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Molecular cloning, expression, and functional analysis of caspase-10 from Japanese flounder *Paralichthys olivaceus*

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Abstract

We isolated and sequenced caspase-10 cDNA and gene from Japanese flounder, *Paralichthys olivaceus*. The Japanese flounder (JF)-caspase-10 cDNA consisted of 2282 bp and encoded 495 amino acid residues. The characteristic death effector domains (DEDs) of caspases were observed in JF-caspase-10 as well as the three aspartic acid residues (D-186, -382 and -392), which are potential cleavage sites for the large and small subunit structures. The amino acid residue (His-325) and pentapeptide (QACQG), which are involved in catalytic activity, were absolutely conserved in Japanese flounder-caspase-10. JF-caspase-10 gene has a length of 6.6 kb and consists of 11 exons and 10 introns similar to that of human. The strong expression of JF-caspase-10 mRNA was detected in the gills, peripheral blood leukocytes, spleen and posterior kidney, while the weak expression was observed in the head kidney, heart, intestine, skin and stomach. The over-expression analysis of JF-caspase-10 in Japanese flounder cell line HINAE was shown to induce apoptosis 24 h post-transfection using TUNEL assay.

Keywords: Caspase-10; Japanese flounder (*Paralichthys olivaceus*); Gene cloning; RT-PCR; Over-expression analysis; TUNEL assay

1. Introduction

Apoptosis is one of the categories of programmed cell death, wherein cell death is induced without inflammation and tissue damage [1,2]. Apoptosis mainly occurs during normal tissue turnover, embryonic development and selection of T cells in the thymus [3]. Apoptosis is also induced by cytotoxic cells during the removal of virus-infected cells or tumor cells [4,5].

Caspases, which are the transmediator of the apoptotic signal, were identified in mammals as homologues of *ced* genes from the nematode *Caenorhabditis elegans* [6]. Caspases are located in the cytoplasm of the cell, and categorized into initiator or effector caspases according to their function [7,8]. Initiator caspases are located up-stream in the caspase cascade and receive the apoptotic signals from mitochondoria or death receptors, and transmit apoptotic

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signals to effector caspases [9,10]. Effector caspases transmit apoptosis induction signal from initiator caspases and activate various enzymes, such as DNase and proteases, which are involved in apoptosis induction [11].

Caspase-10 is classified as an initiator caspase, and comprised of a pro-domain that contains two death effector domains (DEDs) at the N-terminus [12]. The DED is a protein interaction domain and mediates the recruitment of two proteins [13]. This domain is highly conserved among the adaptor proteins involved in induction of the apoptosis signal [13]. Fourteen caspases have been cloned and identified from mammals, and their protein sequences have been well characterized, however, DED is observed only in caspase-8 and -10 [14].

The cross-linking of death ligands to their specific receptors on the surface of the cell membrane, such as Fas ligand and Fas antigen, causes formation of a protein complex consisting of death receptor, FADD and caspase-10 in the cytoplasm [15]. In this protein complex, caspase-10 digests itself by autoproteolytic activity at specific aspartic acid residues, resulting in the formation of active caspase-10, which is a heterotetramer of two of each of the large and small subunits [16]. The mature caspase-10 acquires protease activity and cleaves effector caspases into biologically active forms [17].

Several amino acid residues and domains are highly conserved and are known to be vital to preserve the catalytic activity of caspase-10. Especially, the pentapeptide QACQG, considered as a catalytic domain, is completely conserved between human and African clawed frog, despite their evolutionary distance [18]. In addition, caspases possess four characteristic amino acid residues in the large and small subunit, which form a carboxylate binding pocket in order to fix and capture the substrate [19]. It is also suggested that the amino acid residues around the carboxylate binding pocket may determine the substrate specificities of caspase [19].

It is reported that caspase-10 plays an important role in maintaining homeostasis and in the immune response, especially through the apoptotic signal from Fas ligand. Caspase-10 is involved in the elimination of the T cells which react to self-antigen, therefore the introduction of mutations in caspase-10 lead to a decrease in the sensitivity to Fas ligand or TRIL-mediated apoptosis, and causes autoimmune diseases [20]. In addition, human immunodeficiency virus (HIV) appears to down-regulate the apoptosis induction pathway by suppression of caspase-10 to propagate themselves in the host cells [21].

In fish, several caspases have been cloned from rainbow trout *Oncorhynchus mykiss*, medaka *Oryzias latipes*, channel catfish *Ictalurus punctatus*, Atlantic salmon *Salmo salar*, sea bass *Dicentrarchus labrax* and zebrafish *Danio rerio* [22–30], however, no homologue of fish caspase-10 has yet been identified. As described above, caspase-10 plays a vital role via inducing apoptosis in the target cells, therefore, identification and elucidation of caspase-10 for fish is essential to understand fish immune response. Fortunately, the partial cDNA fragment, which showed similarity to mammalian caspase-10, was obtained by the EST analysis from a previous study [31]. In this study, the full-length Japanese flounder-caspase-10 cDNA and gene were determined and their structure characterized. The gene expression analysis was conducted in several tissues from healthy fish, and its expression pattern was compared with Japanese flounder *Paralichthys olivaceus* Fas ligand. Furthermore, the over-expression analysis was performed to investigate the apoptosis induction activity by introducing JF-caspase-10.

2. Materials and methods

2.1. Determination of cDNA and gene sequence

In our previous EST study, we obtained the partial fragment of Japanese flounder-caspase (JF-caspase)-10 from cDNA library, which was constructed from peripheral blood leukocytes (PBLs) stimulated with concanavalin A (70 μg/ml) and phorbol myristate acetate (0.35 μg/ml) [31]. To determine the entire cDNA sequence of JF-caspase-10, 5′-RACE PCR was conducted using SMART RACE cDNA amplification kit (Clontech, USA) following the manufacturer's instructions. The sequence of JF-caspase-10 specific primer was 5′-AGTGTCACTCAG GATGTCCA-3′. PCR was performed with an initial denaturation step for 5 min at 95 °C, and then 30 cycles were run as follows: 30 s of denaturation step at 95 °C, 30 s of annealing step at 55 °C, and 2 min of extension step at 72 °C. Amplified PCR products were ligated into pGEM-T Easy vector (Promega, USA) and DNA sequences were determined using Thermo sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences, USA) following the manufacturer's instructions.

Phylogenetic analysis was conducted to analyze which cluster JF-caspase-10 belongs. Predicted amino acid sequence of JF-caspase-10 and reported caspase-8 and -10 were aligned by Clustal W software version 1.83