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Molecular identification of disk abalone (*Haliotis discus discus*) tetraspanin 33 and CD63: Insights into potent players in the disk abalone host defense system

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ABSTRACT

Tetraspanins are a superfamily of transmembrane proteins involved in a diverse range of physiological processes including differentiation, adhesion, signal transduction, cell motility, and immune responses. In the present study, two tetraspanins, CD63 and tetraspanin 33 (TSPAN33) from disk abalone (AbCD63 and AbTSPAN33), were identified and characterized at the molecular level. The coding sequences for *AbCD63* and *AbTSPAN33* encoded polypeptides of 234 and 290 amino acids (aa) with predicted molecular mass of 25.3 and 32.5 kDa, respectively. The deduced AbCD63 and AbTSPAN33 protein sequences were also predicted to have a typical tetraspanin domain architecture, including four transmembrane domains (TM), short N- and C- terminal regions, a short intracellular loop, as well as a large and small extracellular loop. A characteristic CCG motif and cysteine residues, which are highly conserved across CD63 and TSPAN33 proteins of different species, were present in the large extracellular loop of both abalone tetraspanins. Phylogenetic analysis revealed that the AbCD63 and AbTSPAN33 clustered in the invertebrate subclade of tetraspanins, thus exhibiting a close relationship with tetraspanins of other mollusks. The *AbCD63* and *AbTSPAN33* mRNA transcripts were detected at early embryonic development stages of disk abalone with significantly higher amounts at the trochophore stage, suggesting the involvement of these proteins in embryonic development. Both *AbCD63* and *AbTSPAN33* were ubiquitously expressed in all the tissues of unchallenged abalones analyzed, with the highest expression levels found in hemocytes. Moreover, significant induction of *AbCD63* and *AbTSPAN33* mRNA expression was observed in immunologically important tissues, such as hemocytes and gills, upon stimulation with live bacteria (*Vibrio parahaemolyticus* and *Listeria monocytogenes*), virus (viral hemorrhagic septicemia virus), and two potent immune stimulators [polyinosinic:polycytidylic acid (poly I:C) and lipopolysaccharide (LPS)]. Collectively, these findings suggest that AbCD63 and AbTSPAN33 are involved in innate immune responses in disk abalone during pathogenic stress.

1. Introduction

The tetraspanin superfamily proteins are transmembrane proteins found in a diverse range of eukaryotic organisms. To date, 33, 37 and 20 distinct tetraspanins have been identified in mammals, fruit flies (*Drosophila* spp.) and nematodes (*Caenorhabditis elegans*), respectively [1]. Most tetraspanins are approximately 200–300 amino acids (aa) in length and share characteristic structural features that include four transmembrane domains (TM), short N- and C- terminal regions, as well as large and small extracellular loops (ECLs) [2,3]. Tetraspanins are easily distinguished from other proteins that also contain four transmembrane domains by the presence of other unique structural features, such as 4–6 conserved cysteine residues in the extracellular domain, a highly

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conserved Cys-Cys-Gly sequence (also referred to as CCG motif) within the large ECL, and the presence of polar residues in the transmembrane domains [1,2]. Particularly, tetraspanins have been shown to be involved in cell differentiation, adhesion, signal transduction, and motility [2,4,5]. Moreover, the involvement of tetraspanins in immune-related functions has been widely described in humans and mice [6–8].

CD63 is a well-known member of the tetraspanin superfamily and is also referred to as lysosomal integral membrane protein [5]. CD63 is commonly found on the granules of various types of blood cells, such as on the cytotoxic granules of T lymphocytes, the alpha granules of megakaryocytes, the crystalloid granules of eosinophils, and the secretory granules of basophilic granulocytes [5]. In addition, CD63 is also present on the plasma membrane and on the membrane of lysosomes in mast cells [9], dendritic cells [10] and on Weibel-Palade bodies in endothelial cells [11]. CD63 participates in several immunological and pathophysiological processes. Specifically, CD63 is involved in the protection of membrane proteins from proteolytic degradation [12], in antigen presentation [13], and in platelet adhesion and spreading [14]. CD63 can directly interact and thereby influence the cellular trafficking of a number of proteins, including the β subunit of hydrogen/potassium ATPase (H^+/K^+ ATPase) [15], phosphatidylinositol 4-kinase (PI 4-kinase) [16], syntenin 1 [17], membrane type 1 matrix metalloprotease (MT1-MMP 1) [18], tissue inhibitor of metalloproteinases 1 (TIMP 1) [19], adaptor protein 3 (AP3) [20], amelogenin [21], and protein-tyrosine kinase (PTK) [22]. Even though CD63 has been extensively studied in many mammalian species, limited information is available for other vertebrates and invertebrate species. In fish, CD63 has recently been identified and characterized in rainbow trout (*Oncorhynchus mykiss*) [23], channel catfish (*Ictalurus punctatus*) [24], and zebrafish (*Danio rerio*) [25]. CD63 homologues in invertebrates have been identified for the coleopteran beetle (*Tenebrio molitor*) [26], amphioxus (*Branchiostoma belcheri tsingtauense*) [27], and Chinese shrimp (*Fenneropenaeus chinensis*) [4]. Moreover, transcriptional modulation of *CD63* mRNA was quantified in coleopteran beetle and Chinese shrimp after stimulation with different live pathogens or pathogen associated molecular patterns (PAMPs). A *CD63*-like gene was also described in oysters (*Crassostrea ariakensis*), and up-regulated mRNA expression was detected in hemocytes after treatment with lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid (poly I:C) *in vitro* [28].

Tetraspanin 33 (TSPAN33) was previously defined as Penumbra, and it was originally identified from a murine, multipotent hematopoietic cell line [29]. This molecule belongs to the TSPANC8 subgroup of tetraspanins, which is considered as a largely unstudied tetraspanin subgroup [30]. Based on the mammalian studies, TSPAN33 is mainly expressed in erythroid precursor cells, activated B cells, and bone marrow cells in mice, whereas its expression is comparatively low in human bone marrow cells [6,29]. It has been reported that TSPAN33 expression is elevated in pathological states that include systemic lupus erythematosus, rheumatoid arthritis, Hodgkin's lymphoma, and diffuse large B cell lymphoma [6]. Moreover, expression of TSPAN33 is induced by Toll-like receptor (TLR) signaling and interferon (IFN)-γ [31]. Like most tetraspanins, TSPAN33 is functionally relevant to the immune system, being specifically involved in inflammation processes through the modulation of pro-inflammatory gene expression and in the activation of different immune cells [31]. To date, few reports are available regarding the functional aspects of TSPAN33 in humans and mice, particularly in relation to its involvement in different diseases [6,29–31]. Moreover, reports on the characterization and functional analysis of TSPAN33 in other vertebrates and invertebrate species are not available.

Disk abalone (*Haliotis discus discus*) has become a commercially important marine gastropod species for the Korean aquaculture industry. However, abalone production has been severely affected by pathogenic infections, such as bacteria [32], virus [33] and parasites [34]. As is the case with all invertebrate species, abalones are totally dependent on their innate immune system to overcome infections because they lack an adaptive immune system. Therefore, the identification of novel innate immune components as well as their mechanisms of action and responses against pathogens are vital for developing appropriate disease prevention strategies in the abalone farming industry. In the present study, we identified and characterized, at the molecular level, the tetraspanin homologues of CD63 and TSPAN33 in disk abalone. Furthermore, the transcriptional modulations of these two newly identified invertebrate tetraspanins were determined in disk abalone hemocytes and gills upon stimulation with two bacterial species (*Vibrio parahaemolyticus* and *Listeria monocytogenes*) or one viral pathogen [viral hemorrhagic septicemia virus (VHSV)], as well as upon treatment with two potent stimulators of the immune system (LPS and poly I:C).

2. Materials and methods

2.1. Identification of CD63 and TSPAN33 coding sequences

The full-length coding sequences for disk abalone *CD63* and *TSPAN33* (designated as *AbCD63* and *AbTSPAN33*) were identified from a previously constructed transcriptome library using the 454 Genome Sequencer FLX system (GS-FLX™, Roche) using the Basic Local Alignment Search Tool [BLAST, National Center for Biotechnology Information (NCBI)] [35].

2.2. Characterization of AbCD63 and AbTSPAN33 sequences

The open reading frames (ORFs) of *AbCD63* and *AbTSPAN33* were identified using UGENE software [36]. The deduced aa sequences of the AbCD63 and AbTSPAN33 proteins were loaded into the ExPASy ProtParam tool to determine their physiochemical properties [37]. Conserved domains and active sites in the protein sequences were detected using ExPASy PROSITE [38], NCBI conserved domain database [39] and motif scan servers [40]. The transmembrane membrane topologies of AbCD63 and AbTSPAN33 were predicted using TMHMM v 2.0 server [41]. Multiple sequence alignments and pairwise sequence alignments were performed using ClustalW [42] and Emboss needle [43] tools, respectively. The presence or absence of signal peptides were predicted using SignalP 4.1 server [44]. Furthermore, the phylogenetic relationships of AbCD63 and AbTSPAN33 with other known tetraspanins were determined by the neighbor-joining (NJ) method using the Molecular Evolutionary Genetic Analysis (MEGA) software version 6.0 [45].

2.3. Collection of different disk abalone embryonic development stages

Obtained Disk abalone (*H. discus discus*) eggs and sperms from the Ocean and Fisheries Research Institute, Jeju, Republic of Korea were fertilized in sea water at 18 °C. Thereafter, different embryonic developmental stages including egg, 16-cell stage, morula, gastrula, trochophore, early veliger, middle veliger, and late veliger were collected after confirmation of their morphologies under the light microscope. The collected embryo samples were washed with Phosphate-buffered saline (PBS) and immediately stored at −80 °C until RNA extraction was performed.