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A β-integrin from sea cucumber *Apostichopus japonicus* exhibits LPS binding activity and negatively regulates coelomocyte apoptosis

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ABSTRACT

Integrins are a family of membrane glycoproteins, which are the major receptors for extracellular matrix and cell–cell adhesion molecules. In this study, a 1038 bp sequence representing the full-length cDNA of a novel β-integrin subunit (designated as *AjITGB*) was cloned from *Apostichopus japonicus*by using combined transcriptome sequencing and RACE approaches. The deduced amino acid sequence of AjITGB shared a conserved tripeptide Arg-Gly-Asp (RGD) binding domain with an S-diglyceridecysteine or N-Palm cysteine residue (C^{31}), a transmembrane domain, and a β-integrin cytoplasmic domain. Spatial distribution analysis showed that *AjITGB* was constitutively expressed in all tested tissues with dominant expression in the muscles and weak expression in the respiratory tree. The pathogen *Vibrio splendidus* challenge and LPS stimulation could both significantly down-regulate the mRNA expression of *AjITGB*. Functional investigation revealed that recombinant AjITGB displayed higher LPS binding activity but lower binding activity to PGN and MAN. More importantly, knockdown of *AjITGB* by specific siRNA resulted in the significant promotion of coelomocyte apoptosis *in vitro*. Results indicated that AjITGB may serve as an apoptosis inhibitor with LPS binding activity during host–pathogen interaction in sea cucumber.

1. Introduction

Cell adhesion molecules in all multicellular animals have a long extracellular domain, a transmembrane region, and a short intracellular region [1,2]. Adhesion molecules play important roles in maintaining the integrity of the organism functions, cell recognition, signal transduction and transfer, cell proliferation, differentiation, maturation, walking in the regulation, inflammation and wound healing [3]. Cell adhesion molecules can be divided into five large families, namely, integrin, calcium-dependent adhesion factors, selectin, immunoglobulin protein superfamily, and CD44, according to their structure and function [4].

Integrins are heterodimeric cell surface receptors with α and β subunits. These molecules participate in mediating cell–matrix and cell–cell interactions as well as in bidirectional signal transduction [5]. Extracellular matrix components, such as collagen and fibronectin molecules, are the main ligands of integrin, which are recognized because of their specific amino acid sequence including Arg-Gly-Asp (RGD). This RGD domain is usually located at the β subunit and exerts its adhesion function in a metal ion-dependent manner [6,7]. After its specific binding, integrin can regulate intracellular signal transduction by enhancing phosphorylation of tyrosine in the intracellular protein [8]. Therefore, integrin β subunit has received much attention both in studies on vertebrates and invertebrates, especially in its functions in innate cellular immune responses such as phagocytosis and apoptosis [9,10]. Some studies have indicated the peptide containing RGD can suppress the binding between integrin and extracellular matrix, thereby blocking integrin-mediated platelet aggregation, infection, inflammation and other biochemical processes [11–14]. In mice, Group A *Streptococcus* (GAS) entry into host cells is mediated by fibronectin bound to surface proteins, M1 or PrtF1, thereby binding β1 integrin.

Abbreviation list: cDNA, Complementary DNA; RACE, Rapid-amplification of cDNA ends; RGD, The tripeptide Arg-Gly-Asp; LPS, Lipopolysaccharide; PGN, Peptidoglycan; MAN, Mannotriose-di-(N-acetyl-D-glucosamine); SUS, Skin ulceration syndrome; qPCR, Quantitative PCR; PAMPs, Pathogen-associated molecular patterns; ERK, Extracellular regulated protein kinases; GAS, Group A *Streptococcus*; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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This interaction leads to cytoskeletal rearrangement and phagocytosis of *Streptococcus* [11]. In addition, collagen-type surface protein expressed in *GAS*, Sel1, can bind with human collagen receptor integrin to promote the adhesion and phagocytosis of pathogens, whereas integrin β together with Fas mediates apoptosis induced by *GAS* pyrogenic endotoxin B (SPEB) [12]. In marine animals, the immune function of integrin is concentrated in Chinese shrimp (*Fenneropenaeus chinensis*) and oyster (*Crassostrea gigas*). A study further demonstrated that an integrin β subunit of *Fenneropenaeus chinensis* may serve as a cellular receptor mediating WSSV infection and the down-regulation of integrins would promote apoptosis by inhibiting intercellular contact [13]. Integrin from *Crassostrea gigas* mediated the phagocytosis toward *Vibrio splendidus* through LPS binding activity [14].

Aquaculture of sea cucumber *Apostichopus japonicus* is one of the largest industries in China with annual production of approximately 180,000 tons and valued at 30 billion Yuan [15]. The development of large-scale culture of this species led to the gradual induction of various diseases caused by virus or bacteria in the aquaculture of *Apostichopus japonicus,* resulting in incalculable loss in the sea cucumber industry [16]. Characterizing the immune-related genes involved in the innate immune defense mechanisms of *A. japonicus* can provide new insights into its sustainable production and disease control in aquaculture. Integrin is considered one of the most promising candidates in this fields. In this context, we characterized the immunological function in innate immunity of AjITGB first by cloning the full-length cDNA of *AjITGB* and investigating the expression patterns after challenges using pathogenic microorganism and LPS. Furthermore, PAMP binding activities of rAjITGB were validated by generating the recombinant protein *in vitro*. Finally, AjITGB modulation of coelomocyte apoptotic activity was also analyzed through RNA interference in cultured coelomocytes.

2. Materials and methods

2.1. Animals and challenge experiment

Healthy adult sea cucumbers (n = 120), weighing 116 ± 14 g, were collected from the Dalian Pacific Aquaculture Company and acclimatized in aerated seawater (salinity, 28; temperature, 16 °C; pH = 8.1) for 3 days. The sea cucumbers were then randomly divided into 6 tanks with each tank containing 20 individuals. Pathogenic microorganism *Vibrio splendidus* was initially isolated from SUS-diseased sea cucumbers and kept in our laboratory. The bacteria were inoculated in liquid 2216E medium at 28 °C and 220 rpm overnight. The cultures were centrifuged at 5000 × *g* for 5 min to harvest the bacteria and then resuspended in filtered seawater to obtain a stock solution of 1×10^{11} CFU mL^{-1}.

For the challenge experiment, the sea cucumbers in three tanks were infected with live *V. splendidus* at a final concentration of 1×10^{7} CFU mL^{-1}. The other three tanks were supplemented with the same volume of seawater and served as the control group. Coelomic fluids were collected at 0, 6, 24, 48, 72, and 96 h post-infection and centrifuged at 800 × *g* at 4 °C for 5 min to harvest the coelomocytes. Three biological replicates were obtained for each group. The samples were stored at −80 °C for RNA extraction and cDNA synthesis.

2.2. Cloning the full-length cDNA of AjITGB

Total RNA was extracted with RNAiso Plus reagent (TaKaRa), and first-strand cDNA was synthesized according to the 3′ and 5′-full RACE core set instructions (TaKaRa). Four gene specific primers,*P*1:5′GGTTCATTGTCAAATCGGAG-3′, *P*2:5′GATTACGTCGCTCTGGTCCA-3′, *P*3: 5′ GGCCACGCGTCGACTAGTACG-3′, and *P*4: 5′ CCAACAATTCTGCAACCTCCG-3′ were designed based on the annotated EST of *AjITGB* and used to amplify the 3′ and 5′ end of *AjITGB*,. The desired PCR products were cloned into the pMD19-T simple vector (TaKaRa) and sequenced at Sangon Biotechnology (Shanghai).

2.3. Sequence analysis of the AjITGB cDNA

The *AjITGB* sequence was analyzed using the BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast. The predicted amino acid sequence was analyzed using the Expert Protein Analysis System (http://www.expasy.org/). The percentage of sequence similarity between AjITGB and integrins from other organisms was calculated using the Identity and Similarity Analysis program (http://www.biosoft.net/sms/index.html). The domain in the AjITGB amino acid sequence was detected using the Simple Modular Architecture Research Tool (SMART) program. A phylogenetic tree was constructed based on the full-length amino acid sequences of the different original integrins using Mega 4.0 (http://www.megasoftware.net/).

2.4. Spatial expression analysis of AjITGB mRNA

The expression patterns of *AjITGB* in five tissues namely, muscles, tentacles, intestines, respiratory trees, and coelomocytes were investigated using the Rotor-Gene 6000 real-time PCR detection system. β-actin served as the internal control to verify successful reverse transcription and calibrate the cDNA template (P5: CCATTCAACCCTAAAGCCAACA, *P*6: ACACACCGTCTCCTGAGTCCAT). Two specific primers for *AjITGB*, P7: CTGTGAGAACCCAGTTTGAGAA and P8: GTTACCGACAGTTAACCTTCCA, were designed to amplify a product of 194 bp. Real-time PCR amplifications were performed in a total volume of 20 μL containing 10 μL of 2 × SYBR Green Mix (TaKaRa), 4 μL of diluted cDNA (1:20), 1 μL of each primer (10 mM), and 4 μL of PCR-grade water. The qPCR parameters included a denaturing step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 25 s. The amplified products were subjected to melting analysis at the end of each PCR to confirm the generation of a single PCR product. The $2^{-\triangle\triangle CT}$ was used to analyze the expression levels of *AjITGB*, and the values represented the *n*-fold difference relative to the calibrator tissue (coelomocytes).

2.5. Time-course analysis of AjITGB in response to V. splendidus challenge

Coelomocytes were selected to analyze the temporal expression profile of *AjITGB* in *Vibrio*-challenged samples from Section 2.1. RNA extraction, cDNA synthesis, and expression analysis were performed according to Section 2.2. The untreated samples served as the control (calibrator). The values obtained denoted the *n*-fold differences relative to the calibrator. Data are presented as mean ± SD ($n = 3$). The results were subjected to one-way ANOVA, followed by Duncan's multiple range tests, to determine the differences between the challenged and control groups at each sampling time. P values less than 0.05 were considered significantly different.

2.6. Cell culture and LPS exposure

Primary coelomocytes were prepared according to our previous work [15]. Briefly, the harvested cells were resuspended in Leibovitz L-15 cell culture medium (Invitrogen, USA) containing