Short communication

Cloning and characterization of 37 kDa laminin receptor precursor in pearl oyster, *Pinctada fucata*

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Abstract

A 1063 bp cDNA clone encoding a putative 37 kDa laminin receptor precursor (37 kDa LRP) is isolated from the mantle tissue of pearl oyster, *Pinctada fucata*. The amino acid sequence predicted from the cDNA sequence is 301 residues long, with a calculated molecular mass of 33.5 kDa. RT-PCR analysis shows that 37 kDa LRP mRNA is especially highly expressed in the mantle while widely expressed in several tissues. In situ hybridization analysis reveals that 37 kDa LRP is expressed in the outer epithelial cells of the mantle edge, suggesting its involvement in cell proliferation and secretion in *P. fucata*. The identification and characterization of 37 kDa LRP in the pearl oyster will help us to further understand the signal transduction in the processes of mantle epithelial cell proliferation and tissue formation.

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1. Introduction

The mantle epithelial cells are directly responsible for pearl formation in pearl oyster, *Pinctada fucata*, by the deposition of aragonitic CaCO₃ crystals and the secretion of organic protein matrix to hold them [1]. Thus the culture of outer epithelial cells of the mantle tissue is important to study the pearl-formation process [2]. However, the establishment of cell lines from marine invertebrates has encountered obstacles, mainly due to the low level of cell proliferation and viability and there is no single permanent cell line from pearl oyster to be successfully established [3–5].

The 67 kDa laminin receptor (67 kDa LR), also termed the 67 kDa laminin binding protein, is a non-integrin receptor of laminin. The mature 67 kDa receptor is formed by homo- or hetero-dimerization of the acylated 37 kDa precursor (37 kDa LRP), by non-covalent bonds [6]. Laminin is the main non-collagenous glycoprotein found in the basement membrane. Various laminin isoforms are involved in many physiological and pathological processes [7–9]. Two laminin binding sites were identified on the 37 kDa LRP [10], the first is called G peptide [11–13], and the second is at the carboxy terminal [14,15]. The 67 kDa laminin receptor therefore recognizes various binding sites on laminin which are different from the sites recognized by integrins [10,12,14,16], allowing for higher overall binding affinity and different signal transduction options. The receptor is involved in several physiological processes such as implantation [17], invasive phenotype of trophoblastic tissue [18], angiogenesis [19,20], T-cell biology [16] and shear stress-dependent endothelial nitric oxide synthase expression [15]. It has also been found that the 67 kDa laminin receptor is identical to the oncofetal antigen protein that is expressed by tumors [21]. The overex-
expression of the receptor has been implicated in laminin-induced cell attachment and migration, as well as in tumor growth, invasion and metastasis [22–25]. In the previous attempts to establish oyster cell line and culture pearl-sac in vitro, a migration and proliferation along the extracellular matrix of mantle epithelial cell was observed [26,27]. Thus, an investigation on the 37 kDa LRP in P. fucata was undertaken.

In this study, a putative 37 kDa LRP was first cloned by RT-PCR and rapid amplification of cDNA ends (RACE) from the mantle tissue of pearl oyster P. fucata. The expression sites of this laminin receptor were examined by RT-PCR and in situ hybridization analysis.

2. Materials and methods

2.1. Preparation of the total RNA

Live individuals of adult oyster P. fucata were obtained from GuoFa Pearl Farm in Beihai, Guangxi Province, China. Total RNA was extracted from mantle tissue with RNAzol RNA isolation kit (Biotex, USA) following the user’s manual. RNA integrity was determined by separation on a 1.2% formaldehyde-denatured agarose gel and stained with ethidium bromide. The quantity of RNA was determined by measuring OD260nm with an Ultrospec 3000 UV/Visible Spectrophotometer (Amersham, USA).

2.2. RT-PCR and RACE

Single-stranded cDNA for all rapid amplification of cDNA end reactions were prepared from the mantle total RNA. 5’-RACE and 3’-RACE were conducted according to the manufacturer’s instructions, using the SMART RACE cDNA Amplification Kit (Clontech) and Advantage 2 cDNA Polymerase Mix (Clontech). The gene-specific primers 5RP (5’-GAT GGT GCC GAG TAT GTG GTA-3’) for 5’-RACE and 3RP (5’-GAY TTY CAR ATG GAR CAG TAT G-3’) for 3’-RACE, respectively, were prepared based on the conserved regions of 37 kDa LRP. The RT reaction and following PCR were performed according to the manufacturer’s instructions on a T gradient Thermocycler (Biometra, Germany). PCR cycles were conducted at the following setting: denaturation at 95 °C for 30 s, 47 °C for 45 s, and then elongation at 72 °C for 10 min. Amplification of GADPH was as the internal reference in PCR with primers GADPH1 (5’-GAT GGT GCC GAG TAT GTG GTA-3’) and GADPH2 (5’-CG TTG ATT ATC TTG GCC AGT G-3’), and negative controls were carried out in the absence of cDNA template to examine the cross contamination of the samples.

2.3. Sequence and phylogenetic analyses

Sequence similarity searches were performed with the BLAST program in GenBank, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Multiple alignments were created using the Clustal W program. The phylogenetic tree was constructed using Clustal W program with the Neighbor-Joining (NJ) algorithm.

2.4. Tissue expression analysis by RT-PCR

Tissue expression of the oyster 37 kDa LRP mRNA was investigated by RT-PCR. Total RNA was isolated from mantle, foot, gill, gonad, digestive gland, and adductor muscle tissues of the adult individual of P. fucata. Equal quantities (2 μg) of total RNA from different tissues were reverse transcribed into cDNA as described above. The generated cDNA was used as the template for PCR with primers of RP1 (5’-GAC TTT CAG ATG GAG CAG TAT G-3’) and RP2 (5’-GGT CAC ACAA CAG ACG AGG CTC-3’). The reaction was performed with a denaturation at 95 °C for 5 min, 20 cycles of 95 °C for 30 s, 47 °C for 30 s, 72 °C for 45 s, and then elongation at 72 °C for 10 min. Amplification of GADPH was as the internal reference in PCR with primers GADPH1 (5’-GAT GGT GCC GAG TAT GTG GTA-3’) and GADPH2 (5’-CG TTG ATT ATC TTG GCC AGT G-3’), and negative controls were carried out in the absence of cDNA template to examine the cross contamination of the samples.

2.5. In situ hybridization of 37 kDa LRP

The mantle was removed from the adult P. fucata and immediately fixed in 4% paraformaldehyde containing 0.1% DEPC (Sigma, USA) overnight. In situ hybridization of oyster 37 kDa LRP mRNA was carried out on frozen sections of the mantle. Digoxigenin-labeled DNA probes were generated from the plasmid with the insertion fragment encoding partial sequence of LRP using a DIG High Prime DNA Labeling and Detection Starter kit I (Roche, Germany). In situ hybridization was performed as described previously with some modification [28]. The hybridization temperature was 45 °C.

3. Results

3.1. Cloning and sequence analysis of cDNA sequence encoding 37 kDa LRP

A cDNA product of 883 bp was obtained by 3’-RACE using total mantle RNA as the template. Based on the sequence, a gene-specific primer was synthesized for...
5'-RACE (5RP). To confirm the sequence obtained by RACE, two-specific primers (FP1 and FP2) corresponding to 5'- and 3'-UTR sequences of putative 37 kDa LRP mRNA were designed and RT-PCR was performed. The PCR products were cloned and sequenced, which matched well the sequence expected from the results of 5'- and 3'-RACE.

As shown in Fig. 1, the complete cDNA sequence including the poly-A tail is 1063 bp. It contains a 67 bp 5'-untranslated sequence, an ORF consisting of 903 bp, a TGA stop codon, a 77 bp 3'-untranslated sequence, and a poly-A tail of 13 nucleotides. A putative polyadenylation signal (AATAAA) was recognized at position 1042 on the upstream of the poly-A tail. This cDNA sequence has been deposited in GenBank with Accession No. EF427937.

The ORF of this cDNA clone encoded a protein consisting of 301 amino acid residues, which shared high similarity with 37 kDa LRP in other species. Comparison of two conserved laminin binding sites of 37 kDa LRP with those from the other organisms is shown in Fig. 2.

A phylogenetic tree was then constructed based on the alignment of the full length amino acid sequences of 37 kDa LRP in *P. fucata* and the other known 37 kDa LRPCs by Clustal W program with NJ algorithm (Fig. 3).

3.2. Gene expression analyses of oyster 37 kDa LRP

To investigate the functions of oyster 37 kDa LRP in vivo, tissue-specific expression of 37 kDa LRP mRNA was examined by RT-PCR with primers RP1 and RP2. Samples of total RNA were prepared separately from mantle, foot, gill, gonad, digestive gland, and adductor muscle tissues of *P. fucata*. The results revealed that 37 kDa LRP transcript was especially highly expressed in mantle while widely expressed in several tissues, suggesting its multifunctional influence on physiological and pathological processes in *P. fucata* (Fig. 4).

To determine the more precise expression site of 37 kDa LRP mRNA in mantle, *in situ* hybridization was performed on frozen sections of the mantle tissue. Strong hybridization signals for 37 kDa LRP mRNA were detected in the outer epithelial cells along the mantle edge (Fig. 5).

4. Discussion

The complete cDNA sequence of 37 kDa LRP including the poly-A tail is 1063 bp, encoding a protein consisting of 301 amino acid residues. The protein has a calculated

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Fig. 1. The cDNA sequence and deduced amino acid sequence of 37 kDa LRP from pearl oyster, *Pinctada fucata*. The start codon, the stop codon, and the putative polyadenylation signal (AATAAA) are boxed. Two putative laminin binding sites are underlined, and the putative transmembrane domain is in shadow.
molecular mass of 33.5 kDa, which is consistent with those in other species, and a little smaller than the identical protein ribosomal p40 [29–31]. Phylogenetic analysis on the relationship between the 37 kDa LRP and p40 indicated that all of these protein sequences were derived from orthologous genes and that the 37 kDa LRP is indeed a ribosomal protein that acquired laminin-binding capability with the appearance of the palindromic sequence LMWWML during evolution [32].

Two laminin binding sites were identified on 37 kDa LRP. One is called G peptide (Fig. 2a), which binds specifically with high affinity (\(K_d = 51.8\) nM) to laminin. It is of interest to note that G peptide contains a palindromic sequence of six amino acids, LMWWML, as the actual binding site. Another is at the carboxyl terminal (amino acids 205–229) (Fig. 2b), which binds to the peptide YIGSR on \(\beta 1\) chain of laminin [14,15]. Comparison of these two con-
served laminin binding sites with those from other organisms showed that these two laminin binding sites were similar, but not the same, with those of other species. There are still several amino acid residues different from the consensus sequence, compared with the high conserved sequences in vertebrates. The palindromic sequence LMWWML was observed as LMWWLL with a mutation of M to L in P. fucata, Drosophila melanogaster, and Acanthamoeba healyi, suggesting that they are genetically close in relationship during evolution. Comparison of full length of these 37 kDa LRPs revealed that all vertebrate proteins are nearly identical, including the C-terminal region, which is the most divergent in other invertebrates. A phylogenetic tree based on it shown in Fig. 3 also illustrates the high identity of vertebrate 37 kDa LRPs with invertebrate 37 kDa LRPs.

According to the previous reports, a membrane-associated domain resides within the region of the 37 kDa LRP from residue 86 to 101. These data defined the two-third of carboxyl terminal of the 37 kDa LRP to be extracellular. Analysis of signal peptide cleavage site carried out using the SignalP v3.0 program showed the absence of a signal peptide, which was consistent with previous reports. The question about how the 67 kDa-LR located finally on the cell membrane is still to be answered. The absence of an N-terminal leader sequence was observed also in some other membrane-associated proteins previously. Notable examples are the transferrin receptor [33] and the asialoglycoprotein receptor [34]. The mechanisms responsible for the integration, insertion, or association of these unusual receptors to the cell membrane are not yet elucidated. One possible membrane association mechanism is that another predicted polypeptide, either alone or in conjunction with the 37 kDa LRP, may possess a signal peptide or a signal patch formed by the juxtaposition of amino acids that are physically separated before protein folding or before association of the two subunits.

Thirty-seven kilo Dalton LRP is considered to be involved in several physiological processes such as implantation, invasive phenotype of trophoblastic tissue, angiogenesis, T-cell biology, mechanical sensing, as well as cell attachment and migration. The tissue expression analysis based on RT-PCR showed that 37 kDa-LRP transcript is widely expressed in mantle, foot, gill, gonad, digestive gland, and adductor muscle tissues of the adult individual. The tissue expression analysis carried out using in situ hybridization, strong hybridization signals for 37 kDa LRP mRNA were detected in the epithelial cells along the mantle edge. The mantle, that is an important part of the molluscan body, is responsible for the formation of the shell. The outer epithelial cells of mantle are active in proliferation and tissue remodeling. Also secretory cells are found in the epithelium layer in this area. As shown in Fig. 5, hybridization signals of 37 kDa LRP mRNA are arranged along the mantle edge in a very regulated way, like an outline sketching of the mantle, suggesting its involvement in the function of these epithelial cells. According to previous reports, 67 kDa LR, the mature form of 37 kDa LRP, could be an upstream functional receptor of the MAPK pathway. Some researches revealed that the basal phosphorylation extent of ERK, JNK and p38 was significantly higher in cell lines expressing reduced 67 kDa LR [35]. MAPK pathway is involved in a variety of processes in cell cycle, such as proliferation and differentiation. The role of 67 kDa LR in this pathway, however, still needs to be further examined.

Pinctada fucata is predominantly used for pearl production in marine water in China as it is abundantly distributed throughout the saltwater habitats of the country. During pearl cultivation, the quality of pearl is directly related to the function of the epithelial cells. Detailed physiological studies on function of epithelial cells, however, have not been conducted adequately, mainly due to unavailability of constantly cultured cell in vitro. In this study, 37 kDa LRP of the pearl oyster P. fucata was first cloned and its tissue expression was examined to deduce the function of it in mantle epithelial cells. The results can be helpful to understand the signal transduction in the mantle epithelial cells, and can provide primary support for the cultivation of mantle epithelial cell and tissue regeneration in vitro.

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References


