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Short communication

An immune deficiency homolog from the white shrimp, *Litopenaeus vannamei*, activates antimicrobial peptide genes

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ABSTRACT

Invertebrates rely on innate immunity as the first line defense against microbes. In *Drosophila*, the inducible antimicrobial peptides (AMPs) regulated by the Toll and immune deficiency (Imd) pathways are important effectors in innate immunity. Here we report an immune deficiency homolog (LvIMD) from the white shrimp, *Litopenaeus vannamei*. The full-length cDNA of LvIMD is 758 bp with an open reading frame of 483 bp that encodes a putative protein of 160 amino acids including a death domain at the C-terminus. LvIMD death domain shows similarity to that of *Drosophila* IMD and human receptor interacting protein 1 (RIP1) of the tumor necrosis factor receptor (TNFR) pathway, with 27.9% and 26.4% identity, respectively. Phylogenetic analysis shows that LvIMD clusters with a predicted protein from the starlet sea anemone (*Nematostella vectensis*) independent to insect IMDs and vertebrates RIP1s. LvIMD mRNA is expressed in most tissues and is induced in hepatopancreas and hemocytes after immune challenge. Luciferase reporter assays confirm that LvIMD is able to induce the expression of AMP genes, including *Drosophila* Attacin A and shrimp Penaeidin 4 in S2 cells. To our knowledge, this is the first report that LvIMD participates in innate signaling to activate the expression of AMP genes in shrimp.

1. Introduction

Without an adaptive immune system, invertebrates rely entirely on innate immunity as the first-line host defense to prevent or combat microbial invaders by activating various genes encoding antimicrobial peptides (AMPs), complement-like proteins and regulators of phagocytosis (Lemaitre and Hoffmann, 2007). In *Drosophila*, the inducible expression of AMPs, which exhibit a broad range of activities in fighting microorganisms, is a critical aspect of the innate immune system. The *Drosophila* Toll and immune deficiency (Imd) pathways control the expression of AMP genes as well as crucial genes in cellular immune responses (De Gregorio et al., 2002; Matova and Anderson, 2006). When the two pathways are inactive, AMPs cannot be induced and the flies become very susceptible to many microbes including those that are normally nonpathogenic (Tanji et al., 2007).

IMD is a death domain protein encoded by the *immune deficiency* (*imd*) gene. The *imd* refers to a mutation that shows severe defects in resistance to Gram-negative bacteria but has normal responses to fungi and Gram-positive bacteria (Georgel et al., 2001; Lemaitre et al., 1995). The *imd* mutant flies could not induce the expression of some AMPs, such as Cecropins, Diptericin and Drosocin, which are activated against Gram-negative bacteria, but the antifungal peptide Drosomycin is still inducible (Lemaitre et al., 1995). Later researches discovered that the Toll pathway is activated by Gram-positive bacteria and fungi, while the Imd pathway responds to Gram-negative bacteria infection (Georgel et al., 2001; Lemaitre et al., 1996; Naitza et al., 2002; Rutschmann et al., 2002). Gram-negative bacteria-derived diaminopimelic acid (DAP)-type peptidoglycan can be recognized by peptidoglycan recognition protein (PGRP)-LE and PGRP-LC receptor complex to activate the adaptor protein IMD, leading to activation of the signaling cascade to activate the NF-κB factor Relish (Takehana et al., 2004). Relish is activated by proteolytic cleavage and translocation of Relish into the nucleus promotes expression of AMPs and other immune-related genes (Stoven et al., 2003).

Abbreviations: ALFs, anti-lipopolysaccharide factors; AMPs, antimicrobial peptides; EST, expression sequence tag; IMD, immune deficiency; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria broth; LPS, lipopolysaccharide; ORF, open reading frame; PGRP, peptidoglycan recognition protein; PI, propidium iodide; proPO, prophenoloxidase; RACE, rapid amplification of cDNA end; RIP1, receptor interacting protein 1; RT-PCR, Reverse Transcriptase-Polymerase Chain Reaction; S2, *Drosophila* Schneider 2; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel; WSSV, white spot syndrome virus; YPD, yeast extract–peptone–D-glucose.

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The white shrimp, *Litopenaeus vannamei*, is the primary farmed shrimp species in China and Southern Asia. However, shrimp diseases have become a major constraint and the most limiting factor for the shrimp culture industry, resulting in high mortality and huge economic losses consequently (Bachere, 2000; Yang et al., 2007). Therefore, it is very important to understand shrimp immunity in order to design better strategies for prevention and control of shrimp diseases. Research has been focusing on AMPs and other immune-related genes in shrimp, including penaeidins, crustins, anionic haemocyanin and anti-lipopolysaccharide factors (ALFs). Penaeidins, crustins and ALFs have a broad spectrum of activities against fungi and bacteria in shrimp (Amparyup et al., 2008; Cuthbertson et al., 2008; de la Vega et al., 2008; Destoumieux et al., 1999; Kang et al., 2007; Padhi et al., 2007; Somboonwiwat et al., 2008, 2005; Supungul et al., 2008; Vargas-Albores et al., 2004). Penaeidins, the largest AMP family found in shrimp, are mainly synthesized in hemocytes and released from hemocyte cytoplasmic granules to kill or inhibit invading microorganisms (Destoumieux et al., 2000). So far, there are four classes of penaeidins, PEN2, PEN3, PEN4 and PEN5 (Padhi et al., 2007). It has been reported that successful responses in shrimps to circumvent *Vibrio* (Gram-negative bacterial pathogens in shrimp) infections are related to the expression level of these immune-related genes, such as PEN2, PEN3, ALF and crustin (de Lorgeril et al., 2008). However, little is known about the regulations of these immune-related genes. In this study, we identify an immune deficiency homolog (LvIMD) from *L. vannamei*, and investigate its function in activating AMP genes.

2. Materials and methods

2.1. Experimental animals

Healthy white shrimp, about 8–9 g in body weight and approximately 8–10 cm in length, were collected from Hengxing shrimp farm in Zhanjiang, Guangdong Province, China. The feeding and supervising modes were similar to those described previously (Yang et al., 2007).

2.2. RNA extraction, cDNA synthesis and genomic DNA extraction

Total RNA was extracted from each tissue using RNeasy Mini Kit (Qiagen, Germany). Residual genomic DNA was removed by RNase-free DNase I (Qiagen, Germany). Total RNA (0.5 μg) was reverse transcribed to cDNA using PrimeScript™ First Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Genomic DNA from shrimp muscles or *Drosophila* S2 cells was extracted using the Universal Genomic DNA Extraction Kit Ver.3.0 (TaKaRa, Dalian, China) according to the manufacturer's instructions.

2.3. Cloning of LvIMD cDNA

Based on an expression sequence tag (EST) sequence in the shrimp cDNA library, which is homologous to *Drosophila* IMD, specific primers (Table 1) were designed to obtain the 3′ and 5′ end cDNA sequences of LvIMD by rapid amplification of cDNA ends (RACE). The cDNA template for RACE-PCR was prepared using the BD SMART RACE cDNA Amplification Kit (Clontech, USA). 5′ RACE1 and 3′ RACE1 primers (Table 1) were used for the first round 5′-end and 3′-end RACE-PCR, using the following program: 94 °C for 2 min, 9 cycles of 94 °C for 45 s, 58 °C for 30 s (decrease 0.5 °C per cycle), 72 °C for 1 min, 24cycles of 94 °C for 45 s, 53 °C for 30 s, 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The conditions for second round 5′-end and 3′-end PCR (using 5′ RACE2 and 3′ RACE2 primers, respectively) were as follows: denaturation at 94 °C for 2 min, then 26 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 1 min, followed by a 10 min extension at 72 °C. The PCR products were cloned into pGEM-T easy vector (Promega, USA) and sequenced.

2.4. Bioinformatics analysis

BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) was used to analyze the nucleotide sequence and search for IMD or receptor interacting protein 1 (RIP1) sequences from other species in the database. Multiple sequence alignments were performed using the ClusterXv1.83 program (Thompson et al., 1997). An NJ phylogenic tree was constructed based on the deduced amino acid sequences of LvIMD and other known IMD or RIP1 proteins by MEGA 4.0 software (Tamura et al., 2007). Bootstrap sampling was reiterated 1000 times. Protein motifs were predicted by PROSITE program (http://expasy.org/prosite/).

2.5. Pathogen preparation and immune challenge in shrimps

Gram-negative *Vibrio alginolyticus* and Gram-positive *Staphylococcus aureus* were cultured in Luria broth (LB) medium overnight at 37 °C. Yeast (*Saccharomyces cerevisiae*) was cultured in yeast extract–peptone–D-glucose (YPD) medium overnight at 37 °C. Then the cells were pelleted at 5000 × *g* for 10 min, washed, and resuspended in sterile saline (10 mM Tris–HCl, pH 7.5, 400 mM NaCl) to a density of 10^7 CFU ml^{-1}, heat-killed by boiling to 100 °C for 30 min. White spot syndrome virus (WSSV) solution was prepared from the homogenates of WSSV-infected white shrimp (Ai et al., 2008). Lipopolysaccharide (LPS) from *Escherichia coli* 055: B5 (Sigma, USA) was diluted in sterile saline to 0.5 μg/μl. For the immune challenged experiments, healthy white shrimps were injected intramuscularly at the third abdominal segment with 50 μl sterile saline, *V. alginolyticus*, *S. aureus*, *S. cerevisiae*, WSSV solution, or LPS. The untreated shrimps were used as controls. Six hours post-injection, five shrimps from each group were randomly selected for dissection. Hemocyte, eyestalk, gill, heart, hepatopancreas, intestine, nerve, muscle, pyloric caecum and epithelium were collected from these shrimps for RNA extraction.

2.6. Tissue distribution of LvIMD mRNA in healthy and immune-challenged shrimps

To investigate distribution of LvIMD mRNA in different tissues of healthy shrimps, one microliter of the transcribed cDNA and a pair of gene-specific primers LvIMD-F and LvIMD-R (Table 1) were used for RT-PCR: 94 °C for 2 min, 32 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, followed by 72 °C for 10 min. β-Actin cDNA fragment was amplified with β-actin-F and β-actin-R primers (Table 1) as an internal control by PCR: 94 °C for 2 min, 24 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, followed by 72 °C for 10 min. For LvIMD mRNA in tissues of the immune-challenged shrimps, RT-PCR conditions were essentially the same as described above except that the cycles were modified as indicated in Fig. 4B.

2.7. Construction of vectors for protein expression and luciferase assays

PQE-30 expression vector (Qiagen, Germany) and PCR products (amplified with pqeLvIMDF and pqeLvIMDR primers, Table 1) were digested with BamHI and HindIII, purified, ligated, and transformed into DH5α competent cells to select positive clones for sequencing. The recombinant expression vector PQE30-LvIMD confirmed by sequencing was used for recombinant protein expression in bacteria. For protein expression in S2 cells, the expression vector pAc5.1-LvIMD was constructed using pAc5.1/V5-His A vector (Invitrogen, USA) and PCR products amplified with pAcLvIMDF and

Table 1
PCR primers used in this study.

Primers	Primer sequences (5′–3′)
cDNA cloning	
5′ RACE1	TCATCATCTCGTACACTCGGTC
5′ RACE 2	GGTTCCCTTGTTGAAAATCAC
3′ RACE 1	AGACAGATTCGGCTCCACTT
3′ RACE 2	CCGAGTGTACGAGATGATGAGT
RT-PCR analysis	
LvIMD-F	TGGGTCCGTGTCCAGTGAT
LvIMD-R	ACAAACAACCACACACAAGCAG
β-Actin-F	GAAGTAGCCGCCCTGGTTG
β-Actin-R	CGGTTAGCCTTGGGGTTGAG
Protein expression	
pqeLvIMDF	CGGGATCCATGGATAATATTAAGACAGATTCGGCT
pqeLvIMDR	CCAAGCTTTCAAGGTGTAAGCTTCTTCAGCACG
pAcLvIMDF	GGTACCATGGATAATATTAAGACAGATTC
pAcLvIMDR	GCGGCCGCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCAGGTGTAAGCTTCTTCAGCACG
Luciferase reporter vector	
pGL3-AttA	
AttAF	GGTACCGCCATCAGGCCACCACCCATTCTG
AttAR	AGATCTGTTGCTGAACTGGATTGCTGGAGCTGAC
pGL3-mAttA	
mAttAR1	TTTCCGGCTCCCCCATCCACGCAAAATAACGTATTGATAAAGCCTGACATCAAGTGAGAAATACGATTTATAACTGTCAAATG
mAttAF2	CATTTGACAGTTATAAATCGTATTTCTCACTTGATGTCAGGCTTTATCAATACGTTATTTTGCGTGGATGGGGGAGCCGGAAA
mAttAR2	TCACATCTGAGCGCTTTGATAAGGCATCCAGGCCGAGATCGGCAATCAGATGAATCATGTCAATCATCAGAAAAGCATCTTGAGGTAT
mAttAF3	ATACCTCAAGATGCTTTTCTGATGATTGACATGATTCATCTGATTGCCGATCTCGGCCTGGATGCCTTATCAAAGCGCTCAGATGTGA
pGL3-PEN4	
PEN4F	GGTACCGCCATCAGGCCACCACCCATTCTG
PEN4R	AGATCTGTTGCTGAACTGGATTGCTGGAGCTGAC
pGL3-mPEN4	
mPEN4R1	GAGTGAATCCTTGTTAATCATCAATTTTGATATCTAAAACCCAAACATAAATAAC
mPEN4F2	GATATCAAAATTGATGATTAACAAGGATTCACTCTATTTCATTTCTACCCTCTGTTCATTT
mPEN4R2	CTCCTACCCAGTTTCCCTCCTATGCATCCCCTTATGCCCTTTCGC
mPEN4F3	GCGAAAGGGCATAAGGGGATGCATAGGAGGGAAACTGGGTAGGAG

pAcLvIMDR primers (Table 1). The recombinant expression vector was then confirmed by sequencing.

The *Drosophila melanogaster* Attacin A (AttA) promoter fragment from −1206 to +33 amplified with AttAF and AttAR primers (Table 1) was inserted into pGL3-Basic luciferase reporter vector (Promega, USA) at KpnI and BglII sites to construct pGL3-AttA luciferase reporter vector. We used primer extension and fusion PCR (with fusion primers, Table 1) to delete all the κB sites, including −42, −114, −397 and −409 κB sites, in the AttA promoter. Then the mutant AttA promoter fragment was inserted into pGL3-Basic luciferase reporter vector at Kpn I and Bgl II sites to construct pGL3-mAttA luciferase reporter vector. The pGL3-Basic luciferase reporter vector and *L. vannamei* PEN4 promoter fragment from −619 to +77 were used to construct the pGL3-PEN4 luciferase vector. We used primer extension and fusion PCR to delete all the κB sites, including −129, −156, −321 and −372 κB sites, in the PEN4 promoter. Then the mutant PEN4 promoter fragment was inserted into pGL3-Basic luciferase reporter vector at KpnI and BglII sites to construct pGL3-mPEN4 luciferase reporter vector. pRL-CMV luciferase reporter vector with the cytomegalovirus promoter (Promega, USA) served as an internal standard for luciferase reporter assays (O'Leary and Gross, 2006).

2.8. Expression and purification of recombinant protein and preparation of antiserum

The recombinant expression vector PQE30-LvIMD was transformed into *E. coli* M15 competent cells. After IPTG induction at 37 °C for 6 h, the recombinant protein was expressed and then purified under denaturing conditions in 8 M urea using nickel–nitrilotriacetic acid (Ni–NTA) resin following the handbook's instruction (Qiagen, Germany).

Purified recombinant LvIMD was further separated by 15% SDS–PAGE and stained with Coomassie Brilliant Blue R250. The gel slice containing recombinant protein was cut off, emulsified in an equal volume of the complete Freund's adjuvant (Sigma, USA), and then used as an antigen to immunize mice subcutaneously for polyclonal antibody production on day 1. On day 8, 15, 22, and 30, booster immunizations were performed to each mouse with the purified protein in gel slice mixed with an equal volume of the Freund's incomplete adjuvant (Sigma, USA). On day 35, antiserum was collected by exsanguinating the mice, analyzed by Western blot, and then stored at −20 °C.

2.9. SDS–PAGE and Western blot analysis

12% or 15% SDS–PAGE was prepared using SDS–PAGE Gel Preparation Kit (Beyotime, China). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 or transferred to a pre-wet nitrocellulose (NC) membrane in electroblotting buffer with constant current of 180 mA for 3 h for Western blot analysis. Anti-His monoclonal antibody (1:5000) and anti-LvIMD polyclonal antibody (1:5000) were used to detect the expression of recombinant protein in *E. coli* M15. Then anti-LvIMD polyclonal antibody (1:1000) was used to detect LvIMD protein in various tissues or different shrimp cell compartments by immunoblotting.

2.10. Immunoblot analysis and immuno-localization of LvIMD in shrimp primary cells

For immunoblot analysis, three shrimps were randomly selected, and hepatopancreas (50 mg from each shrimp) was collected and mixed with 1.5 ml PBS by pipetting. Hepatopancreas

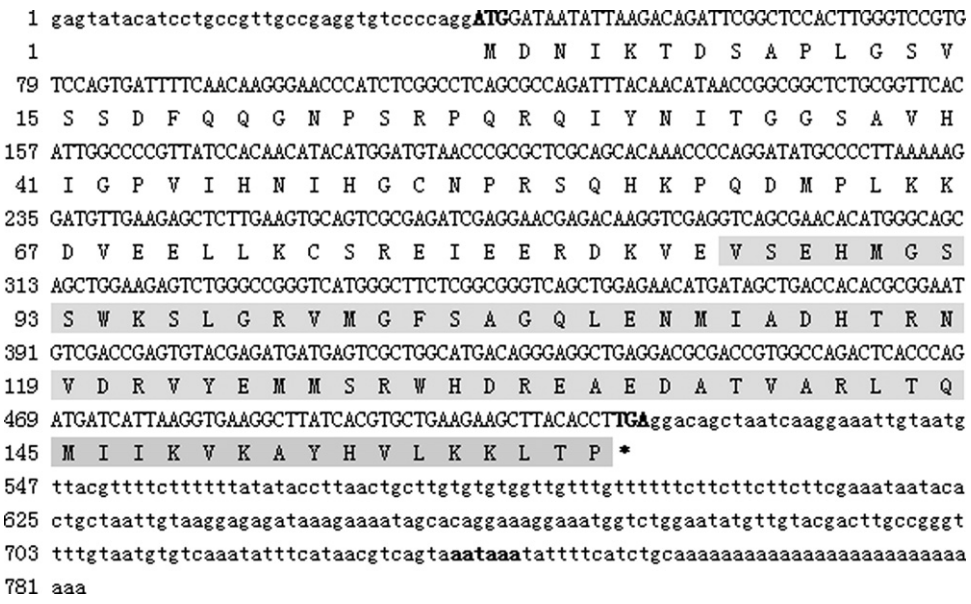

Fig. 1. Nucleotide and deduced amino acid sequences of LvIMD from *L. vannamei*. The open reading frame of the nucleotide sequence is shown in upper-case letters, the 5′- and 3′-UTRs are in lower-case letters. Nucleotides and amino acids are numbered on the left of the sequences. The C-terminal death domain is shaded. The initiation codon, stop codon and the poly (A) signals (AATAAA) are in bold.

cells were collected by centrifugation at 500 × *g* at 4 °C for 10 min to remove supernatant. The cellular compartment fractionation of cells was carried out using the Qproteome Cell Compartment Kit (Qiagen, Germany), and the protein concentration was determined by the BCA protein assay kit (Pierce, USA). The cytosolic, membrane and nuclear proteins were analyzed by 12% SDS–PAGE, and then transferred to a pre-wet NC membrane for Western blot analysis using mouse anti-LvIMD polyclonal antibody (1:1000).

Hepatopancreas cells were also collected from healthy shrimps by centrifugation and then suspended in 1 ml L15 medium (Gibco, USA) containing 10% Penicillin–Streptomycin (Invitrogen, USA). The cell suspension (1 ml) was placed on one well of a 24-well culture plate that contained an additional 1 ml culture medium with 40% fetal bovine serum (FBS) (Invitrogen, USA), and each well was paved with a poly-L-lysine-treated cover-slip. After cultured at 28 °C for 6–8 h, monolayer adherent cells were formed and then rinsed with PBS, fixed with 4% paraformaldehyde at 37 °C for 30 min. Cells were then permeabilized with 0.2% Triton X-100 for 10 min, blocked in 5% BSA for 1 h at room temperature and incubated overnight at 4 °C with mouse anti-LvIMD polyclonal antibody diluted (1:200) in PBST containing 1% BSA. The next morning, cells were washed and incubated with FITC-conjugated goat anti-mouse IgG antibody (1:150) in PBS containing 1% BSA for 1 h at 37 °C. After stained with PI (propidium iodide) in PBS for 15 min at 37 °C, the cover-slip glasses were taken out, the cells were observed using Axio Imager Z1 (Carl Zeiss, Jena, Germany, http://www.zeiss.com) and analyzed using Axio Vision software version 4.6 (Carl Zeiss).

2.11. Cell culture, transfection and luciferase assays

Since no shrimp cell line is available at present, *Drosophila* Schneider 2 (S2) cells were used to analyze the effect of LvIMD on the activation of AMP genes. S2 cells were maintained at 28 °C in *Drosophila* serum-free medium (Invitrogen, USA) supplemented with 20 mM L-glutamine, 10% FBS (Invitrogen, USA) and 5% Penicillin–Streptomycin (Invitrogen, USA). Twenty-four hours prior to transfection, the cells were seeded in a 24-well culture plate in 2 ml medium at 1×10^6 cells/ml. Transfections were conducted using Effectene Transfection Reagent (Qiagen, Germany) following the protocols. In S2 cells, pAc5.1-LvIMD vector was co-transfected with pGL3-AttA or pGL3-PEN4 luciferase vector to analyze the Attacin A or PEN4 promoter activity. To test the importance of promoter κB sites in activating the promoter of AMP genes, pGL3-mAttA reporter and pGL3-mPEN4 reporter, in which the promoter κB sites were all deleted, were co-transfected with pAC5.1-LvIMD in S2 cells, respectively. The pRL-CMV luciferase vector was used as an internal control. Cells were harvested and lysed 36 h after transfection for examination of protein expression and dual luciferase activities using the dual luciferase reporter assay system (Promega, USA). Values were expressed as mean relative stimulations, and data from a representative experiment of three separate experiments were used to generate the figures.

3. Results and discussion

3.1. cDNA cloning and sequence analysis of LvIMD

A full-length cDNA of LvIMD (758 bp) was isolated by RACE-PCR, with an open reading frame (ORF) of 483 bp, a 5′ untranslated region of 36 bp, and a 3′ untranslated region of 264 bp (Fig. 1). Sequence analysis indicates that LvIMD contains a predicted death domain of 75 amino acids and belongs to the death domain superfamily that plays a very important role in host immune responses (Park et al., 2007). Further analysis indicates that invertebrate IMDs and vertebrate RIP1s all have a death domain in the C-terminus (Fig. 2). Moreover, RIP1s also have a kinase domain in the N-terminus (Fig. 3A), and this kinase domain is not necessary for NF-κB activation (Hsu et al., 1996). So the death domain is essential for the functions of these proteins. As for the kinase domain, it might emerge later in the evolution.

3.2. Phylogenetic analysis

To investigate the relatedness between LvIMD and its homologues, the conserved death domains were subjected to phylogenetic analysis. The result shows that these death domain containing proteins can be divided into three groups: group 1 contains vertebrate RIP1 proteins, group 2 contains insect IMD proteins, and group 3 includes LvIMD and a *Nematostella vectensis* predicted protein

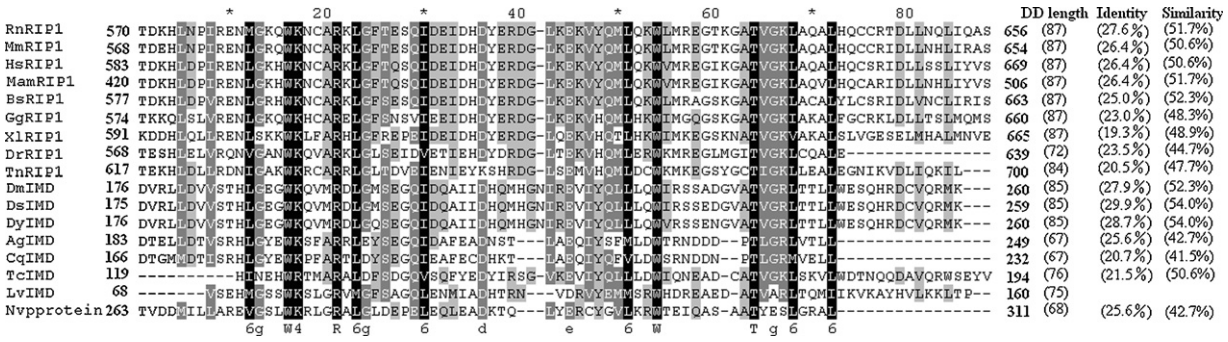

Fig. 2. Multiple sequence alignment of the death domains. LvIMD, *L. vannamei* IMD (accession no. FJ592176); AgIMD, *A. gambiae* IMD (accession no. XP_001688608); BsRIP1, *Bos taurus* RIP1 (accession no. NP_001030184); CqIMD, *Culex quinquefasciatus* IMD (accession no. XP_001861391); DmIMD, *D. melanogaster* IMD (accession no. NP_573394); DrRIP1, *Danio rerio* RIP1 (accession no. CAP09375); DsIMD, *D. simulans* IMD (accession no. AAQ64720); DyIMD, *D. yakuba* IMD (accession no. XP_002091920); GgRIP1, *Gallus gallus* RIP1 (accession no. BAC75633); HsRIP1, *Homo sapiens* RIP1 (accession no. NP_003795); MamRIP1, *Macaca mulatta* RIP1 (accession no. XP_001091986); MmRIP1, *Mus musculus* RIP1 (accession no. NP_033094); Nvpprotein, *N. vectensis* predicted protein (accession no. XP_001618286); RnRIP1, *Rattus norvegicus* RIP1 (accession no. NP_001100820); TcIMD, *Tribolium castaneum* IMD (accession no. XP_971829); TnRIP1, *Tetraodon nigroviridis* RIP1 (accession no. CAF91598); XlRIP1, *Xenopus laevis* RIP1 (accession no. NP_001089189). Alignment was done by Clustal X. Identical residues were indicated in black, and similar residues were in gray. The death domain length (DD length), identities and similarities were also indicated.

(Nvpprotein) (Fig. 3B). Group 3 is close to group 2 that contains most insect IMD proteins, including TcIMD (*Tribolium castaneum*), AgIMD (*Anopheles gambiae*), CqIMD (*Culex quinquefasciatus*), DyIMD (*D. yakuba*), DsIMD (*D. simulans*) and DmIMD (*D. melanogaster*). In the mean time, group 2 and group 3 form a larger cluster, which is distant from group 1 (Fig. 3B). The result also indicates that LvIMD is in a new group with an *N. vectensis* predicted protein (Nvpprotein) independent to current known IMD proteins. *N. vectensis* is an very ancient species belonging to the phylum Cnidaria, which has been in existence since about 1300 million years ago (Pinto et al., 2007). These results suggest that these death domain proteins have an ancient origin.

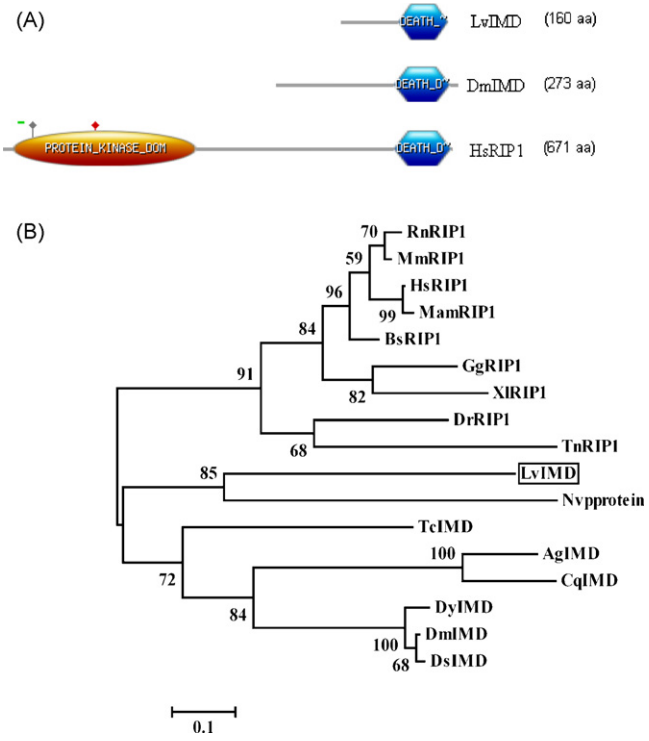

Fig. 3. (A) Schematic representation of the structural motifs of LvIMD, DmIMD and HsRIP1. (B) Phylogenetic tree based on the death domains (indicated in Fig. 2) showing the relationship between LvIMD and other known death domain containing proteins. The rooted tree was constructed by the"neighbor-joining" method and was bootstrapped 1000 times. 0.1 indicates the genetic distance. LvIMD is boxed.

3.3. Expression of LvIMD mRNA in healthy and immune-challenged shrimps

Semi-quantitative RT-PCR was performed to detect the distribution of LvIMD mRNA in healthy shrimps and its induced expression in tissues after immune challenge. The results indicate that in healthy shrimps, LvIMD mRNA is highly expressed in nerve, gill, intestine and pyloric caecum, and moderately expressed in eyestalk, hemocyte, muscle, heart and hepatopancreas (Fig. 4A). In hepatopancreas, LvIMD mRNA is induced by LPS (from *E. coli*) and Gram-negative *V. alginolyticus*, but not by Gram-positive *S. aureus*, Yeast (*S. cerevisiae*) or WSSV (Fig. 4B, (a)). In hemocytes, expression of LvIMD mRNA is strongly induced by LPS, *V. alginolyticus* and WSSV, but not by Yeast (*S. cerevisiae*) or *S. aureus* (Fig. 4B, (b)). In gill, LvIMD mRNA is not induced after immune challenge, though it is expressed at a high level (Fig. 4B, (c)). Induced expression of LvIMD gene in some special tissues (hepatopancreas and hemocytes) is similar to that of *Drosophila* IMD (Georgel et al., 2001), suggesting that LvIMD may have a function similar to *Drosophila* IMD.

3.4. LvIMD is localized in the cytoplasm of hepatopancreas cells

We have produced mouse polyclonal antibody to recombinant LvIMD. To determine whether anti-LvIMD polyclonal antibody could recognize native LvIMD, the recombinant LvIMD and native LvIMD in healthy shrimp tissues were detected by Western blot analysis. We found that the recombinant LvIMD reacted with mouse polyclonal antibody strongly (Fig. 5A, (b)), while native LvIMD was detected in hepatopancreas and intestine although the signal was weaker, but LvIMD was not detected in gill and hemocyte (Fig. 5A, (c)). These results indicated that the anti-LvIMD polyclonal antibody is able to recognize native LvIMD. Then, cellular localization of LvIMD was investigated by Western blot analysis and indirect immunofluorescence. The cytosolic, membrane and nuclear proteins from healthy shrimp hepatopancreas were separated by SDS–PAGE and analyzed by immunoblotting using mouse anti-LvIMD polyclonal antibody. A protein band around 22 kDa was detected in the cytosolic proteins (Fig. 5A, (d), lane 3), but not in the membrane or nuclear proteins (Fig. 5A, (d), lanes 1 and 2). To determine the localization of LvIMD at the single-cell level, immuno-localization assay was performed in shrimp hepatopancreas cells. We observed that the nuclei were stained red with PI and LvIMD was stained green (with FITC-conjugated (green) second antibody) in the cell cytoplasm (Fig. 5B). The cells incubated with pre-immune mouse serum were stained red with PI in the

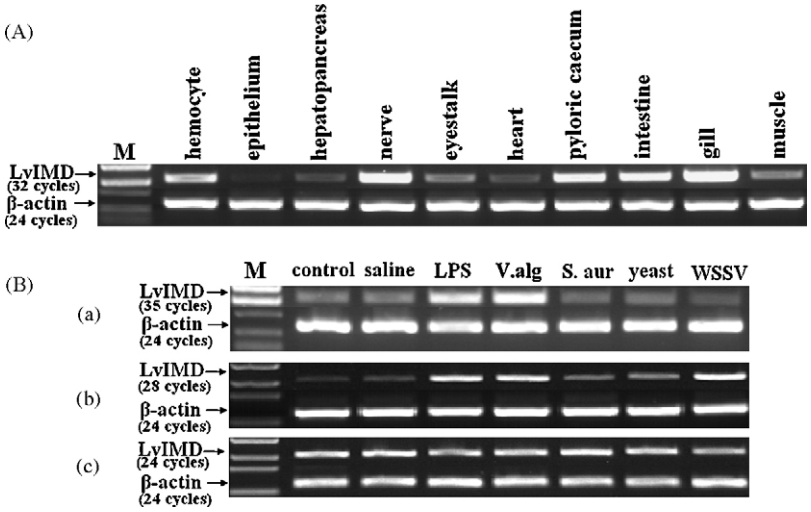

Fig. 4. Expression of Lv-IMD mRNA in healthy and immune-challenged shrimps. (A) Tissue distribution of LvIMD mRNA in healthy shrimps by RT-PCR analysis. (B) Induced expression of LvIMD mRNA in hepatopancreas (a), hemocyte (b) and gill (c) from shrimps injected with saline, LPS, Gram-negative *V. alginolyticus*, Gram-positive *S. aureus*, yeast (*S. cerevisiae*), or white spot syndrome virus (WSSV). The untreated shrimps were used as controls.

nuclei but no green fluorescence was observed in the cell cytoplasm (data not shown). Localization of the components in the IMD pathway is very important for our understanding of the protein function and molecular organization (Lemaitre and Hoffmann, 2007). *Drosophila* IMD is believed to be localized in the cytoplasm. Our results show that LvIMD is localized in the cytoplasm, and Western blot also shows that LvIMD is detected in the cytoplasmic proteins, but not in the membrane or nuclear proteins (Fig. 5). This result also correlates with LvIMD's function in the signal transduction pathway downstream of a trans-membrane receptor PGRP-LC.

3.5. Luciferase reporter assays

To investigate the function of LvIMD in activation of AMP genes and signal transduction pathway, luciferase report assays were performed. The results show that expression of LvIMD could induce *Drosophila* Attacin A expression by 6.4-fold and increase shrimp

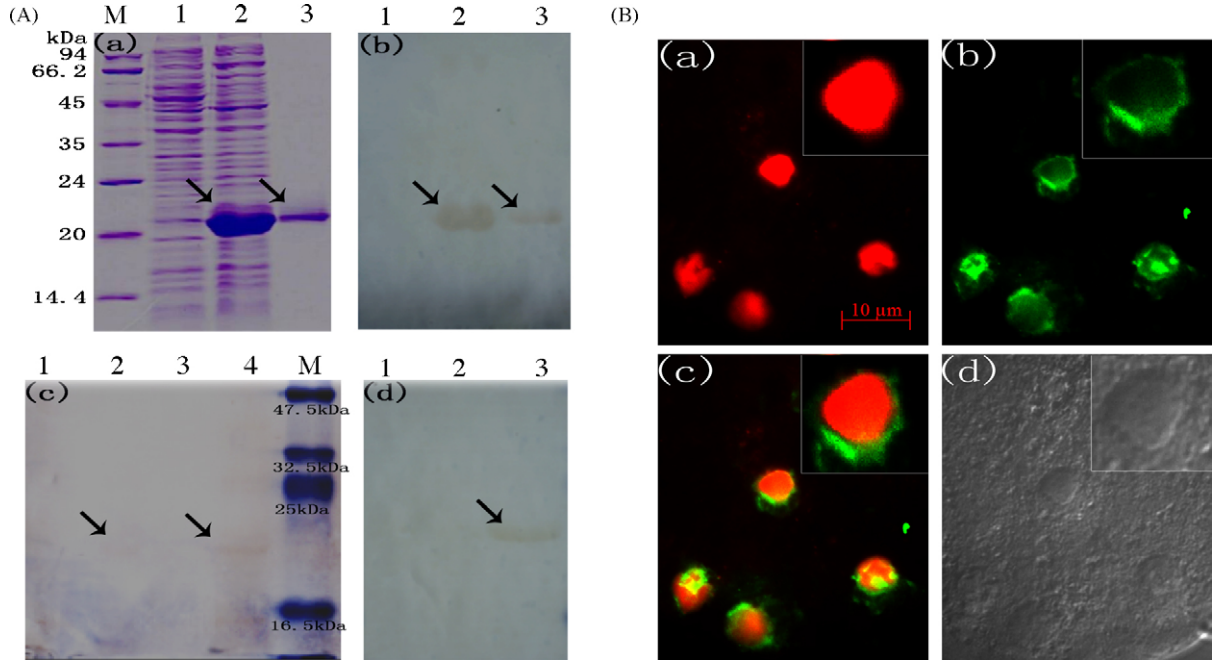

Fig. 5. Immunoblot analysis and immuno-localization of LvIMD. (A) SDS–PAGE and Western blot analyses of recombinant LvIMD and native LvIMD. (a) SDS–PAGE analysis of recombinant LvIMD. Lane 1: bacterial lysate without IPTG induction; lane 2: bacterial lysate with IPTG induction; lane 3: purified recombinant LvIMD (~5 μg). (b) Western blot analysis of recombinant LvIMD in (a) with mouse polyclonal anti-LvIMD antibody as the primary antibody. (c) Western blot analysis of native LvIMD in hemocyte (lane 1), intestine (lane 2), gill (lane 3), and hepatopancreas (lane 4). Proteins (40 μg total proteins) were separated on 15% SDS–PAGE and transferred to a NC membrane for Western blot analysis using mouse polyclonal anti-LvIMD antibody as the primary antibody. (d) Western blot analysis of native LvIMD in the membrane (lane 1), nuclear (lane 2), and cytoplasmic proteins (lane 3). Each well was loaded with 50 μg total proteins, and LvIMD was identified by Western blot using mouse polyclonal anti-LvIMD antibody. (B) Immuno-localization of LvIMD in shrimp hepatopancreas cells. LvIMD was visualized by FITC-labeled secondary antibody (green) in the cell cytoplasm, and the nuclei of hepatopancreas cells were stained with PI (red). (a) The nuclei of hepatopancreas cells were stained with PI; (b) LvIMD in the cell cytoplasm was stained green; (c) merge of (a) and (b); (d) differential interference contrast (DIC) view of hepatopancreas cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

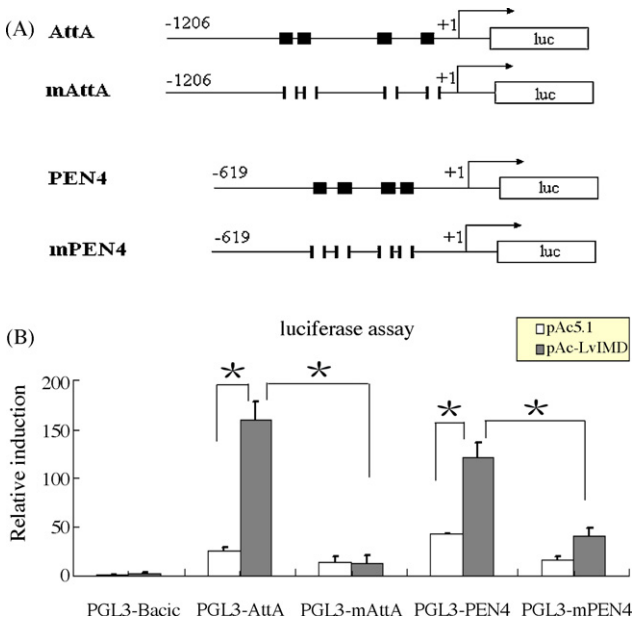

Fig. 6. Functional study of LvIMD in activation of *Drosophila* Attacin A and shrimp PEN4 promoters. (A) Schematic diagram of Attacin A and PEN4 promoter regions in the luciferase reporter gene constructs. The mutant promoters of Attacin A and PEN4 with the κB binding sites deleted are also indicated. +1 denotes the transcription initiation site for Attacin A and PEN4 genes, and −1 indicates 1 bp before the translation initiation site. Luc denotes the firefly luciferase reporter gene. The putative κB binding sites are indicated with fill boxes, while the deleted κB sites are indicated with open boxes. (B) Relative luciferase activity in the S2 cells. The bars indicate mean ± S.D. of the luciferase activity ($n = 3$). The significance of differences was calculated by the *t*-test (* indicates $p < 0.05$).

PEN4 expression by ∼3-fold (Fig. 6B). These results indicate that LvIMD could serve as an adaptor protein to activate expression of Attacin A and PEN4 in S2 cells. When the κB sites in the Attacin A and PEN4 promoters were deleted, activation of the Attacin A and PEN4 by LvIMD was dramatically reduced (Fig. 6B). So the κB binding sites in the promoters of Attacin A and PEN4 are important for their induced expression by LvIMD, a result correlates well with the studies of Busse and colleagues (Busse et al., 2007). In *Drosophila*, many immune-related genes including AMP genes contain clusters of κB sites in their promoters (Senger et al., 2004). Some shrimp AMP genes, such as PEN4 from the white shrimp *L. vannamei*, crustin-like AMP from the black tiger shrimp *Penseus monodon*, and ALF from *P. monodon*, also contain κB binding sites in their promoters (Amparyup et al., 2008; O'Leary and Gross, 2006; Tharntada et al., 2008). So it is very likely that LvIMD could induce expression of AMP genes in shrimp.

Our current study reveals that LvIMD can induce expression of some AMP genes, and its expression in hepatopancreas and hemocytes is in response to immune challenge, suggesting that a similar IMD pathway may regulate the expression of AMP genes and maybe some other immune-related genes in shrimp. These observations may help us to further understand innate immunity in invertebrates, particularly in shrimp, in response to pathogen infection. Further investigations should focus on functional studies of other components in the IMD pathway to demonstrate that the IMD pathway plays an important role in innate immune responses, especially in activation of AMP genes and some other immune-related genes, in shrimps to fight against pathogens.

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