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Cloning, characterization and comparative analysis of four death receptorTNFRs from the oyster *Crassostrea hongkongensis*

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ABSTRACT

Apoptosis plays an important role in homeostasis of the immune systems. The tumor necrosis factor receptors (TNFRs) play critical roles in the extrinsic apoptosis pathways and in determining cell fate. In this study, four death receptors (DR) named *Ch*EDAR, *Ch*TNFR27, *Ch*TNFR5, and *Ch*TNFR16 were identified from the oyster *Crassostrea hongkongensis*. These *Ch*DRs proteins had 382, 396, 414 and 384 amino acids, respectively, with the typical domains of death receptors, such as the signal peptide (SP), transmembrane helix region (TM) and death domains. Phylogenetic analysis showed that the *Ch*DR proteins clustered into three distinct groups, indicating that these subfamilies had common ancestors. mRNA expression of the *Ch*DRs were detected in all 8 of the selected oyster tissues and at different stages of development. Furthermore, expression of all the genes was increased in the hemocytes of oysters challenged with pathogens or air stress. Fluorescence microscopy revealed that the full-length proteins of the *Ch*DRs were located in the plasma membrane of HEK293T cells. Over-expression of the *Ch*DRs activated the NF-κB-Luc reporter in HEK293T cells in a dose-dependent manner. These results indicate that the *Ch*DRs may play important roles in the extrinsic apoptotic pathways in oysters.

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1. Introduction

Programmed cell death, also known as apoptosis, is very important in immunity and other biological processes. Apoptosis is characterized by a series of remarkable changes in cell morphology, including membrane blebbing, chromatin condensation and DNA fragmentation [1]. The extrinsic apoptotic pathways and the mitochondrial apoptotic pathway are the major signal transduction pathways of apoptosis. The extracellular TNFs and their cell-surface receptors, such as TNFR family, initiate and activate the extrinsic pathway, while the activation of the BCL-2 families mediates the mitochondrial pathway [2,3].

TNFR family members play pivotal role in diverse biological processes, such as development and organogenesis, host immune defense, inflammation, environmental stress, and others [11,12]. The TNF receptor (TNFR) family has many members and plays important roles in apoptosis. In humans, 18 TNF family ligands and 29 receptors have been identified [4]. Among the members of the TNFR families, the death receptors have recently been identified as a subgroup of the TNFR superfamily with a major function in induction of apoptosis. The receptors are characterized by an intracellular region, called the death domain, which is required for the transmission of cytotoxic signals [5]. To date, eight different death receptors have been identified in humans, including TNFR1, FAS, DR3, DR4, DR5, DR6, NGFR, and EDAR [6]. All these receptors are type I membrane proteins that contain one to four cysteine-rich

Abbreviations: ORF, open reading frame; UTR, untranslated region; qPCR, real-time quantitative PCR; RACE, rapid amplification of cDNA ends; EGFP, enhanced green fluorescent protein; hpi, hours post infection; TNFRSF, tumor necrosis factor receptor superfamily; EDAR, anhidrotic ectodysplasin receptor; DR, death receptor; NGFR, nerve growth factor receptor; NF-κB, nuclear factor kappa B; SP, signal peptide; TM, transmembrane helix region; DISC, death-inducing signaling complex; PBS, phosphate-buffered saline.

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extracellular domains (TNFR) and a motif named the 'death domain' (DD) in their cytoplasmic tails. The DRs selectively bind TNFs by the TNFR domains, and the TNFRs then transmit the extrinsic TNF superfamily signals to execute different activities [7]. The death domain (DD) is a homotypic protein interaction module composed of a bundle of six alpha-helices, which recruits various proteins, such as caspase-8 or 10, FADD, FLIP and others, that mediate both death and proliferation of the cells. These proteins in turn recruit other proteins via their DDs or death effector domains. In humans, these DRs cause apoptosis through caspase activation. The DRs interact with caspase-8 or -10 through the DD in the C-terminal. These caspase proteins consist of two death effector domain (DED) motifs at the N-terminus, which promote homotypic interactions and can trigger cell death following death effector domain-mediated recruitment to the "death-inducing signaling complex" (DISC) [8]. Furthermore, the DD mediates the self-association of these DRs and causes them to form oligomers to induce apoptosis [9]. The DRs were found to be involved in the regulation of apoptosis and inflammation through their activation of caspases and NF-κB, thus leading to downstream events, including death and proliferation of the cells [10].

Most members of the TNFR superfamily have been extensively studied in the vertebrates, such as humans, and 29 receptors have been identified in humans. However, fewer studies have been performed in invertebrates, although TNFR has been studied for a long period of time, since the first member of the TNFR superfamily with a deficiency in the DD domain was identified from invertebrates, Wengen, in *Drosophila* [16]. Recently, the importance of the study of invertebrate TNFRs, especially those in marine mollusks, was substantially increased. In the mollusk, many TNF family member genes were found to be involved in the host immune defense, such as *Ab*FasL and *Ab*TNF-a [13,14]. In the Zhikong scallop *Chlamys farreri*, the TNFR-like proteins *Cf*TNFR1 and *Cf*TNFR2, which have TNFR and DD domains, were identified and shown to be increased following challenge with *L. anguillarum* [7,17]. In general, the classification and the exact functions of mollusk TNFRs remain far from understood.

Oyster is a powerful genetic model for studying the *in vivo* role of genes and their physiological regulation in mollusks [15]. They live in the intertidal zone, and transcriptomic analysis has indicated that air stress induced a large number of genes, including many apoptosis-related genes, such as TNFR, indicating that exposure to air is a major stressor and that oysters have evolved an extensive set of genes involved in defense [15]. All TNFRs were computationally predicted to be found in *Crassostrea gigas*. Recently, we identified four death receptors, the TNFR superfamily member with a DD domain, from a *C. hongkongensis* hemocyte EST library. The aims of this study were as follows: (1) to clone and characterize new types of TNFRs from *C. hongkongensis*, (2) to provide new insights into the evolution and function of these important, wide spread and functionally diverse proteins, (3) to investigate the expression of these genes during development in the embryo and different tissues and the temporal expression after microorganism challenge and air stress, (4) to determine the subcellular localization and the involvement of intracellular signaling pathways of these genes.

2. Materials and methods

2.1. cDNA cloning and recombinant plasmid construction of ChDRs

Using a homologue search of the *C. hongkongensis* hemocyte EST library with the BLAST program (http://www.ncbi.nlm.nih.gov/blast), four ESTs were found to be homologous to the TNFRs of *C. gigas* (GenBank# EKC21561.1, GenBank# EKC38398.1, GenBank# XP_011419990 and GenBank# XP_011419989.1) and designated *Ch*TNFR16, *Ch*TNFR5, *Ch*EDAR, and *Ch*TNFR27. To obtain the full-length sequence of the *Ch*DRs, RACE-PCR was performed using cDNAs from *C. hongkongensis* and the BD SMART RACE cDNA Amplification Kit (Clontech, USA). Based on the identified EST sequences, *Ch*DRs gene-specific primers for RACE amplification were designed. *Ch*DRs gene-specific primer pairs include 5′RACE-OU/IN and 3′RACE-OU/IN for 5'- and 3′-RACE (Table 1), respectively. To reconstruct a full-length *Ch*DR cDNA, sequences were obtained from over lapping ESTs and the fragments amplified via RACE. Using the full-length cDNA sequence, the open reading frames of the *Ch*DRs were amplified with the upstream primer ORF-up and the downstream primer ORF-down (Table 1). PCR products were cloned into the pGEM-T easy vector (Promega, USA) for sequencing using an ABI 3730 DNA sequencer (Applied Biosystems, USA).

Amino acid sequences were deduced using DNAStar. Protein domains were predicated with the program SMART (http://smart.embl-heidelberg.de). The amino acid sequences of the *Ch*DRs were aligned with sequences from representative invertebrate and

Table 1
Primers used in this study.

Primer name	Sequence(5′–3′)
ChTNFR16 5′RACE-OU	TCTTTTCTTTGATTCTGGAATACTG
ChTNFR16 5′RACE-IN	TATTTTTCCCTAACTGTTGTTCGGT
ChTNFR16 3′RACE-OU	TGATGAAGGAACCTCAGACGGAAG
ChTNFR16 3′RACE-IN	ACAGAGCCAGATGTTGGTTTGAAT
ChTNFR55′RACE-OU	ACACTGGGAATGTCGGTCACAATAG
ChTNFR55′RACE-IN	AGTGCTCGTCAATACAGGAGGTGGT
ChTNFR53′RACE-OU	AGTGGGCATTGTGGTGGGGGTCGTA
ChTNFR53′RACE-IN	ATGTGTCGCAGGCAAGGCTGTCTAC
ChEDAR5′RACE-OU	GGAACACAGACCAAGAATAGCACCA
ChEDAR 5′RACE-IN	CGCCTTCGGTACATTTTAAGCAGTC
ChEDAR 3′RACE-OU	CAAGAAGTACCCAATGACAGAGAGC
ChEDAR 3′RACE-IN	ACGCACGTTTAGGTCGCGAACTGGG
ChTNFR27 5′RACE-OU	AATCTTCGCTGGCAGTTGATAAATA
ChTNFR27 5′RACE-IN	GTGAAAATCAGAATCATTAGGAAGA
ChTNFR27 3′RACE-OU	TACCGTCGTTCCACTAGTGATTT
ChTNFR27 3′RACE-IN	CGCGGATCCTCCACTAGTGATTTCACTATAGG
ChTNFR16 ORF-up	CATTTTAAACGGGGACTTGC
ChTNFR16 ORF-down	AAATAAAAAGGAGCAAAAACAAAAG
ChTNFR5ORF-up	TACTGTGAACATAAATAAACGGACT
ChTNFR5ORF-down	CAAACACAAGAATCACAGTTATGGC
ChEDAR ORF-up	CTGGTACGATTTAAAGAACACATGA
ChEDAR ORF-down	CAGAAACCCTCCACATAAAACAAC
ChTNFR27ORF-up	TTTGTCAACAGCCAGAGACTATGCA
ChTNFR27ORF-down	AATCAATTGCCGCGACCCTTATGCT
ChTNFR16-qPCR F	GCAAGGCAACTCGGATACAC
ChTNFR16-qPCR R	TTGTGGTCAGTCTTTGAGCCC
ChTNFR5-qPCR F	GTAATAGAGGAACACGAGGCG
ChTNFR5-qPCR R	TGGGAAACAGCACAGTGAAAC
ChEDAR -qPCR F	GCGCAGTGGTGCTATTCTTGGTCT
ChEDAR -qPCR R	TCGCCGCCATCCTGATCTTTTA
ChTNFR27-qPCR F	AAGCAGCCCTCAACAAACACAACA
ChTNFR27-qPCR R	TACATCATTCCCCGTATTCAACCTC
ChGAPDH-qPCR F	GGATTGGCGTGGTGGTAGAG
ChGAPDH-qPCR R	GTATGATGCCCCTTTGTTGAGTC
ChTNFR16 His F	TAGTCCAGTGTGGTGGAATTCATGGGACTAATGAATGCCAC
ChTNFR16 His R	GAAGGGCCCTCTAGACTCGAGCACAATATGAACATGATTGT
ChTNFR16 GFP F	CTACCGGACTCAGATCTCGAGATGGGACTAATGAATGCCAC
ChTNFR16 GFP R	GTACCGTCGACTGCAGAATTC AATATGAACATGATTGTGGT
ChTNFR5 His F	TAGTCCAGTGTGGTGGAATTC ATGTTGAGGTGGTATCTGT
ChTNFR5 His R	GAAGGGCCCTCTAGACTCGAGCTGGTTTCTTGGAACGTCA
ChTNFR5 GFP F	CTACCGGACTCAGATCTCGAGATGTTGAGGTGGTATCTGTA
ChTNFR5 GFP R	GTACCGTCGACTGCAGAATTCGGTTTCTTGGAACGTCACTG
ChEDAR His F	TAGTCCAGTGTGGTGGAATTCATGGGCTTTCCGCCGGTCC
ChEDAR His R	GAAGGGCCCTCTAGACTCGAGAGGATCGTCTTTCAGAATA
ChEDAR GFP F	ATGGGCTTTCCGCCGGTCCTAGTTCTTCTGTCAGTGGCTGT
ChEDAR GFP R	GTACCGTCGACTGCAGAATTCGATCGTCTTTCAGAATATCA
ChTNFR27 His-F	TAGTCCAGTGTGGTGGAATTCATGCAGTGGCAGTCGGGAG
ChTNFR27 His R	GAAGGGCCCTCTAGACTCGAGTTTTCGACTGGACGGATTG
ChTNFR27 GFP F	CTACCGGACTCAGATCTCGAGATGCAGTGGCAGTCGGGAG
ChTNFR27 GFP-R	GTACCGTCGACTGCAGAATTCTTCGACTGGACGGATTGTTA

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