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A thioredoxin domain-containing protein 12 from black rockfish *Sebastes schlegelii*: Responses to immune challenges and protection from apoptosis against oxidative stress

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ABSTRACT

Thioredoxin (TXN) superfamily proteins are identified by the presence of a thioredoxin active site with a conserved CXXC active motif. TXN members are involved in a wide range of biochemical and biological functions including redox regulation, refolding of disulfide containing proteins, and regulation of transcription factors. In the present study, a thioredoxin domain-containing protein 12 was identified and characterized from black rockfish, *Sebastes schlegelii* (RfTXNDC12). The full length of RfTXNDC12 consists of a 522-bp coding region encoding a 173-amino acid protein. It has a 29-amino acid signal peptide and a single TXN active site with a consensus atypical WCGAC active motif. Multiple sequence alignment revealed that the active site is conserved among vertebrates. RfTXNDC12 shares highest identity with its *Epinephelus coioides* homolog. Transcriptional analysis revealed its ubiquitous expression in a wide range of tissues with the highest expression in the ovary. Immune challenges conducted with *Streptococcus iniae* and poly I:C caused upregulation of *RfTXNDC12* transcript levels in gills and peripheral blood cells (PBCs), while lipopolysaccharide injection caused downregulation of *RfTXNDC12* in gills and upregulation in PBCs. Similar to TXN, RfTXNDC12 exhibited insulin disulfide reducing activity. Interestingly, the recombinant protein showed significant protection of LNCaP cells against apoptosis induced by H_2O_2-mediated oxidative stress in a concentration dependent manner. Collectively, the present data indicate that RfTXNDC12 is a TXN superfamily member, which could function as a potential antioxidant enzyme and be involved in a defense mechanism against immune challenges.

1. Introduction

Cellular reactive oxygen species (ROS) are produced as by-products during normal cellular metabolism, upon microbial infection as antimicrobial agents or in response to various stimuli including cytokines, neurotransmitters, growth factors, and hormones. Oxidative stress to a cell can be defined as by the presence of ROS or oxidants in excess of the cell's ability to mount an effective antioxidant response (Ray et al., 2012). Many antioxidant enzymes, including peroxidases, superoxide dismutase, catalase, glutathione, and thioredoxins (TXNs), protect cells through redox homeostasis against oxidative stress (Abele and Puntarulo, 2004). Among these enzymes, TXNs are known to be one of the main intracellular redox regulatory agents involved in a wide range of biochemical and biological functions (Matsuo et al., 2001; McEligot et al., 2005; Moriarty-Craige and Jones, 2004). TXN has been identified in both prokaryotic and eukaryotic cells and has been shown to facilitate the refolding of disulfide-containing proteins (Liu et al., 2003). The intramolecular disulfide bond in the oxidized TXN is reduced by thioredoxin reductase and NADPH. In turn, two thiol groups in the reduced TXN can catalyze disulfide bond formation in multiple substrate proteins. Hence, TXN is involved in many thiol-dependent cellular processes including gene expression, signal transduction, and proliferation (Matsuo et al., 2001). In addition, human TXN was reported to function as a growth factor and an oxidative stress indicator in different cancers (Nakamura et al., 1999; Welsh et al., 2002).

The TXN superfamily is a group of enzymes characterized by the presence of TXN-fold structure and TXN or TXN-like active sites with two conserved cysteine residues (CXXC) that are separated by two amino acid residues [6,7]. Trx can be a cytosolic (Trx-1) or a mitochondrial (Trx-2) form (Holmgren, 1985). In addition, disulfide bond formation in

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the endoplasmic reticulum (ER) is known to be catalyzed by several TXN superfamily enzymes including protein-disulfide isomerases (PDI) (Haugstetter et al., 2005; Noiva, 1999; Sevier and Kaiser, 2002), all of which are comprised of at least one TXN-like domain (Ellgaard and Ruddock, 2005). To date, nearly 20 human PDI or PDI-related family members have been identified (Ellgaard and Ruddock, 2005), which include ERdj5 (Cunnea et al., 2003), thioredoxin-related transmembrane protein 2 (TMX2) (Meng et al., 2003), ERp44 (Anelli et al., 2003), EndoPDI (Sullivan et al., 2003), ERp19, ERp46 (Knoblach et al., 2003), ERp28 (Ferrari et al., 1998), ERp72, PDI-related P5 (Ferrari and Soling, 1999), and thioredoxin domain-containing (TXNDC) protein 12 (previously reported as ERp16, ERp18 (Alanen et al., 2003), ERp19, or hTLP19). However, functional properties of these proteins are not fully understood (Ellgaard and Ruddock, 2005).

TXNDC proteins have one or more thioredoxin active sites and have significant roles in redox regulation, defense against oxidative stress, refolding of sulfide containing proteins, and regulation of transcription factors (Jeong et al., 2008; Liu et al., 2003; Wei et al., 2012). Human TXNDC12, localized in ER (Do et al., 2004), is composed of a TXN-fold with a consensus active-site sequence (CXXC) (Liu et al., 2003). It catalyzes thiol-disulfide exchange reactions (Ellgaard and Ruddock, 2005; Jeong et al., 2008) and plays a crucial role in the cellular defense against prolonged ER stress (Do et al., 2004). At present, there are limited information regarding the structure and function of TXNDC12 in lower vertebrates, except for a study on the orange spotted grouper (Wei et al., 2012). In the present study, we describe the molecular characterization and functional properties of TXNDC12 from black rockfish, *Sebastes schlegelii*, by using a transcriptional profile and biological activities.

2. Material and methods

2.1. cDNA library and identification of RfTXNDC12

A cDNA library of black rockfish was constructed by 454-GS-FLX™ sequencing technique (Droege and Hill, 2008). In brief, total RNA was extracted from blood, liver, head kidney, gill, intestine, and spleen tissues of three fish (~100 g) challenged with immune stimulants, including *Edwardsiella tarda* (10^7 CFU/fish), *Streptococcus iniae* (10^7 CFU/fish), lipopolysaccharide (LPS; 1.5 mg/fish) and polyinosinic:polycytidylic acid (poly I:C; 1.5 mg/fish) using TRIzol reagent (TaKaRa, Japan) according to the manufacturer's instructions. The extracted RNA was then cleaned by an RNeasy Mini Kit (Qiagen, USA) and assessed for quality and quantified by using an Agilent 2100 Bio-analyzer (Agilent Technologies, Canada), resulting in an RNA integration score (RIN) of 7.1. Subsequently, a GS-FLX™ 454 shotgun library was constructed, and the cDNA database was established by using fragmented RNA from the aforementioned RNA samples (Macrogen, Korea). The complete sequence of RfTXNDC12 was identified form the cDNA library by the Basic Local Alignment Search Tool (BLAST) algorithm in NCBI (http://www.ncbi.nlm.nih.gov/BLAST).

2.2. In silico analysis of RfTXNDC12

In silico analysis of RfTXNDC12 was performed by using standard bioinformatic tools. The open reading frame (ORF) and amino acid sequence of RfTXNDC12 were determined by DNAssit (version 2.2). This sequence was subjected to a BLAST search to identify orthologous sequences for comparison. The amino acid sequence of RfTXNDC12 was analyzed using tools available in the ExPASy database (http://www.expasy.org). The putative conserved domains were identified by the ScanProsite tool (http://prosite.expasy.org/cgibin/prosite). Disulfide bonds were predicted by DISULFIND, a disulfide bonding state and cysteine connectivity prediction server (http://www.disulfind.dsi.unifi.it). Sequence similarity analysis and pairwise and multiple alignments (MSA) were carried out using the ClustalW program in the BioEdit Sequence Alignment Editor package. The presumed tertiary structure of the RfTXNDC12 protein was generated by SWISS-MODEL (http://swissmodel.expasy.org). A phylogenetic tree was constructed by the neighbor-joining method using the MEGA 5.0 package (http://www.megasoftware.net/). To deduce the confidence value for the phylogenetic analysis, bootstrap trials were replicated 5000 times.

2.3. Experimental animals and tissue collection

Healthy black rockfish with an average body weight of 200 g were obtained from the Marine Science Institute of Jeju National University, Jeju Self Governing Province, Republic of Korea and were kept in 400 L flat-bottom tanks filled with aerated, sand-filtered seawater at 22 ± 1 °C. All fishes were acclimatized for one week prior to experiments. During acclimatization period fish were fed with commercial fish feed twice a day and stopped two days prior to the experiment. In order to examine the tissue-specific expression of *RfTXNDC12* mRNA, peripheral blood was collected from caudal veins of five healthy fish using sterile syringes coated with 0.2% heparin sodium salt (USB, USA). Peripheral blood cells (PBCs) were immediately harvested by centrifugation at 3000 × *g* at 4 °C for 10 min. Other tissues, such as gills, liver, spleen, head kidney, kidney, skin, muscle, heart, brain, intestine, testes, and ovary, were also excised from five healthy individuals. These samples were immediately snap-frozen and stored at −80 °C for RNA isolation.

2.4. Immune challenge and tissue collection

To determine the immune response of *RfTXNDC12*, acclimatized fish were divided into four groups and subjected to the following challenges: a bacterial strain *S. iniae* (1×10^5 CFU/μL), the immunostimulants LPS and poly I:C (1.5 μg/μL). Each of these stimulants was separately resuspended in 200 μL of phosphate-buffered saline (PBS) and intraperitonially administrated as a single dose to the respective group of fish. Meanwhile, 200 μL of PBS was injected to the control group. Then, PBCs and gill tissue samples were collected at 3, 6, 12, 24, and 48 h post-injection (p.i.) from five fish from each group (n = 5), snap-frozen in liquid nitrogen, and stored at −80 °C.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from tissues of five individual fish (40 mg/fish) using QIAzol® (Qiagen), and the RNA quality was assessed by 1.5% agarose gel electrophoresis and then quantified spectrophotometrically at 260 nm in a micro-drop plate (Thermo Scientific). The first strand cDNA synthesis was carried out using 2.5 μg of extracted total RNA by using PrimeScript™ II First-Strand cDNA Synthesis Kit (TaKaRa) and diluted 40× in nuclease free water and stored at −20 °C until use.

Table 1
Description of primers used in this study.

Name	Primer sequence (5′ → 3′)	Description
*RfTXNDC12*_FW1	AGTACTTCTACAGCACCGCAGAACA	qPCR forward primer
*RfTXNDC12*_RV1	TCTCCAGTGTGACCCTGCTTGAA	qPCR reverse primer
*RfTXNDC12*_FW2	GAGA GAgaattcGCCAGCAGCAAAGGGTTTGG	Cloning primer with *EcoRI* restriction site
*RfTXNDC12*_RV2	GAGA GAaagcttTCACAGCTCGTCTCCAGTGTGA	Cloning primer with *HindIII* restriction site
*EF1A*_FW	AACCTGACCACTGAGGTGAAGTCTG	qPCR internal reference gene
*EF1A*_RV	TCCTTGACGGACACGTTCTTGATGTT	qPCR internal reference gene