GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Cloning and expression analysis of the 37-kDa laminin receptor precursor gene from *Hyriopsis cumingii*

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Genet. Mol. Res. 12 (4): 6130-6139 (2013)
Received July 18, 2013
Accepted October 10, 2013
Published December 2, 2013
DOI http://dx.doi.org/10.4238/2013.December.2.10

ABSTRACT. *Hyriopsis cumingii* is an economically important freshwater pearl mussel with high pearl quality that is endemic in China. Investigation of genes relevant to shell formation is important for increased pearl output. The substances that form mollusk shells are secreted by epithelial cells in the mantle, the proliferation of which influences secretion ability. This study focused on the proliferation-related 37-kDa laminin receptor precursor (*37LRP*) of *H. cumingii*. The full-length cDNA (1133 bp) encoding this 300-amino acid protein was cloned from the mantle. Quantitative fluorescence analysis showed that *37LRP* expressed in eight tissues, with the highest expression observed in the liver, and its expression pattern in the mantle reflected shell repair. During repair, *37LRP* expression was higher in the experimental shell repair group than that in the control group, exhibiting an initial increase followed by a decrease in expression, and returning to basal levels on completion of the repair. A similar trend was also observed with respect

to immunity and cellular metabolism. Expression of the 37LRP protein in the experimental group was significantly higher than that in the control group at the first and second days after shell injury. After 4 days, *37LRP* expression in the experimental group was lower than that in the control group. *In situ* hybridization revealed a strong positive signal corresponding to the *37LRP* mRNA at the horny grooves of the mantle, evagination, and in epithelial cells of the velum, which implicated these areas in the repair and formation of the cuticle, prismatic layer, and nacre.

Key words: *Hyriopsis cumingii*; Laminin receptor precursor; Expression analysis; *In situ* hybridization; Shell repair

INTRODUCTION

It is widely acknowledged that the substances forming mollusk shells are secreted by the epithelial cells in the mantle (McKenna et al., 2001), and that the shell is formed by ordered deposition of $CaCO_3$ crystals under the regulation of a protein matrix also secreted by the epithelial cells in the mantle (Donaldson et al., 2000). Fu et al. (2008) analyzed the 37-kDa laminin receptor precursor gene (*37LRP*) of *Pinctada fucata* and speculated that the encoded protein was involved in mantle epithelial cell proliferation and nacre secretion (Livak and Schmittgen, 2001). The *37LRP*, which is the precursor of the 67-kDa laminin receptor (LR), has two high-affinity laminin (LN) binding sites (Malinda et al., 1999): the G peptide (Castronovo et al., 1991; Taraboletti et al., 1993; Menard et al., 1997), and another, located at the C-terminal (Magnifico et al., 1996; Satoh et al., 1999). The 67-kDa LR also has a strong binding affinity for LN, which is an important non-integrated LR used as a marker for tumor detection, is important in cell proliferation and migration, and combines with various extracellular signals, such as extracellular matrix, viruses, and bacteria (Vacca et al., 1993; Sanjuan et al., 1996; Bai et al., 2009; Li et al., 2010). Based on its important role in cell proliferation, and the differences in the survival environments of marine and freshwater mussels, as well as the crystal form of nacre and prismatic layers, we studied the *37LRP* gene of *Hyriopsis cumingii.* This is the first report on *37LRP* in a freshwater mussel.

The *37LRP* cDNA was first cloned from the mantle of *H. cumingii* and then sequenced. Its expression during shell repair was analyzed by real-time polymerase chain reaction (PCR) to verify its function. The shell is usually made up of three layers: the corneum, the prismatic layer, and the nacreous layer. The outermost corneum (horny layer) is formed by secretion from horny groove cells between the middle and outer pleats. The internal prismatic layer is formed by secretion from epithelial cells in the mantle near the ventral region. The nacre layer is formed from secretion of epithelial cells in the mantle near the dorsal region (Suzuki et al., 1991; Kibbey et al., 1992; Awaji and Suzuki, 1995; Miyamoto et al., 1996; Nomizu et al., 1998; Coggin et al., 1999). *In situ* hybridization was performed to investigate the function of this gene in shell repair.

MATERIAL AND METHODS

Material and RNA isolation

Two-year-old *H. cumingii* were obtained from the Shanghai Aquaculture Engineering

Center (coastal). According to the requirements of the experiments, the mantle, blood, gill, foot, liver, kidney, intestine, and adductor muscle were isolated, snap-frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted from the mantle, blood, gill, foot, liver, kidney, intestine, and adductor muscle using Trizol according to manufacturer instructions (TaKaRa, Dalian, China).

Amplification, cloning, and sequencing of *37LRP* cDNA

Primers of 5'- and 3'-rapid amplification of cDNA ends (RACE) were designed using Primer Premier 5.0 software and the BLAST program according to the labeled expressed sequence tags in the constructed mantle cDNA library (Ardini et al., 1998) and synthesized (Sangon, Shanghai, China). The 3'-end was amplified using the TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa) with the upstream primer L-F (5'-CCGTTCTCAAGTTTGCCTCC-3') and the downstream primer (M13 Primer kit) M4 (5'-GTTTTCCCAGTCACGAC-3'). The 5'-end was amplified using the SMART™ RACE cDNA Amplification Kit (BD) and the Advantage® 2PCR Enzyme System kit (Clontech, Santa Clara, CA, USA). The downstream primer (L-R), designed using Primer Premier 5.0, was 5'-GACGAAGCACCTCCCTTGCCAGC-3'. The upstream primer from the kit was 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTAT CAACGCAGAGT-3' (long); 5'-CTAATACGACTCACTATAGGGC-3' (short). The RACE-amplified products were extracted and separated by 1.5% agarose gel electrophoresis. The PCR product of the target gene was purified using the TIANgel Midi Purification Kit (TIANGEN, Beijing, China). The recombinant plasmid was obtained by ligating the purified RACE products with the pMD19-T vector (TaKaRa). The resulting construct was transformed into competent *Escherichia coli* DH5α cells and cultured on Luria-Bertani plates [containing ampicillin, isopropyl-b-D-1-thiogalactopyranoside (IPTG), and X-gal]. Blue/white screening was used to detect the inserted fragment. The positive clone obtained was sequenced (Shanghai Branch, Beijing Luhe, China).

Sequencing

The similarity of the nucleotide and amino acid sequences was analyzed using the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST). The correct open reading frame (ORF) was determined using the ORF Finder program (http://www.ncbi.nlm.nih.gov/projects/gorf/) (Gloe et al., 1999). The physical parameters of the amino acid sequences were predicted using ProtParam and SignalP 4.0 servers. Multiple comparisons of the amino acid and coding nucleotide sequences were performed using ClustalW1.8 and BioEdit softwares.

Quantitative real-time PCR (qPCR) analysis of *37LRP* expression in eight tissues

Total RNA was extracted from the blood, gill, mantle, foot, adductor muscle, liver, kidney, and intestine as previously described. qPCR analysis of *37LRP* expression was conducted using the following primers designed using Primer Premier 5.0: *37LRP*, upstream primer Lrt-F: 5'-TCACCTGGGAGCCACAAA-3' and downstream primer Lrt-R: 5'-GCAGAGATGAC GCAAACATC-3'; β-actin-F: 5'-ACGGATAACACAAGGAAAGGAAAC-3' and β-actin-R: 5'-ATGGATGGAAACACGGCTCT-3'. β-actin expression in *H. cumingii* was analyzed as the internal reference. Each sample was analyzed in triplicate using the iScript™ cDNA Synthesis

Kit and iQ™ SYBR® Green Supermix (BIO-RAD, USA) in a 20-μL reaction system containing 10 μL iQTM SYBR® Green Supermix, 0.8 μL 10 M primers, 6.8 μL RNase-free water, and 1.6 μL cDNA. Amplification was performed using a BIO-RAD CFX96™ under the following conditions: 95°C (pre-denaturation) for 30 s, 95°C (degeneration) for 5 s, and 60°C (annealing) for 30 s (40 cycles) with a temperature gradient of 0.5°C per cycle from 65° to 95°C. The relative expression of the target gene and reference gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Landowski et al., 1995) and analyzed with the SigmaPlot10.0 software.

qPCR analysis of *37LRP* expression during shell repair

Healthy adult *H. cumingii* (N = 60) of similar shape were randomly and equally divided into the experimental shell repair and control groups. The shell of *H. cumingii* in the experimental group was damaged (a V-shaped gap near the edge of the adductor muscle was formed) and randomly divided into 10 groups (N = 3 per group). The control group was also divided into 10 groups. *H. cumingii* from the experimental and control groups (N = 3 from each group for each time point analyzed during the shell repair process) were placed together in a mesh bag, suspended, and maintained at the Shanghai Aquaculture Engineering Center (Binhai). The mantle and liver were collected at specific time points after shell damage (2, 6, 12, 24 h, and 2, 4, 7, 15, and 30 days). Tissues were snap-frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted for qPCR analysis of *37LRP* expression during shell repair.

In situ hybridization of *37LRP* in the mantle

The mantle was collected from living *H. cumingii*, fixed in 4% paraformaldehyde for 24 h, rinsed with 1X phosphate-buffered saline (prepared with diethylpyrocarbonate-treated water), dried, fixed with methanol, and stored at -20°C. The fixed tissues were dehydrated, embedded in paraffin, and sectioned for evaluation. For hybridization, sections were incubated with hybridization solution and probe at 55°C overnight and then incubated with NBT-BCIP in the dark for 12 h. Sections were dehydrated, mounted, and photographed. The DIG RNA Labeling Kit (SP6/P7) and DIG Nucleic Acid Detection Kit (Roche, Mannheim, Germany) were used for the synthesis and detection of the digoxin-labeled RNA probe, respectively.

RESULTS

cDNA and amino acid sequence of *37LRP* and homology analysis

The full-length cDNA sequence (1133 bp) of the *37LRP* (accession No. JX441866) was obtained after amplification and found to comprise a 5'-untranslated region (UTR) of 71 bp, a 3'-UTR of 159 bp, and an ORF of 903 bp, encoding 300-amino acid residues (Figure 1). The total atomic weight was 4625 and the molecular formula was $C_{1477}H_{2291}N_{395}O_{452}S_{10}$ (molecular weight was approximately 33.1 kDa). Comparisons of the full-length *H. cumingii* 37LRP protein with that of other species using the ClustalW software (Figure 2) showed a high degree of similarity. The amino acid sequence of *H. cumingii 37LRP* exhibited the highest similarity (80%) with those of *P. fucata*, *Bos taurus*, *Chlorocebus aethiops*, and *Rattus norvegicus* (73%), and with *Homo sapiens* and *Xenopus laevis* (72%).

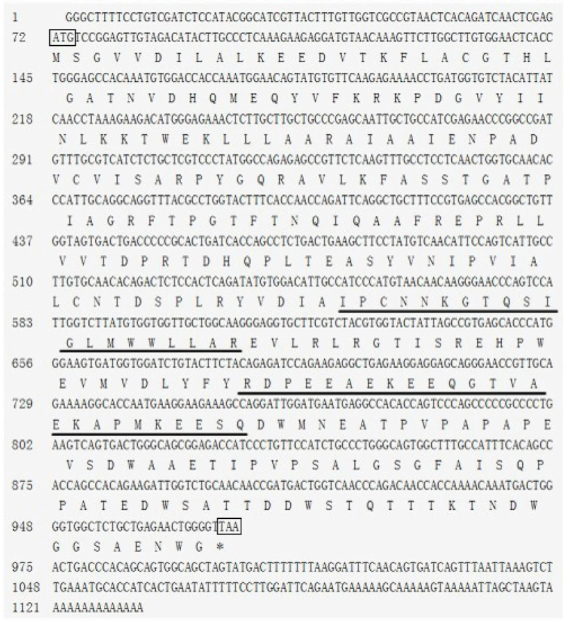

Figure 1. Full-length cDNA and deduced amino acid sequences of *37LRP* from *Hyriopsis cumingii*. Box: initiator codon and stop codon; underlined: laminin-binding sites.

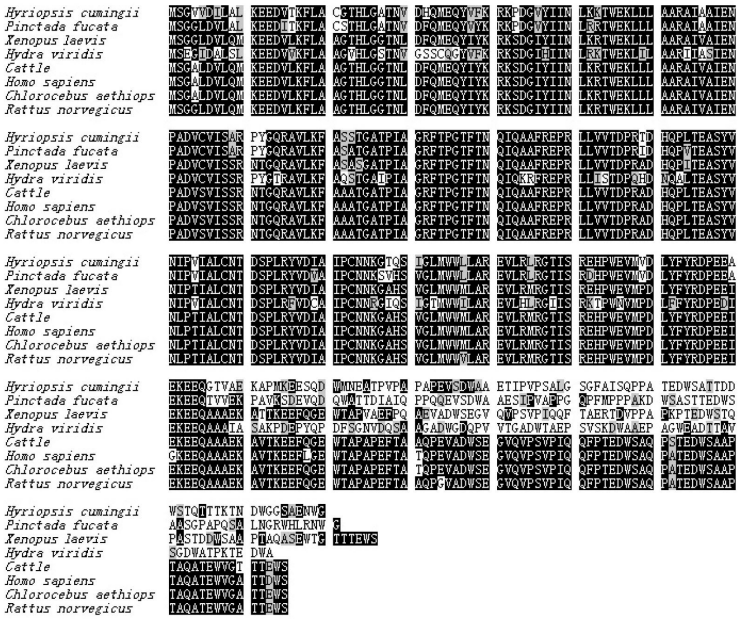

Figure 2. Comparison of amino acid sequences at the putative LN binding sites in the *37LRPs* among *Hyriopsis cumingii* and other species. The black line above the acid sequences shows the two LN binding sites in *37LRPs*.

Expression of *37LRP* in eight tissues in *H. cumingii*

Quantitative PCR analysis showed that *37LRP* was expressed in the mantle, blood, gill, foot, liver, kidney, intestine, and adductor muscle tissues. Highest expression was detected in the liver, lowest in the blood, and similarly low levels in all other tissues evaluated (Figure 3).

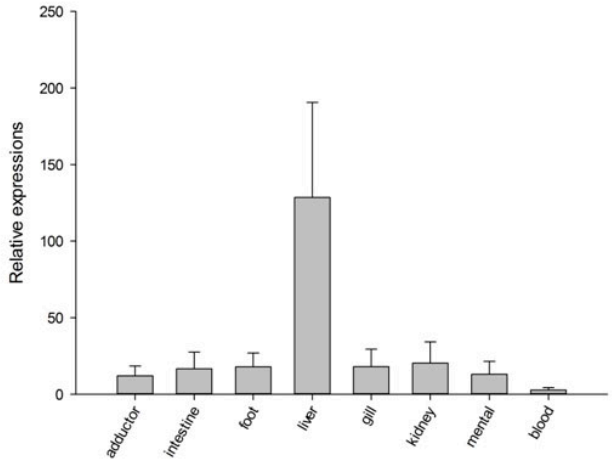

Figure 3. Tissue distribution of *37LRP* mRNA in *Hyriopsis cumingii*.

Expression of *37LRP* in the liver and mantle during shell repair

During shell repair, expression of *37LRP* in the liver was initially increased, followed by decreased expression and a subsequent increase. Expression in the experimental group was significantly higher than that in the control group during the first and second days after shell damage, and was lower than that in the control group after four days (Figure 4).

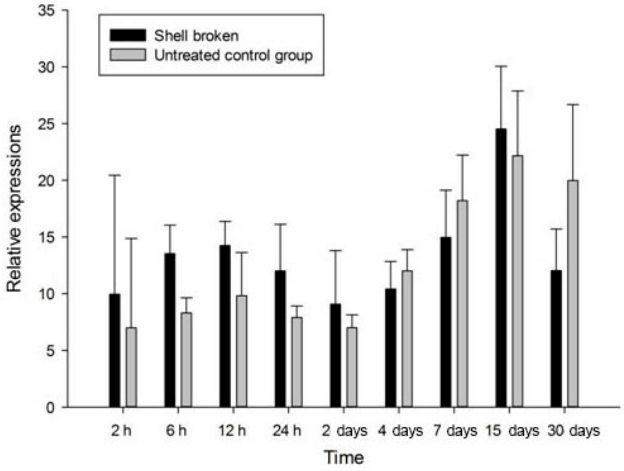

Figure 4. Relative expression of *37LRP* in liver after shell damage at each sampling time point.

Expression of *37LRP* in the mantle initially increased and subsequently decreased during shell repair. Its expression in the experimental group was higher than that in the control group at each time point, with highest expression at 24 h and recovery to normal levels upon repair completion (Figure 5). These data indicated that *37LRP* plays an important role in shell repair and mantle growth.

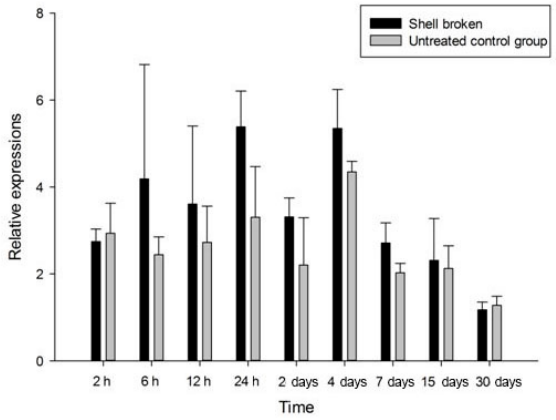

Figure 5. Relative expression of *37LRP* in the mantle after shell damage at each sampling time point.

In situ hybridization of *37LRP* in the mantle

A strong positive signal for expression of *37LRP* was observed in the external epithelial cells in the mantle, internal and external epithelial cells in the evagination, and in the epithelial cell layer in the peritoneum (Figure 6).

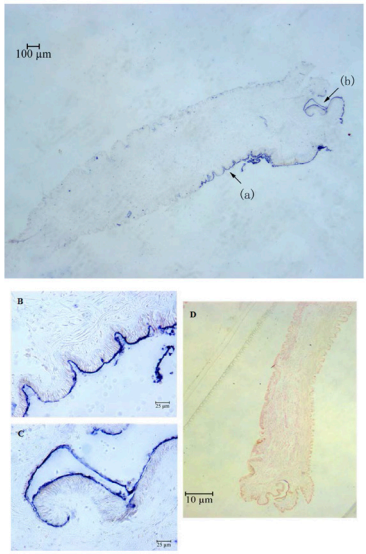

Figure 6. *In situ* hybridization of *37LRP* in tissues. **A. B. C.** Positive *in situ* hybridization. **D.** Negative control. B is the magnified image of the arrow (a) in A; C is the magnified image of the arrow (b) in A. Blue-violet indicates a positive hybridization signal.

DISCUSSION

The 37-kDa LRP is the precursor of the 67-kDa LR, which is a highly conserved protein with many important functions. The cloned *37LRP* from *H. cumingii* exhibited high similarity with those of other species, such as *P. fucata* (80%) and mammals and amphibians (over 70%), which confirmed its high degree of conservation. Ardini et al. (1998) analyzed the relationship between *37LRP* and its homologous protein, ribosomal protein p40, and found that both had a common ancestor, although *37LRP* is slightly smaller than p40 and has a particular palindromic sequence, LMWWML, with the ability to combine with LN (Acosta-Salmon and Southgate, 2005). The *37LRP* of *H. cumingii* has two LN binding sites, one being designated the G peptide, which contains the particular palindromic sequence and binds LN with high-affinity, and another, located at the C-terminal (residues 205-229), which combines with the YIGSR sequence of the LN β1 chain (Awaji and Suzuki, 1995; Miyamoto et al., 1996) (Figure 2). The amino acid sequences are not identical in different species, although a high degree of conservation was observed among vertebrates and slight variation was observed among invertebrates. For *H. cumingii*, *P. fucata*, and *Drosophila*, the palindromic sequence in the G peptide was altered from LMWWML to LMWWLL with only one amino acid residue difference. This indicates that the LN binding function of *37LRP* is important, and is therefore evolutionarily conserved.

qPCR results showed that *37LRP* was generally expressed in various tissues of *H. cumingii*, with highest expression in the liver and lowest in blood. In order to explore the influence and function of *37LRP* on shell and pearl formation, shell damage was inflicted in *H. cumingii* and *37LRP* expression in the mantle and liver were analyzed by qPCR. The result showed that expression of *37LRP* in the experimental group and the control group exhibited the same trend in the liver from the early stage of the injury to completed repair. This trend was characterized by an initial increase followed by decreased expression and a subsequent increase. The liver is a tissue associated with abundant secretions and high metabolic activity. At the early stage of injury, the liver can enhance its anabolic function and generate an immune response resulting in increased *37LRP* expression. The intermediate period was associated with shell repair and reduced liver metabolism, resulting in decreased *37LRP* expression. Once the shell repair was complete, liver metabolism recovered to normal levels resulting with concomitant recovery of normal *37LRP* expression levels. In the mantle, expression of *37LRP* was initially increased following shell injury and then decreased. At 2 h post-injury, there was no difference in the expression of *37LRP* between the experimental and control groups. Between 6 h and 15 days, its expression was significantly higher in the experimental group than that in the control group. Subsequently, expression was recovered to normal levels due to basic completion of the repair of the shell and mantle, with no significant difference between the two groups. Studies have shown that the secretory capacity of the mantle is recovered following injury (Gardner et al., 2011) with development of the epithelial and glandular cells for 15 days post-injury, which was consistent with our results.

In situ hybridization studies showed that *37LRP* mRNA was expressed in the horny groove (Figure 6C), that is, the external epithelial cells in the central pleats and the internal epithelial cells in outer pleats. The surface of this structure secretes the polysaccharide-protein complexes with the adhesion ability to form the cuticle (Sudo et al., 1997). It was speculated that the expression of *37LRP* was related to cuticle formation. As shown Figure 6A [box (a)

and arrow], *37LRP* mRNA was also expressed in the external epithelial cells in the outer pleats and the border membrane, which are considered to be associated with the formation of the shell prismatic and nacreous layers (Sudo et al., 1997; Tsukamoto et al., 2004; Takeuchi and Endo, 2006; Fu et al., 2008). *In situ* hybridization analysis of *37LRP* expression in *P. fucata* performed by Fu et al. (2008) demonstrated a strong positive signal at the edge of the mantle and in the epithelial cells of the border membrane. Expression was predominantly distributed in the flat epithelial tissue, in which cells showed stronger secretion and proliferation. The *37LRP* has been shown to be involved in epithelial cell proliferation and nacre secretion (Gloe et al., 1999), which is consistent with its detection in the mantle by *in situ* hybridization. Combined with the fluorescence quantitative results in broken shells, the 37-kDa LRP can be considered to be involved in the formation of the cuticle and the prismatic and nacreous layers.

ACKNOWLEDGMENTS

Research supported by the Shanghai University Knowledge Service Platform (#ZF1206).

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