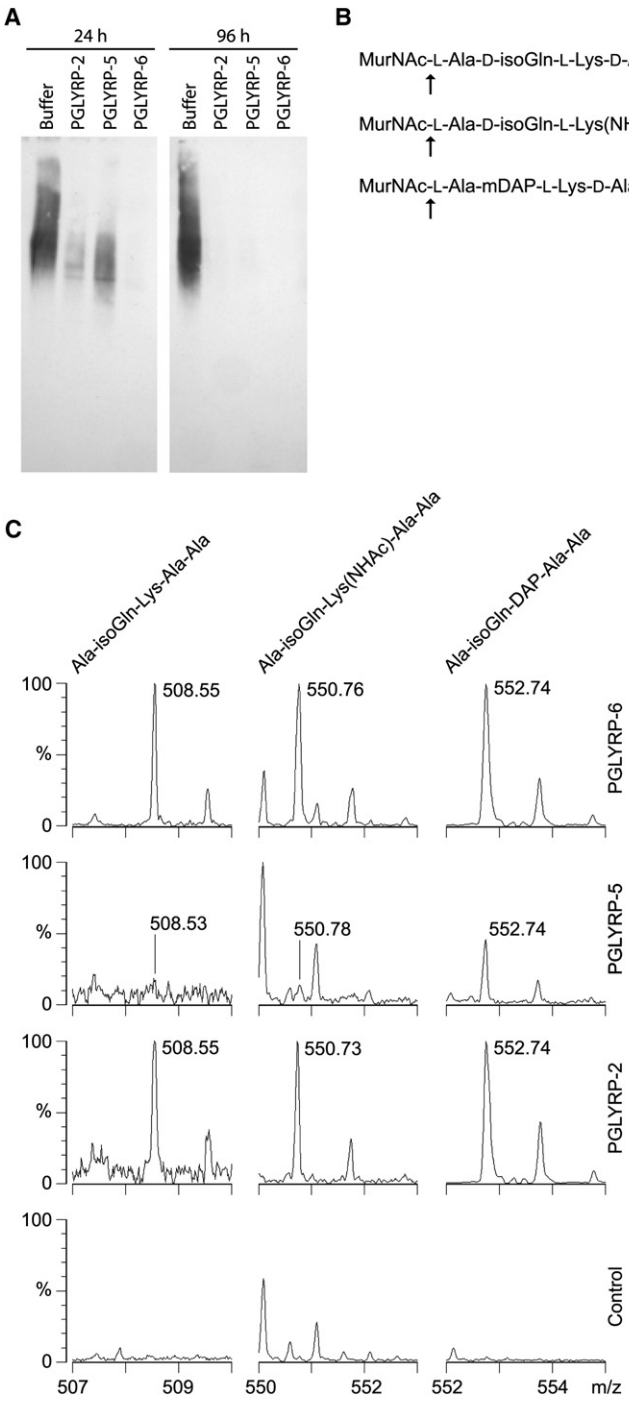

Figure 4. Zebrafish PGLYRP Proteins Are Enzymes with *N*-Acetylmuramoyl-L-Alanine Amidase Activity

(A) *S. aureus* soluble polymeric peptidoglycan, labeled with biotin on terminal Gly, was incubated in buffer alone or with the indicated PGLYRPs for 24 or 96 hr. High-molecular-weight polymeric biotin-peptidoglycan was detected on an immunoblot with streptavidin-peroxidase. Hydrolysis of the peptide from the glycan chain removes the biotin-labeled peptide. The results are from one of two similar experiments.

(B and C) The muropeptides indicated in (B) were incubated in buffer alone (control) or in the presence of the indicated PGLYRPs, and the hydrolysis products were identified with MALDI-TOF MS; peaks for the indicated peptides and observed masses ($[M + Na]^+$) are shown in (C). The vertical scale in the left-hand PGLYRP-5 and PGLYRP-2 panels was expanded so that minor peaks are revealed. The unlabeled peaks are either matrix or buffer adduct peaks that are the same in both digested and undigested samples or reflect natural isotopic distribution of the digestion products. K^+ adducts of the same peptides were also detected (not shown). The hydrolyzed bond is indicated in (B) by an arrow. Identical hydrolysis products were generated by incubation of the same substrates with human PGLYRP-2, a proven *N*-acetyl-muramoyl-L-alanine amidase (Wang et al., 2003), as a positive control (not shown). The results are from one of two similar experiments.

because adaptive immunity matures later. In order to identify the role of PGLYRPs in the embryo during bacterial infection, we first analyzed the expression of these proteins in zebrafish embryos at different stages of development. PGLYRP-2 protein is expressed in eggs and in embryos (Figure 6A), whereas PGLYRP-5 protein is not expressed in the eggs, is weakly expressed in the 24 hr postfertilization (hpf) embryo, and is strongly expressed in the 72 hpf embryo (Figure 6B). PGLYRP-6 protein could not be detected in the eggs or in the early stages of development.

We next determined the role of PGLYRP proteins in resistance to bacterial infection in the developing embryo. We initially selected PGLYRP-2 and PGLYRP-5 for these experiments because both proteins are expressed in the developing embryos (Figures 6A and 6B). Morpholinos that bind mRNA and block translation were designed for both PGLYRP-2 and PGLYRP-5. The morpholino designed for blocking PGLYRP-5 specifically inhibited translation of this protein, and there was no inhibition with the control morpholino (Figure 6C). PGLYRP-2 protein,

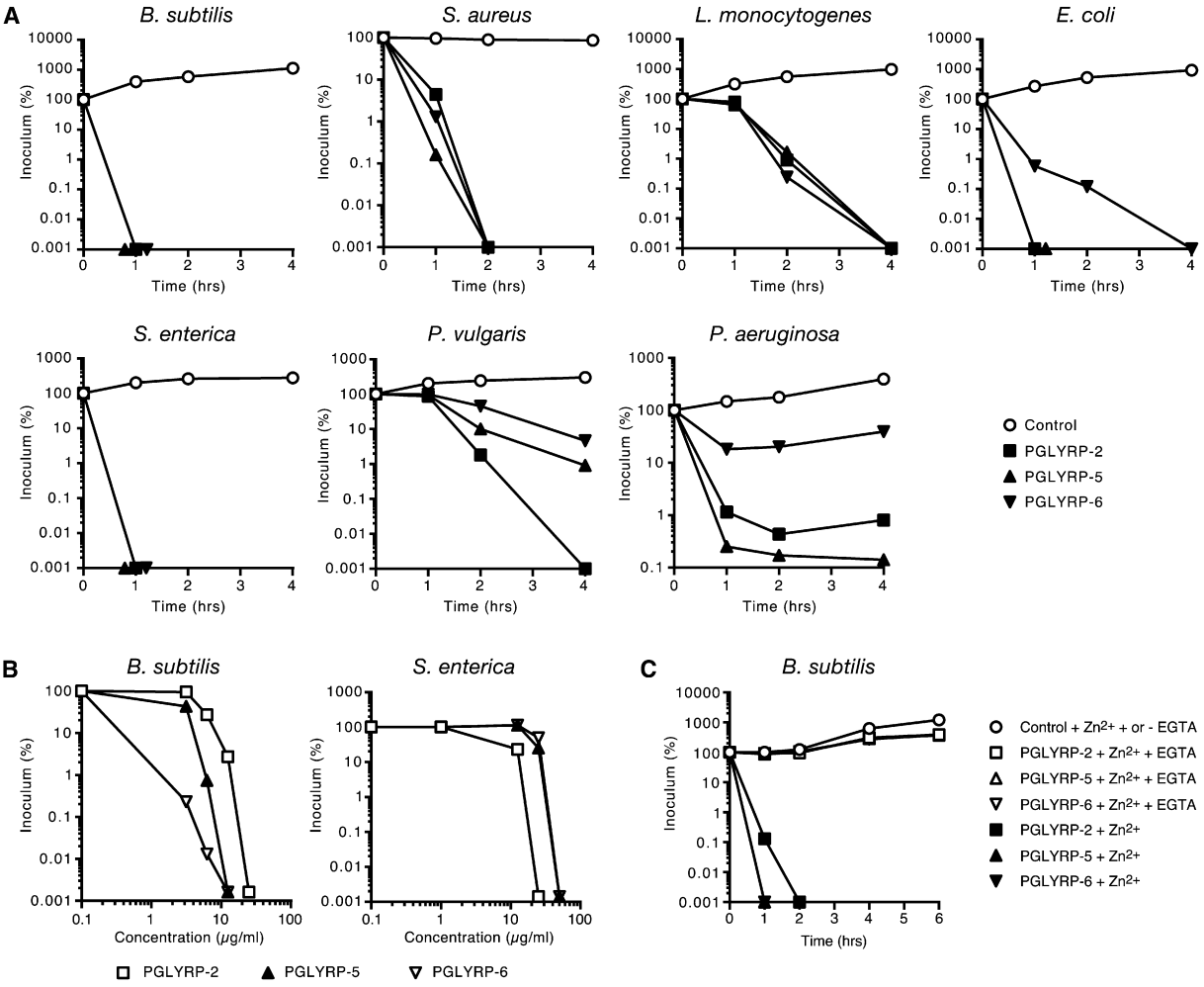

Figure 5. Zebrafish PGLYRP Proteins Are Bactericidal for Several Gram-Positive and Gram-Negative Bacteria and Require Zn^{2+} for Bactericidal Activity

Zebrafish PGKYRP proteins kill 99% of *B. subtilis* at ~1.3–13 μg/ml (0.02–0.2 μM) and *S. enterica* at ~15-33 μg/ml (0.2–1.0 μM). The indicated bacteria were incubated with different PGLYRPs or BSA as a control at (A and C) 100 μg/ml for the indicated time or (B) the indicated concentrations for 1 to 2 hr. In addition, PGLYRPs were treated or not treated with EGTA, as indicated (C). The numbers of bacteria were determined by colony counts. The results are means of three (mostly) or two experiments; the average standard errors were 15% of the mean (ranged from 8%–24%) and are not shown because the error bars were mostly smaller than the size of the symbols in the figures. The significance of differences was calculated with the Student's t test, and all differences in bacterial numbers greater than 1 $\log_{10}$ were statistically significant at $p < 0.05$.

however, was already synthesized in large amounts in the developing oocytes, and its concentration could not be reduced by injection of morpholinos (data not shown).

Embryos, in which the expression of the endogenous PGLYRP-5 protein was inhibited by an injection of a specific morpholino, were then tested for resistance to infections with the Gram-negative (*S. enterica*) and the Gram-positive (*B. subtilis*) bacteria. Embryos injected with either a control (C Mo) or the PGLYRP-5 (P5 Mo) morpholino were subsequently challenged with *S. enterica* and assayed for survival at 4 hr postinfection (hpi). Embryos injected with control morpholino had 94% survival, whereas embryos injected with PGLYRP-5 morpholino had 0% survival (Figure 6D, $p < 0.0001$, control versus PGLYRP-5 morpholino). To confirm that the increased mortality of the embryos was due to the developing infection (manifested by increasing numbers of bacteria in the embryos), we determined the numbers of *S. enterica* recovered from live embryos, which were lysed at 2–3 hpi. Embryos injected with PGLYRP-5 morpholino had significantly ($p < 0.0001$) higher numbers of bacteria than the embryos injected with a control morpholino (Figure 6E). The latter embryos were able to eliminate virtually all injected bacteria within 4 hr (Figure 6E).

Similar results were obtained in infection experiments with *B. subtilis*. Embryos injected with a control morpholino or with PGLYRP-5 morpholino followed by *B. subtilis* infection had 100% and 10% survival, respectively (Figure 6F, $p < 0.0001$, control versus PGLYRP-5 morpholino). To determine the numbers of *B. subtilis* recovered, we

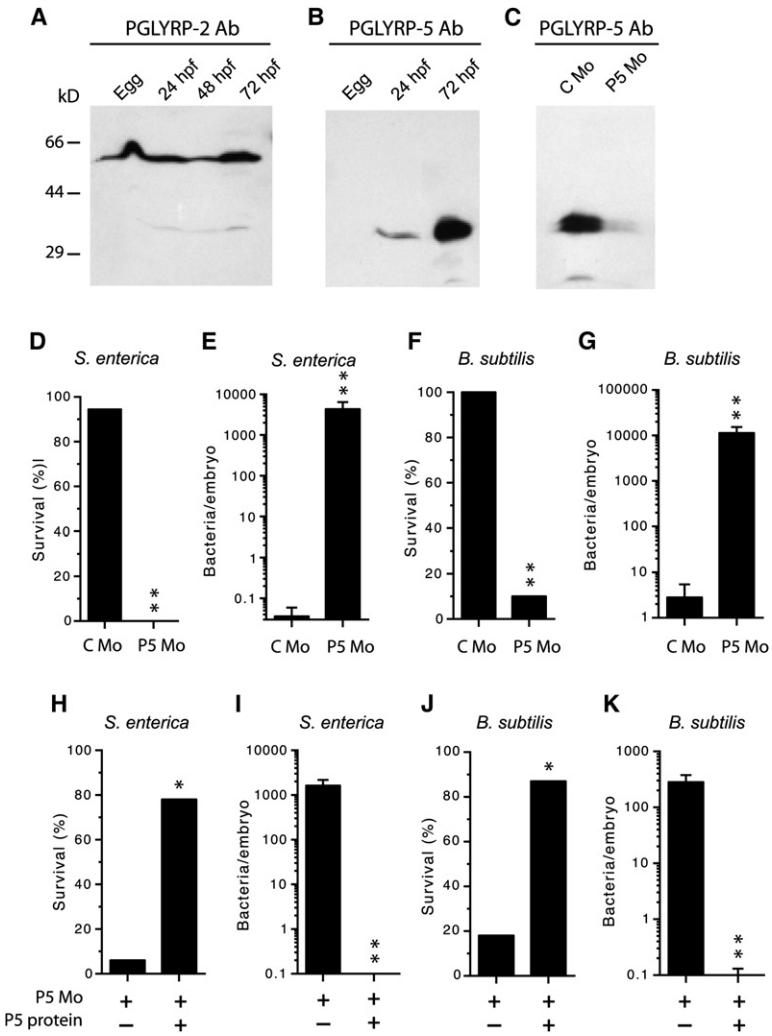

Figure 6. PGLYRP-5 and PGLYRP-2 Are Expressed in the Developing Embryos, and PGLYRP-5 Is Essential for In Vivo Protection against Infection

(A–C) Cell lysates from eggs or from embryos at the indicated developmental stage were analyzed by immunoblotting with PGLYRP-2 (A) or PGLYRP-5 (B and C) antibody. Size markers are shown on the left. The results are from one of three similar experiments.

(D–K) Eggs at the two- to eight-cell stage were injected with 1 nl of 0.5 mM PGLYRP-5 morpholino (P5 Mo) or a control morpholino (C Mo). At the onset of circulation (~30 hpf), embryos were injected with *S. enterica* (D and E) or *B. subtilis* (F and G) or with *S. enterica* (H and I) or *B. subtilis* (J and K) and PGLYRP-5 protein (P5 protein) or buffer. Embryos were assayed for survival at 4 hpi or lysed for determining numbers of recovered bacteria per embryo at 2–3 hpi. The data in each panel are from two to three experiments with the following total numbers of embryos with C Mo and P5 Mo or − and + P5 protein, respectively: (D), 18 and 24; (E), 35 and 43; (F), 24 and 19; (G), 24 and 21; (H), 16 and 9; (I), 16 and 12; (J), 16 and 15; (K), 20 and 28. The survival and the numbers of recovered bacteria were significantly different at $p = 0.0002$ (*) or $p < 0.0001$ (**) between PGLYRP-5 morpholino- and control morpholino-injected groups or between PGLYRP-5 protein-injected and buffer-injected groups. The bactericidal results are means ± SE from two to three experiments.

lysed live embryos at 2–3 hpi. Embryos injected with PGLYRP-5 morpholino had significantly ($p < 0.0001$) higher numbers of bacteria than the embryos injected with a control morpholino (Figure 6G). The latter embryos were able to eliminate virtually all injected bacteria within 4 hr (Figure 6G).

To confirm the in vivo role of PGLYRP-5 in eliminating bacteria and in the survival of the infected embryos, we then performed rescue experiments. Embryos were first injected with PGLYRP-5 morpholino so that the production of endogenous PGLYRP-5 protein could be suppressed. These embryos were then injected with purified PGLYRP-5 protein or buffer alone at the time of *S. enterica* or *B. subtilis* challenge. Injection of PGLYRP-5 protein, but not buffer, significantly ($p = 0.0002$) increased the survival of the embryos infected with *S. enterica* (Figure 6H) and *B. subtilis* (Figure 6J). Similarly, injection of PGLYRP-5 protein, but not buffer, enabled the embryos to kill the infecting bacteria (*S. enterica*, Figure 6E; and *B. subtilis*, Figure 6K, $p < 0.0001$). Thus, these results confirm that PGLYRP-5 protein is responsible for eliminating both *Salmonella* and *Bacillus* from the embryos and for the survival of the embryos during bacterial infections.

DISCUSSION

Our results demonstrate the essential role of innate immunity in defense against infections in fish and identify the component of their innate immune system responsible for this effect (bactericidal PGRPs). Because fish eggs and developing embryos have to survive in bacteria-containing water before adaptive immunity develops, during that time they have to rely solely on innate immunity for defense against infections and survival. Up until now, this requirement was assumed, but to the best of our knowledge, there was no experimental evidence for it. Moreover, our results demonstrate an absolute requirement for a PGRP molecule in defense and survival against bacterial infections in vertebrates.

Zebrafish PGLYRP-2, PGLYRP-5, and PGLYRP-6 proteins (which we identified and cloned) are highly conserved and have one PGRP domain, which is homologous

to the PGRP domains of other invertebrate and vertebrate PGRPs. Zebrafish PGLYRPs, similar to human PGLYRPs, are selectively expressed in a wide range of tissues including the liver, intestine, pancreas, maturing oocytes, gills, and skin and are secreted in the lumen of the sinusoids in the liver and in the blood vessels of the gills. All three proteins are expressed in the developing oocytes, although there may be quantitative differences in their expression. PGLYRP-2 is also strongly expressed in the eggs, and both PGLYRP-2 and PGLYRP-5 are expressed in the developing embryo. This expression of PGLYRPs in a wide range of tissues suggests an important role for this family of proteins in defense of adult zebrafish against bacterial infections. Furthermore, the expression profile of PGLYRPs would allow the fish to combat bacteria both in the surrounding water and internalized bacteria during a systemic infection. Expression of PGLYRPs in the eggs also equips the developing embryo with innate immune mechanisms to fight bacterial infections before the development of adaptive immunity.

We have demonstrated that PGLYRP-2, PGLYRP-5, and PGLYRP-6 digest polymeric peptidoglycan. Furthermore, the three proteins specifically hydrolyze the lactylamide bond between the MurNAc and L-Ala, and this hydrolyzation confirms that PGLYRP-2, PGLYRP-5, and PGLYRP-6 are *N*-acetylmuramoyl-L-alanine amidases, similar to mammalian PGLYRP-2 and *Drosophila* PGRP-SC1, PGRP-LB, and PGRP-SB1 (Wang et al., 2003; Gelius et al., 2003; Kim et al., 2003; Mellroth et al., 2003; Sang et al., 2005; Mellroth and Steiner, 2006).

In addition to the amidase activity, zebrafish PGLYRP-2, PGLYRP-5, and PGLYRP-6 are also bactericidal. The three proteins kill several different Gram-positive bacteria equally efficiently but show somewhat differential killing of several Gram-negative bacteria. Some of these bacteria are human pathogens and are also pathogenic for zebrafish, producing symptoms similar to those in humans (van der Sar et al., 2003; van der Sar et al., 2004). However, zebrafish PGLYRPs are different from human bactericidal PGLYRP-1, PGLYRP-3, and PGLYRP-4 because the human proteins have no amidase activity. Human PGLYRP-2 is an amidase; however, it is not known whether it is also bactericidal. One insect PGRP, *Drosophila* PGRP-SB1, has both amidase and antibacterial activities (Mellroth and Steiner, 2006). However, the spectrum of antibacterial activity of PGRP-SB1 was very narrow and limited to only *B. megaterium*, unlike the broad spectrum of antibacterial activity by human (Lu et al., 2006; Wang et al., 2007) and zebrafish PGLYRPs (this study). The bactericidal activity of PGLYRP-2, PGLYRP-5, and PGLYRP-6 requires zinc, similar to human PGLYRPs (Lu et al., 2006; Wang et al., 2007).

The killing mechanism by human bactericidal PGRPs most probably targets the bacterial cell wall and does not involve peptidoglycan hydrolysis or permeabilization of the cytoplasmic membrane (Lu et al., 2006; Wang et al., 2007). Zebrafish PGRPs, unlike human PGRPs, have both amidase and bactericidal activities. Thus, their mechanism of action may be different.

We have further demonstrated that PGLYRP-5 is required in the zebrafish embryo for eliminating bacteria and for survival of the embryo during bacterial infections. We selected *S. enterica* and *B. subtilis* for these experiments because both bacteria are highly sensitive to the bactericidal activity of zebrafish PGLYRPs, are free-living and found in water and thus infect aquatic life such as fish and turtles, are not highly pathogenic for zebrafish, and are rapidly eliminated in the normal developing embryo. The ability of the host to eliminate bacteria with innate immune mechanisms may be an important property that differentiates less pathogenic from more pathogenic bacteria. PGLYRP-5 is expressed in the developing embryo at an estimated concentration of 100 μg/ml at 24 hpf. This concentration of PGLYRP-5 protein is based on an estimated total volume of 0.3 μl/embryo (24 hpf) and the amount of endogenous PGLYRP-5 protein detected on the western blot (30 ng per 24 hpf embryo). This concentration of PGLYRP-5 is more than sufficient to kill 1 to 5 × 10^7 bacteria/ml (100–500 bacteria/embryo)—the numbers of bacteria that were injected into the embryo. This calculation is based on our in vitro bactericidal assays, in which LD_{99} of PGLYRP-5 is 6 μg/ml for *B. subtilis* and 33 μg/ml for *S. enterica* at 1 to 5 × 10^6 bacteria/ml, and was verified in our rescue experiments with recombinant PGLYRP-5 injected into PGLYRP-5-depleted embryos (Figure 6H-K).

To our knowledge, this is the first demonstration of an essential role for a PGLYRP protein in the survival of vertebrates and contrasts with the results of two previous studies: One study showed partial requirement for PGLYRP-1 in resistance to infection in mice (Dziarski et al., 2003), and the second study concluded that PGLYRP-2 was not essential for immunity in mice (Xu et al., 2004). Our results also demonstrate the importance of innate immunity molecules in the defense against bacterial pathogens, especially during the early stages of development when there is no adaptive immunity.

In conclusion, we have cloned a family of PGLYRP proteins from an earlier vertebrate, the zebrafish, and we have identified the function of PGLYRP-2, PGLYRP-5, and PGLYRP-6 proteins. Zebrafish PGLYRP proteins are expressed in several tissues in adult zebrafish and are differentially expressed in the developing embryos. These proteins are unique compared to other vertebrate PGLYRPs because they have both amidase activity and a broad-spectrum bactericidal activity. Furthermore, zebrafish PGLYRP-5 is essential for defense against bacterial infections in the developing embryos. Thus, our results demonstrate that in zebrafish, innate immunity (and PGLYRP proteins in particular) is essential for defense against infections and survival of developing embryos.

EXPERIMENTAL PROCEDURES

Maintenance of Zebrafish

Adult zebrafish, *Danio rerio*, were obtained from the Zebrafish International Resource Center (supported by grant #RR12546 from NIH-NCRR), University of Oregon. Zebrafish were housed and maintained

in our facilities according to the established protocols described in the "The Zebrafish Book," University of Oregon (www.zfin.org). All experiments with fish were approved by the Institutional Animal Care and Use Committee at Indiana University School of Medicine Northwest.

Cloning of Zebrafish *pglyrp* Genes

We identified four zebrafish PGLYRP genes in the GenBank databases and have cloned cDNAs for three PGLYRPs. All cloning was done by RACE reactions and by RT-PCR with total RNA isolated from adult zebrafish tissues. The cDNAs for the three PGLYRPs were initially cloned with RACE nested primers (see Table S1) on the basis of the following EST clones: CF999082 and CK029852 (PGLYRP-2); CO92224 (PGLYRP-5); and AL922460 (PGLYRP-6). RACE reactions were done with the First-Choice RLM-RACE kit (Ambion). The 5′ RACE products for the three PGLYRPs were extended at the 5′ end with primers on the basis of the GenBank predicted protein sequences XM691972 (PGLYRP-2), XM697301 (PGLYRP-5), and XM695448 (PGLYRP-6) for producing full-length cDNAs. The 3′ RACE reactions resulted in clones with poly(A) tails. All clones were analyzed by restriction digestion and sequencing. Final sequences were analyzed for signal peptides with the SPScan program (Genetic Computer Group) and for transmembrane domains with the Swiss TMpred program. Multiple sequence alignments were performed with the ClustalW program with MacVector (Genetic Computer Group).

In Vitro Transcription and Digoxigenin-Labeled RNA Probes

Zebrafish PGLYRP cDNAs were amplified with primers (Table S1) and subcloned into the pGEM-T vector (Promega). This vector has dual-opposed promoters that allow in vitro transcription of both strands of the cloned DNA. For generating RNA probes, the clones were digested with NcoI or NdeI, and the fragments were purified on an agarose gel and used for separate in vitro transcription reactions with the Digoxygenin RNA Labeling Kit (Roche). Transcription was performed with SP6 RNA polymerase and T7 RNA polymerase so that antisense and sense RNA probes could be generated.

Expression and Purification of PGLRP Proteins

The coding regions for zebrafish PGLYRPs were amplified (Table S1) and subcloned into the expression vector pMT/BiP/V5-His vector (Invitrogen). Stable S2 cell lines were generated, expression of PGLYRPs was induced with 500 μM $CuSO_4$, and proteins were purified as described before (Lu et al., 2006). All purification buffers included 40 μM $ZnSO_4$; elution buffer also included 10% glycerol; and dialysis buffer included 10 μM $ZnSO_4$ and 10% glycerol (Wang et al., 2007). The purity of the proteins was detected on Coomassie-blue-stained blots (Figure S3). For removal of divalent cations, proteins were treated with 100 μM EGTA for 10 min at room temperature and then used for bactericidal assays. To test whether zebrafish PGLYRPs are glycosylated, we treated proteins with the N-glycosidase, PNGase, and analyzed them by western blots as described (Lu et al., 2006).

RNA and Northern Blots

Total RNA from eggs and 24 hr hpf, 48 hpf, and 72 hpf embryos or adult zebrafish skin, muscle, liver, eggs, gills, intestine, brain, liver, and eyes was purified with Trizol reagent (Invitrogen). A total of 30 μg of RNA from each sample was analyzed by Northern blots. PGLYRP cDNA clones were amplified with primers (Table S1) and used as a probe. The 18S ribosomal clone, IMAGE ID 5604426, was used as a control. DNA was labeled with ^{32}P with RadPrime DNA Labeling System (Invitrogen), and hybridizations were done in ExpressHyb solution at 68°C (Wang et al., 2005). All probes were highly specific because they did not crosshybridize with other members of the PGLYRP family (data not shown).

In Situ Hybridization

Adult zebrafish were fixed in Bouin's Reagent for 3 hr, rinsed in 70% ethanol, embedded in paraffin, and 5-μm-thick, longitudinal sections, were prepared by Transgenic Pathology Laboratory (University of California, Davis). The sections were incubated in prehybridization buffer for 4 hr at 60°C and an overnight incubation at 55°C in hybridization buffer containing 10 ng of digoxigenin labeled RNA probe. The sections were washed at 55°C in the following series of wash solutions: 2× SSC, 2× SSC with 50% formamide, 2× SSC with 25% formamide, 1× SSC, and 0.1× SSC and then blocked with 2% normal sheep serum. The sections were incubated with sheep anti-DIG-alkaline phosphatase antibody overnight, and this was followed by color development with nitroblue tetrazolium and 5-bromo-chloro-3-indolyl phosphate. The sections were counterstained with fast red.

Antibodies and Western Blots

PGLYRP antibodies to the following peptides: CETETHPKIKERLN for PGLYRP-2, CSVLPKLRDRLQNNE for PGLYRP-5, and CVRRNLNQDERKKLD for PGLYRP-6 were made and affinity purified (Lu et al., 2006). These antibodies were specific for each PGLYRP, as demonstrated by their reactivity on western blots with only one PGLYRP from which the peptide was derived, and no reactivity with the other PGLYRPs (Figure S3). An antibody to an unrelated peptide, which did not react with zebrafish PGLYRPs, prepared and purified by the same method, was used as a negative control. V5 mouse monoclonal antibody was obtained from Invitrogen.

Total cell protein was prepared from eggs and embryos in SDS sample buffer (The Zebrafish Book, www.zfin.org). Lysates from 20 eggs or embryos were analyzed by SDS-gels and western blots, with antibodies specific for zebrafish PGLYRPs.

Immunohistochemistry

Adult zebrafish were fixed in Bouin's Reagent and sectioned as described above for in situ hybridization. After standard deparaffinization, rehydration, and quenching of endogenous peroxidase by 30 min incubation in 0.3% H_2O_2, the slides were incubated with 1 μg/ml of anti-PGLYRP-2, anti-PGLYRP-5, anti-PGLYRP-6 or control antibody (obtained and tested for specificity as described above) overnight; this was followed by biotinylated second antibody and Vectastain Elite ABC kit (Vector) with 3,3′ diaminobenzidene as a substrate (this generates a brown reaction product) and counterstaining with hematoxylin (blue).

Peptidoglycan Digestion and Amidase Assays

Soluble uncrosslinked peptidoglycan, purified by vancomycin-affinity chromatography from *S. aureus* Rb, was labeled with biotin on the N-terminal glycine of its interpeptide bridge, and its hydrolysis was measured as described previously (Wang et al., 2003). Peptidoglycan-biotin (500 ng) was incubated for 24 or 96 hr at 37°C with 500 ng of recombinant PGLYRP-2, PGLYRP-5, PGLYRP-6, or buffer alone. Enzyme-digested peptidoglycan-biotin was subjected to SDS-PAGE and blotted on Immobilon-P, and high M_r peptidoglycan-biotin was detected with streptavidin-peroxidase and enhanced chemiluminescence as described previously (Wang et al., 2003).

For determining which bond in peptidoglycan is hydrolyzed by zebrafish PGLYRPs, 10 μg of synthetic peptidoglycan fragments (Kumar et al., 2005) were incubated for 72 hr in 4 mM TRIS (pH 7.6), with 60 mM NaCl, 4 μM $ZnSO_4$, and 4% glycerol, with 2 μg zebrafish PGLYRPs, or human PGLYRP-2 as a positive control, or in buffer alone as a negative control. The samples were analyzed by MALDI-TOF mass spectrometry, and digestion products were identified as described previously (Wang et al., 2003).

Antimicrobial Assay

Bactericidal activity of zebrafish PGLYRP proteins was assayed as described (Lu et al., 2006; Wang et al., 2007). In brief, PGLYRP proteins purified from S2 cell supernatants, or BSA as a control, were incubated with bacteria in a buffer containing 5 mM TRIS, pH 7.6, 150 mM NaCl, 5 μM $ZnSO_4$, 5% glycerol, and 1% LB (50% Schaeffer's medium was used for *B. subtilis* instead of LB). The following bacteria were assayed for sensitivity: *B. subtilis*, *L. monocytogenes*, *S. aureus* (clinical isolate Rb), *E. coli* K12, *P. vulgaris*, *P. aeruginosa*, and *S. enterica*. The number

of viable bacteria was determined by colony counts. All other controls were performed as described previously (Lu et al., 2006). Bactericidal activity is defined as an at least 2 $\log_{10}$ decrease in the number of inoculated bacteria in 6 hr; bacteriostatic activity is defined as an inhibition of growth of bacteria or a decrease in the number of inoculated bacteria of less than 2 $\log_{10}$ in 6 hr. The significance of differences was calculated with the Student's t test, and all differences in bacterial numbers greater than 1 $\log_{10}$ were statistically significant at $p < 0.05$.

Infection of Zebrafish Embryos

Zebrafish eggs were rinsed with 0.003% sodium hypochlorite, suspended in embryo medium, and transferred into an agarose injection plate (Grabher et al., 2004). PGLYRP-5 (AAGATGAAGAAACTGTGCT GCATGG) and control (CCTCTTACCTCAGTTACAATTTATA) morpholinos (Gene Tools) were at 0.5 mM concentration. We used a PV830 Pneumatic PicoPump (World Precision Instrument) to pressure inject 1 nl of morpholino solution into the yolk of two- to eight-cell-stage developing embryos. The embryos were placed at 28°C in embryo medium and observed under the microscope so that normal development could be ensured. In initial experiments, embryos were observed for ~60 hpf (this is ~26 hr longer than the infection experiments), and there were no obvious morphological differences between the PGLYRP-5 and control morpholino-injected embryos. At 28–32 hpf, the embryo heart was beating and the circulation had commenced. Embryos similar in development were selected from PGLYRP-5, and control morpholino-injected groups and the embryo yolk was injected with 1 nl of an exponentially growing *S. enterica* or *B. subtilis* culture, diluted so that 100–500 bacteria per injection could be produced. In initial experiments, the entry of the bacteria into the circulation was monitored with phenol red mixed with bacteria. The injected embryos were observed under the microscope, and the sample was seen rapidly entering the circulation. We verified the dose of bacteria injected into each embryo by dispensing an identical volume onto LB plates just before and after each injection group. Subsequent culture of these plates showed the same numbers of bacteria injected into each embryo treatment group. After bacterial injections, the embryos were scored for survival after 4 hr or were individually lysed and plated on LB agar for determining the numbers of recovered bacteria at 2–3 hr. Longer time points were not used because all PGLYRP-5 morpholino-injected embryos challenged with bacteria were dead. For rescue experiments, PGLYRP-5 purified protein (0.4 ng/embryo) was injected into embryos with *S. enterica* or *B. subtilis*, and individual embryos were assayed as described above. We used two types of control injections: (1) control morpholinos, which had no effect on PGRP expression, embryo development, and the course of infection and (2) vehicle or buffer injections, instead of morpholinos, bacteria, or PGRP protein, which had no harmful effects on the embryos (buffer-injected embryos developed normally or had normal course of infections if subsequently challenged with bacteria). The survival results were analyzed for significance of differences by the chi-square test, the numbers of recovered bacteria are presented as geometric means, and the significance of differences was determined by the Mann-Whitney U test.

Supplemental Data

Additional Results and three figures are available at http://www.immunity.com/cgi/content/full/27/3/518/DC1/.

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Accession Numbers

The nucleotide sequences for PGLYRP-1, PGLYRP-5, and PGLYRP-6 have been submitted to GenBank, and their accession numbers are DQ447202, DQ447203, and DQ447204, respectively.