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Antibacterial activity and immune responses of a molluscan macrophage expressed gene-1 from disk abalone, *Haliotis discus discus*

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ABSTRACT

The membrane-attack complex/perforin (MACPF) domain-containing proteins play an important role in the innate immune response against invading microbial pathogens. In the current study, a member of the MACPF domain-containing proteins, macrophage expressed gene-1 (MPEG1) encoding 730 amino acids with the theoretical molecular mass of 79.6 kDa and an isoelectric point (*p*I) of 6.49 was characterized from disk abalone *Haliotis discus discus* (AbMPEG1). We found that the characteristic MACPF domain (Val^{131}–Tyr^{348}) and transmembrane segment (Ala^{669}–Ile^{691}) of AbMPEG1 are located in the N- and C-terminal ends of the protein, respectively. Ortholog comparison revealed that AbMPEG1 has the highest sequence identity with its pink abalone counterpart, while sequences identities of greater than 90% were observed with MPEG1 members from other abalone species. Likewise, the furin cleavage site KRRRK was highly conserved in all abalone species, but not in other species investigated. We identified an intron-less genomic sequence within disk abalone *AbMPEG1*, which was similar to other mammalian, avian, and reptilian counterparts. Transcription factor binding sites, which are important for immune responses, were identified in the 5′-flanking region of *AbMPEG1*. qPCR revealed *AbMPEG1* transcripts are present in every tissues examined, with the highest expression level occurring in mantle tissue. Significant up-regulation of *AbMPEG1* transcript levels was observed in hemocytes and gill tissues following challenges with pathogens (*Vibrio parahemolyticus*, *Listeria monocytogenes* and viral hemorrhagic septicemia virus) as well as pathogen-associated molecular patterns (PAMPs: lipopolysaccharides and poly I:C immunostimulant). Finally, the antibacterial activity of the MACPF domain was characterized against Gram-negative and -positive bacteria using a recombinant peptide. Taken together, these results indicate that the biological significance of the *AbMPEG1* gene includes a role in protecting disk abalone through the ability of *AbMPEG1* to initiate an innate immune response upon pathogen invasion.

1. Introduction

The formation of transmembrane channels on the surface of invading pathogenic cells is an important role of the host immune system because it destroys the target pathogen by disrupting its phospholipid bilayer [1]. The proteins of the membrane-attack complex (MAC) (which consists of the complement system C6, C7, C8α, C8β and C9) along with the perforin comprise the major family of pore-forming molecules. These proteins share a common MAC/perforin (MACPF) domain [2]. Over 500 MACPF domain-containing proteins have been reported in a wide range of living organisms including bacteria, human, plants, protozoa and fungi [2,3].

The formation of pores takes place via the assembly of complement components C6, C7, C8α, C8β and C9, followed by construction of MAC on foreign cell membranes [4]. In contrast, perforin is released by cytotoxic T lymphocytes and natural killer cells and creates holes in the cell membrane of virus-infected and transformed cells [5,6].

Macrophage expressed gene-1 (MPEG1) is another important MACPF domain-containing protein. Originally discovered in human

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and murine macrophages [7], MPEG1 has since been identified in a wide range of animals. As to the role of MPEG1, various studies have demonstrated that it is involved in the innate immune response. Others have shown that a *MPEG1* member from the demosponge *Suberites domuncula* induced a significant elevation of transcripts via the MyD88-dependent signal transduction pathway, as well as a lytic effect on gram-negative bacteria [8]. In a recent study, MPEG1 from *Crassostrea gigas* demonstrated antibacterial activity against both gram-negative and -positive bacteria [9]. The involvement of MPEG1 in the innate immune response has also been reported in a few other molluscs, including *Mytilus galloprovincialis* [10] and several abalone species: *Haliotis corrugata*, *Haliotis rufescens* [11], *Haliotis diversicolor* [12] and *Haliotis midae* [8].

Disk abalone, *Haliotis discus discus*, is one of the most economically important marine gastropod mollusc species in Eastern Asian countries, including the Republic of Korea. Due to the filter feeding and sedentary habits of disk abalone, it is always exposed to an aquatic environment that is rich in potential microbial pathogens. Mass mortalities of abalone have been reported in the past few decades as the result of various pathogenic microbial infections [13–16].

Similar to other invertebrates, disk abalones have to solely rely on a strong innate immune defense system because they do not possess an adaptive immune system. Attempts to establish the mechanisms involved in the immune responses of disk abalone upon microbial pathogenic invasion is limited. To better understand the functional role of MACPF domain-containing proteins in gastropods, we have isolated and characterized a homolog of *MPEG1* from the disk abalone *H. discus discus* (*AbMPEG1*) at cDNA and genomic levels. Subsequently, we determined the spatial distribution of *AbMPEG1* in different tissues and the transcriptional modulation of *AbMPEG1* in gills and hemocytes in response to bacterial and viral infections. The antibacterial activity of the MACPF domain was also examined by means of a recombinant protein.

2. Materials and methods

2.1. Disk abalones and immune challenge

Disk abalones with an average weight of 50 g were purchased from the Youngsoo commercial abalone farm in Jeju Island, Republic of Korea, and acclimatized for at least one week prior to the experiments. The sand-filtered seawater was aerated continuously and a constant temperature (20 ± 1 °C) and salinity (34 ± 0.6 psu) were maintained throughout the experiment. Disk abalones were fed with fresh marine seaweed (*Undaria pinnatifida*) during this period. After the acclimatization period, the disk abalones were randomly separated into seven groups.

Two groups were challenged with 100 μL of live *Vibrio parahemolyticus* and *Listeria monocytogenes* that had been resuspended in saline (0.9% NaCl) at a concentration of 1×10^4 CFU mL^{-1}. One group of disk abalone was administered with 100 μL of 1×10^8 PFU mL^{-1} of viral hemorrhagic septicemia virus (VHSV) that had been isolated from olive flounder (*Paralichthys olivaceus*) as previously described [17]. For the immune stimulant challenge experiment, two groups were injected with 100 μL of lipopolysaccharides (LPS) (5 μg μL^{-1} isolated from *Escherichia coli* 055:B5, Sigma) and poly I:C immunostimulant (5 μg μL^{-1}, Sigma) dissolved in saline. Similarly, 100 μL of saline was injected into the sixth group of disk abalones to serve as a negative control. Four individuals were randomly sampled from each challenged- and saline-injected group at 3, 6, 12, 24, 48, 72, and 120 h after injection, and hemolymph and gills were collected at each time point. The remaining untreated abalone group was used as the blank (0 h) and for the tissue distribution analysis. Several tissues including digestive tract, gills, muscle, gonad, hepatopancreas and mantle were dissected from four untreated individuals to determine the native tissue distribution pattern of *AbMPEG1*. Hemolymph was collected from each challenged and unchallenged individual, and immediately centrifuged at 3000 × *g* for 10 min at 4 °C to collect the hemocytes. All the isolated tissues were snap frozen in liquid nitrogen and stored at −80 °C.

2.2. Identification and characterization of MPEG1 from disk abalone

A 2584 bp contig identified from the disk abalone cDNA library, which was constructed in our laboratory as previously reported [18], revealed high homology with other MPEG1 variants in our BLAST analysis. To obtain the genomic sequence of *AbMPEG1*, an abalone bacterial artificial chromosome (BAC) library established in our laboratory [19] was screened using gene-specific primers (Supplementary Table 1). Based on cDNA to gDNA alignment, the exon–intron boundaries were inferred and further genomic structures were compared with other known structures. The cDNA and deduced amino acid sequences of AbMPEG1 were analyzed with the NCBI BLAST algorithm using default parameters (http://www.ncbi.nlm.nih.gov/blast). The characteristic domain architecture features of *AbMPEG1* were recognized by several tools in the Expert Protein Analysis System (http://www.expasy.org/). Signal peptide was predicted by the SignalP 4.0 program (http://www.cbs.dtu.dk/services/SignalP/). The NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) was used to predict the potential N-glycosylation sites in AbMPEG1 sequence. The SMART program (http://smart.embl-heidelberg.de/) and NCBI CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi) were applied to determine the location of conserved domains. Transmembrane helices in the AbMPEG1 protein were predicted by the TMHMM Server v. 2.0 (http://cbs.dtu.dk/services/TMHMM/). Multiple alignments of AbMPEG1 with other homologs were performed with the ClustalW2 program (http://www.Ebi.ac.uk/Tools/clustalw2) [20]. A neighbor-joining phylogenetic tree was constructed by means of the MEGA version 5.05 program [21] with 5000 bootstrap replicates. The putative promoter region of *AbMPEG1* located upstream of the transcription initiation site was analyzed for the presence of transcription factor binding sites using tools such as TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html).

2.3. RNA isolation, cDNA synthesis, and quantification of AbMPEG1 transcripts

Total RNA was extracted from tissues pooled from four abalones by using Tri Reagent™ (Sigma–Aldrich, Missouri, USA). The purity of the RNA was assessed by measuring the absorbance at 260 nm and 280 nm. The PrimeScript™ first-strand cDNA synthesis kit (TaKaRa, Japan) was used to synthesize the first-strand cDNA. Briefly, 2.5 μg of total RNA, 50 μM of oligo dT primer, 10 mM of dNTP mix and RNase free water were mixed together and incubated at 65 °C for 5 min. The mixer was incubated on ice for at least one minute and 5× PrimeScript™ buffer, 20 U of RNase inhibitor and 200 U of PrimeScript™ RTase were added to a final volume of 20 μL. The final reaction mixture was incubated at 42 °C for 1 h followed by incubation at 70 °C for 15 min to stop the reverse transcription reaction. Finally, the mixture was diluted by 40-fold.

The quantification of *AbMPEG1* transcript levels in healthy and immune-challenged abalones was performed via quantitative real time PCR (qPCR) using the Real Time System TP800 Thermal Cycler Dice™ (TaKaRa, Japan) with the fluorescent agent SYBR Green. The MIQE guidelines [22] were adopted to design the gene-specific primers for *AbMPEG1*, and the amplicon size, GC content and T_m