Developmental and Comparative Immunology 34 (2010) 538–545

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Identification and functional characterization of a human sTRAIL homolog, CasTRAIL, in an invertebrate oyster *Crassostrea ariakensis*

Shoubao Yang [a,b], Xinzhong Wu [a,*]

[a] Laboratory of Marine Life Science and Technology, College of Animal Sciences, Zhejiang University, No. 268, Kaixuan Road, Hangzhou, Zhejiang 310029, China
[b] Shaoxing University, No. 900, South City Road, Shaoxing, Zhejiang, China

ARTICLE INFO

Article history:
Received 14 October 2009
Received in revised form 23 December 2009
Accepted 23 December 2009
Available online 6 January 2010

Keywords:
Oyster *Crassostrea ariakensis*
Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)
CasTRAIL
Anti-RLO immune

ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) is one of the tumor necrosis factor (TNF) superfamily members, participating in many biological processes including apoptosis and immune responses. In present study, a novel human soluble TRAIL (sTRAIL) homolog, CasTRAIL was identified from the oyster, *Crassostrea ariakensis*. CasTRAIL has a 99% and 98% similarity to human sTRAIL over the cDNA sequence and the amino acid sequence, respectively. It mostly distributes in tissues of the oyster defense system and was mainly localized at cell membrane, and has no cytotoxicity to normal hemocytes of oyster. The phosphorylation state of MAP kinases revealed that CasTRAIL induced a rapid increase in the phospho-ERK and phospho-p38 levels, which indicated that the MAPK pathway was involved in CasTRAIL-mediated signaling. In addition, CasTRAIL also showed an ability of anti-RLO infection which might be through the p38-MAPK activation pathway. Present studies provide an understanding and insight of the biological functions of CasTRAIL.

1. Introduction

Members of the TNF ligand superfamily play multiple roles in many important physiological and pathological processes, including immune response, cell growth, survival or apoptosis [1]. In mammals, these proteins include TNF-α, Fas ligand (CD95 ligand) and tumor necrosis factor (TNF) related apoptosis-inducing ligand (Apo2L/TRAIL) [2,3]. Like many other TNF family ligands, membranous TRAIL can be cleaved by specific metalloproteases into soluble form (sTRAIL) [4], which forms a homotrimeric complex with potent apoptosis-inducing activity [2,5]. So, TRAIL cannot only exists as a type II membrane protein but also a biologically active, soluble form (sTRAIL) in human cells [2,5,6], it can interact with two death receptors (DR4 and DR5) and selectively induce apoptosis in many human tumor cell lines but showed little or no cytotoxicity to normal cells [2,6–9]. In addition, many studies revealed that TRAIL can be induced by many kinds of stimuli including LPS, viruses and so on [10–12], which proved that it also plays the important roles in immune responses [13].

Although TRAIL and its roles in pro-apoptosis have been reported in vertebrates from fishes to mammals including grass carp, mandarin fish and human [2,14,15], however, there is still no information available on the existence and function of TRAIL in other lower order species, especially in invertebrates up to date. On the contrary, several kinds of TNF Members have been reported in invertebrates, for example, Eiger and its receptor, Wengen have been characterized and their role in the JNK-dependent pathway was reported in *Drosophila* [16,17]. Then, four TNF ligand superfamily members were found in the sea urchin (*Strongylocentrotus purpuratus*) genome [18,19]. More recently, a TNF ligand (CsTL) was identified in the invertebrate *Ciona savignyi* [20], and in disk abalone (*Haliotis discus discus*) a TNF-α homologue (AbTNF-α) and a Fas ligand (AbFas) were isolated and characterized [21,22]. Besides, other TNF-α-like molecules such as LPS-induced TNF-α factor (LITAF) were also characterized in bivalve *Chlamys farreri* [23], *Crassostrea gigas* [24] and *Pinctada fucata* [25].

Rickettsia-like organism (RLO), an obligate intracellular Gram negative bacterium, multiply only within host cells [26], and has been reported as the species of pathogen in many aquatic animals, including fish, molluscs, and crustaceans [26–28]. The oyster, *C. ariakensis* has been cultured with wide range in southern part of China. It has been proved that the mass mortality of *C. ariakensis* occurred and caused by a new pathogen—rickettsia-like organism (RLO) in recent years [29]. More recently, immune-related genes including CREB and Tolloid-like gene (CaTLL) involved in response to RLO challenge were reported [30,31]. And an outer membrane protein encoded

* Corresponding author. Tel.: +86 571 86971960; fax: +86 571 86971960.
E-mail address: wuxz@zju.edu.cn (X. Wu).

0145-305X/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2009.12.014

by *omp*R gene of rickettsia-like organism was identified. The role of this protein in promoting the immune response was characterized through analyzing the interaction between RLO ompR and oyster [32]. However, molecular mechanisms of immunology in the oysters have not been clearly characterized until now. So, the study of the interaction between the host oyster, *C. ariakensis* and pathogen RLO will contribute to the prevention and control of RLO disease. In this study, we report for the first time the molecular cloning, expression of a soluble TRAIL gene in the oyster, *C. ariakensis* (CasTRAIL, GenBank accession no. EF541151, NCBI). CasTRAIL was highly homologous to human sTRAIL (amino acids 114–281 of human TRAIL) both from the cDNA sequence and the deduced amino acid sequences. The following potential biological functions of oyster sTRAIL, for example, subcellular location, responses to RLO challenge and cytotoxicity to normal oyster hemocytes as well as actions in the MAPKs signaling pathway were involved in present study.

2. Materials and methods

2.1. Challenge with rickettsia-like organisms (RLOs)

Moribund oysters aged 2–3 years were sampled from Guangdong Province, China. The RLOs have been purified directly from infected oyster tissues by the use of differential speed centrifugation and renografin density gradient centrifugation according to previously described methods [33]. 10 oysters were used in each experimental condition. Oysters were challenged by injection into the adductor muscle with 100 μl of RLOs (10^9 bacteria per ml), or 100 μl of saline as control. All experiments were conducted in conformity with the National Guidelines for Biological Research involving animals.

2.2. RNA purification

Hemolymph was collected from the adductor muscle of oysters at different times post-injection and immediately centrifuged at 1000 × *g* for 10 min at 4 °C. Hemocyte pellets were collected and ground immediately under liquid nitrogen for RNA extraction. Total RNA was extracted using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions.

2.3. Cloning and sequencing

First strand cDNA was synthesized using total RNA from hemocytes of RLO-challenged and unchallenged oysters with a First-Strand System Kit (Promega, USA). PCR was performed as the following conditions: initial denaturation at 94 °C for 4 min; followed by 35 cycles of amplification (94 °C for 30 s, 56 °C for 30 s, 72 °C for 90 s) and 1 cycle of 10 min at 72 °C.

Searching of nucleotide and amino acid sequences in GenBank databases was carried out using the Blast at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The open reading frame (ORF) was acquired with ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/). Domains/Motifs were predicted by the domain prediction program simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/). Multiple sequence alignment and phylogenetic tree were performed using neighbor-joining method with MEGA4.0 package. The reliability of the tree was bootstraped with 1000 replicates.

2.4. In situ hybridization

Tissues from the RLO-challenged and unchallenged *C. ariakensis* were removed and immediately fixed in 4% (w/v) paraformaldehyde in PBS (contains 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4) for 4 h. The digoxigenin-labeled RNA probe was generated from the initially cloned cDNA sequence of CasTRAIL using the DIG Labeling Kit (Roche, Hong Kong, China), the sequence was: 5′-ATGTATGCCCCCAGCCTTCATGATGGTGATTAG-CCTGGATC-3′. RNA in situ hybridization was performed according to previously described [34].

2.5. Protein purification and polyclonal antibody production

The ORF sequence of the CasTRAIL gene was amplified from the cDNA mixture with primers TRAILf (5′-CTGCATATGGTGAGAGAAA-GAG-3′) and TRAILr (5′-CGCTCGAGTTAGCCAACTTAAAAG-3′), the PCR product was inserted into the NdeI and XhoI site of pET28a (the restriction sites were underlined, Novagen, Germany) and transformed into *E. coli* strain BL21 (DE3, Invitrogen, Germany). Protein expression was induced with 0.1 mM isopropyl D-thiogalactoside (IPTG, Sigma, USA) at 30 °C. The recombinant fusion proteins were purified by His-bind nickel column chromatography according to the manufacturer's instructions (Qiagen, Germany). Two New Zealand white rabbits were immunized with 2 mg of purified proteins each homogenized in complete Freunds adjuvant (Sigma, USA) for two times at 2-week intervals. A booster injection with 1 mg of purified proteins in incomplete Freunds adjuvant (Sigma) was given after another 2 weeks. Rabbit serum was collected at week 8. Antiserum was prepared according to the method described in Ref. [35], and stored at −20 °C.

2.6. Hemocyte monolayers

Hemocyte monolayers were prepared as previously described [36,37] with a little modification. Briefly, hemolymph was extracted from the posterior adductor muscle of 10 oysters for each experiment and pooled to about 15 ml samples. Hemolymph serum was obtained by centrifuging at 500 × *g* for 5 min, and the supernatant was sterilized through a filter of 0.22 μm pore size. 1 ml of hemolymph (about 10^6 cells) was dispensed into sterile Petri dishes and prepared as hemocyte monolayers, then incubated at 15 °C for 30 min and added with 2 ml of hemolymph serum before use.

2.7. Real-time PCR

Equal quantities (3 μg) of each total RNA sample was used in quantitative Real-time PCR to measure the distribution and expression of CasTRAIL in various tissues under RLO challenged. The primers were Casf (5′-CTGACCCTATAT TGTTGATG-3′), and Casr (5′-GTCCATGTCTATCAAGTGC-3′).

Hemocyte monolayers were incubated with recombinant CasTRAIL proteins (5 μg/ml) for different periods of time. The gene specific primers for p38 (GenBank accession no. EF392834, NCBI) were p38f (5′-GGTGCGGATCTGAACAACA-3′) and p38r (5′-ACAGTCTTCATTCACACCG-3′); for 28S rDNA (GenBank accession no. AF137052, NCBI). The internal control was 28Sf (5′-CACC-GAATCCCTCATCCT-3′) and 28Sr (5′-CTGCTCTGGACTGTCCTA-3′). All data were presented as the standard errors of the mean (S.E.M.). Differences were considered statistically significant when p values were less than 0.05.

2.8. Western blotting

Purified recombinant proteins and proteins extracted from hemocytes were subjected to 10% SDS-PAGE. After transfer, the polyvinylidene difluoride (PVDF) membrane (Sigma, USA) was blocked with PBST (PBS pH 7.4, containing 0.1% Tween-20) containing 5% skim milk for 3 h at room temperature, and then incubated with primary antibodies (1:2500) diluted in

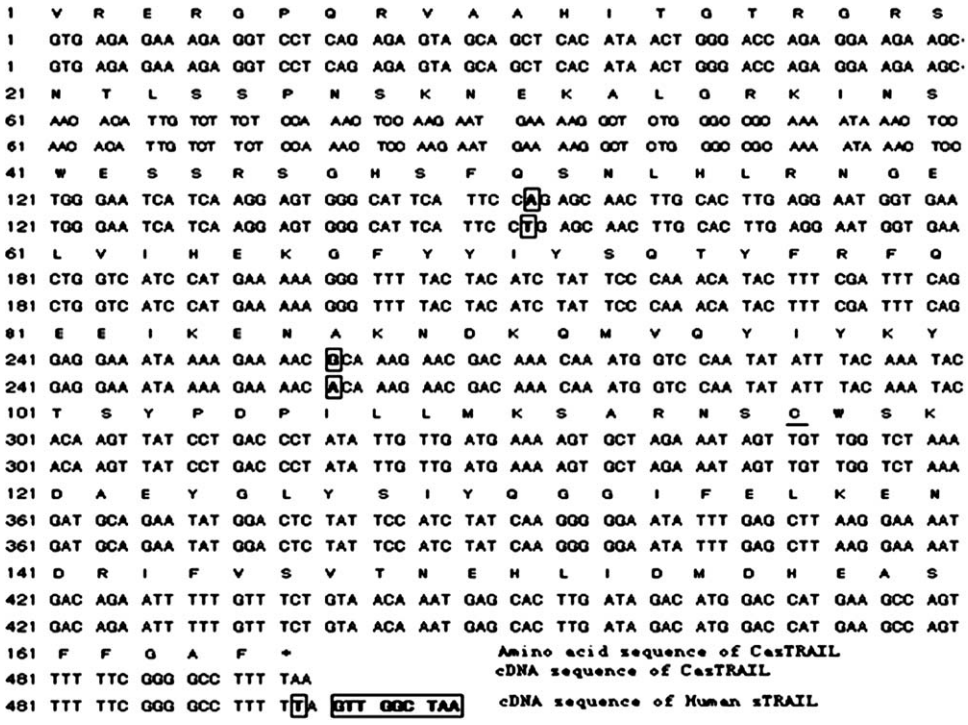

Fig. 1. Nucleotide and amino acid sequence of CasTRAIL and comparison with human sTRAIL cDNA. The sites not conserved were boxed. Nucleotide and amino acid numbers, are starting from the guanine and valine, respectively. Underline shows the unpaired cysteine residue C117 of CasTRAIL. Asterisk indicates the stop codon.

blocking buffer. After washing three times with PBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated IgG (Tiangen, Germany) diluted 1/2000 in PBST at room temperature for 1 h. The immune complexes were detected with an HRP-DAB Detection Kit (Tiangen, Germany). Quantification for total protein was performed using the Bradford method [38].

2.9. Immunofluorescence

Oyster hemocytes were collected and fixed in 4% paraformaldehyde–PBS for 20 min at 37 °C. After that, they were permeabilized in 0.2% Triton X-100 PBS for 3 min and washed three times for 5 min with PBS (contains 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4), and then cells were immunostained with anti-CasTRAIL primary antibodies raised with recombinant CasTRAIL and FITC-labeled secondary antibody. Images were obtained by a fluorescence microscopy using the software ACT-1.0.

2.10. Antibodies

Phosphorylated antibodies, anti-p38, anti-ERK and anti-JUK were purchased from Promega, USA. β-Actin and other antibodies were purchased from Santa Cruz, USA.

2.11. Flow cytometric analysis and DNA fragmentation

Hemocyte monolayers were prepared as described above and incubated with recombinant CasTRAIL proteins (5 μg/ml) for 0–24 h. The CasTRAIL induced-hemocytes were incubated with annexin V-fluorescein and propidium iodide staining (Annexin V-FITC Apoptosis Detection Kit, Merck, Germany), and analyzed by Flow cytometry. Oyster genomic DNA was extracted by a DNA Isolation Kit (Shaibaisheng, China) according to the manufacturer's instructions and subjected to electrophoresis in a 2% agarose gel to detect the formation of DNA ladder.

3. Results

3.1. Sequence analysis and schematic representation of CasTRAIL

Partial sequence of TRAIL cDNA was obtained from the oyster, *C. ariakensis* (GenBank accession no. EF541151, NCBI) when cloning immune-related genes used RT-PCR technology, which includes an open reading frame of 501 nucleotides encoding a hypothetical protein of 167 amino acids with a molecular mass about 18.4 kDa (Fig. 1).

Comparison of CasTRAIL with extracellular domains of TRAIL protein from vertebrates including human and various animals (mice, chicken, platypus and grass carp) revealed a similarity ranging from 42% to 98% (Fig. 2a and b). But oyster TRAIL has only 15% to 20% similarity with other types of TNF domains from invertebrate species including *Drosophila melanogaster* Eiger (GenBank accession no. BAC00950, NCBI), *Ciona savignyi* TNF-α (GenBank accession no. EU216599, NCBI), *H. discus discus* TNF-α (GenBank accession no. EU863217, NCBI) and *H. discus discus* Fas ligand (GenBank accession no. ACJ12607, NCBI) (Fig. 2c).

Interestingly, CasTRAIL was highly homologous to sTRAIL of human. It has a 99% similarity over the cDNA sequence and 98% over the amino acid sequence between them (Figs. 1 and 2a). On the contrary, it was only 42% of similarity compared to grass carp (*Ctenopharyngodon idella*), which suggests that CasTRAIL is a homolog to human sTRAIL (Fig. 2a). The results of the phylogenetic analysis also supported this conclusion (Fig. 2b).

In addition, an unpaired cysteine residue (C230) is essential for the structure and pro-apoptotic activity of human TRAIL [39], CasTRAIL also has a cysteine residue (C117) at the similar site (Fig. 1), indicating that they might have the similar biological function.

3.2. Recombinant protein and polyclonal antibody production

The complete ORF of CasTRAIL was cloned into the pET28a expression vector. A protein with a molecular weight about 20 kDa was expressed in *E. coli* BL21 (DE3) with His-tag at carboxyl terminus and purified using the Ni-NTA affinity columns. Purified

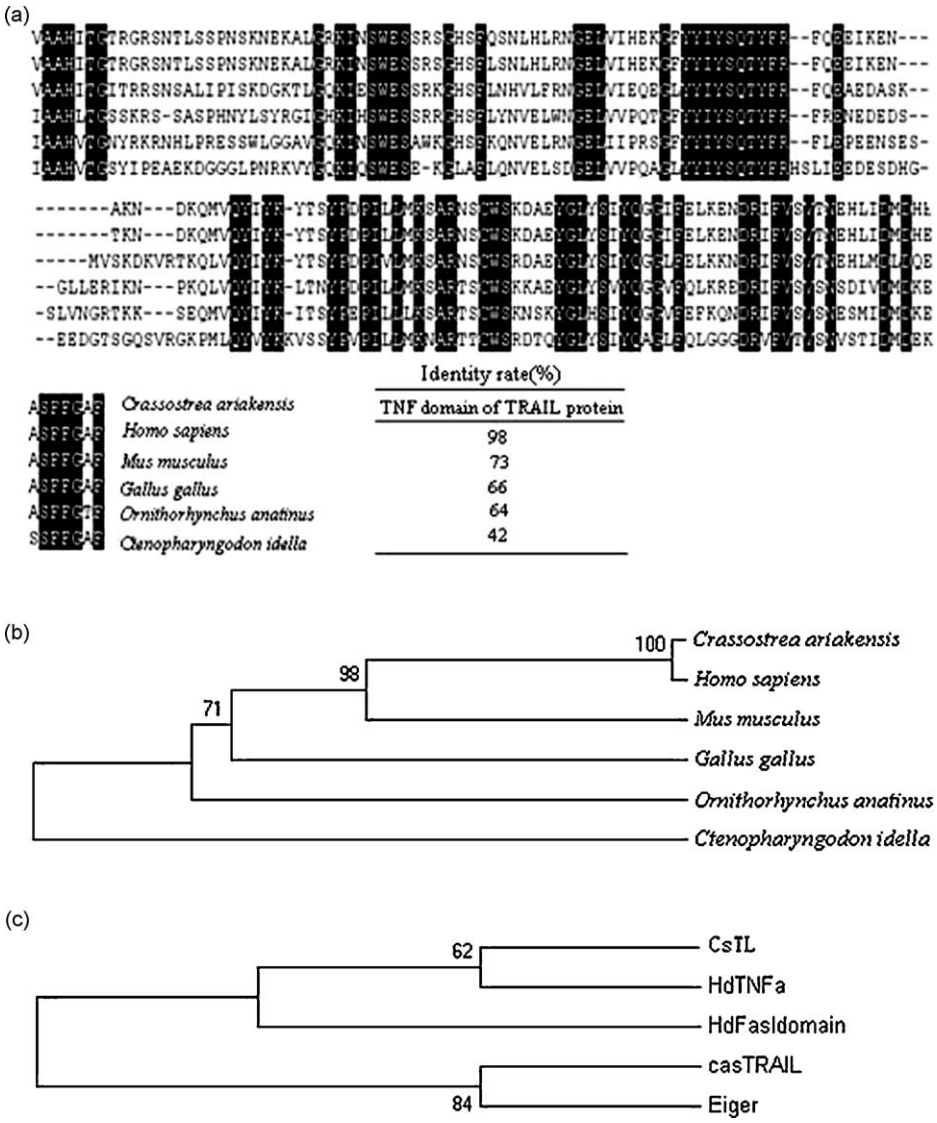

Fig. 2. Sequence and phylogenetic analysis of TNF domain of TRAIL protein. (a) Comparisons of TNF domain from CasTRAIL with other TRAIL proteins and identity levels between TNF domain and other compared proteins. (b) Phylogenetic tree for CasTRAIL and other TRAIL proteins. The unrooted tree was built using the neighbor-joining method based on the alignment of TNF domain amino acid sequences. Numbers indicate the bootstrap percentage (1000 replicates). (c) Phylogenetic tree for oyster TRAIL and other TNF member proteins of invertebrates.

protein showed expected molecular weight as detected by SDS-PAGE and was confirmed by Western blotting using an anti-His antibody and anti-CasTRAIL rabbit antiserum (Fig. 3).

3.3. Tissues distribution of oyster TRAIL

To investigate the potential function of oyster TRAIL in vivo, distribution and the expression levels of TRAIL were investigated by in situ hybridization (ISH) in different tissues including gills, gonad, hemolymph, mantle and posterior adductor muscle. The results showed that TRAIL was ubiquitously expressed in all examined tissues although very few were in gonad (Fig. 4a and c). In oyster, gills, hemolymph and mantle are main components of oyster defense system [32]. TRAIL constituently expressed in these tissues implied that it might play important roles in oyster by providing a first line of defense against pathogenic invasion.

3.4. Subcellular location of oyster TRAIL protein

To better understand the regulation and function of oyster TRAIL, immunostaining was carried out to determine the subcellular locations of TRAIL protein. Oyster hemocytes were immunostained with the anti-CasTRAIL primary antibodies raised with recombinant CasTRAIL and a FITC-labeled secondary antibody. As shown in Fig. 4d, the brightest staining was shown in the cell membrane of oyster hemocytes and some of them in the cytoplasm, but not in the nucleus, proving that similar to human TRAIL, TRAIL in oyster, *C. ariakensis* was also primarily located at the cell membrane, and may be also expressed as a soluble form (sTRAIL) in the cytoplasm and other organelle [2,5,6].

3.5. Functional analysis of oyster TRAIL involved in inflammation and immune responses

3.5.1. The observation of anti-RLO defense function of oyster TRAIL

In order to investigate anti-RLO defense function of oyster TRAIL in vivo, tissues from oysters 6 h after RLO challenge were analyzed by ISH and Real-time PCR as described above. The positive TRAIL mRNA hybridization signals are in red. As shown in Fig. 4a, increased positive signals were detected in mantle, hemolymph and muscle, while decreased in gill, and no obvious changes were found in gonad (Fig. 4b). Results of Real-time PCR showed that the expression levels

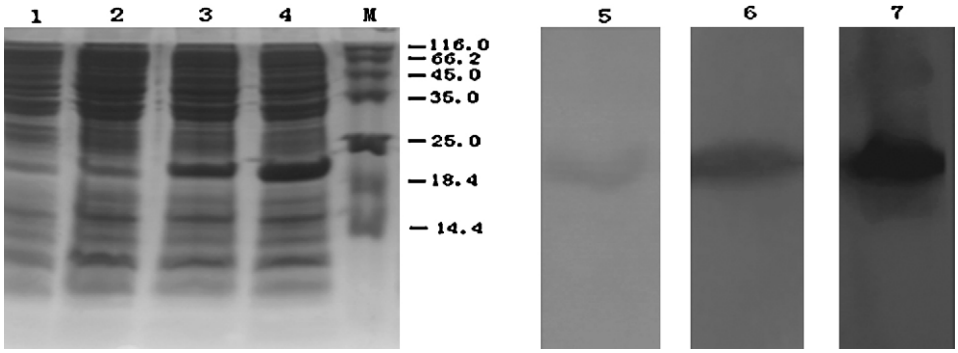

Fig. 3. SDS-PAGE and Western blotting of CasTRAIL. Bacterial proteins and recombinant fusion proteins were separated on 10% SDS-PAGE gels. After electrophoresis, CasTRAIL protein was revealed by Coomassie blue R-250 staining or blotted onto PVDF membranes. A band with a molecular weight of about 20 kDa was detected by Western blotting using anti-His antibody or anti-CasTRAIL rabbit antiserum. Lane 1, before induction; Lane 2–4, after induction by 1.0, 0.5 and 0.1 mM IPTG, respectively; M, molecular weight marker; Lane 5, Western blotting of purified recombinant proteins using anti His-tag antibody; Lane 6, purified recombinant proteins and the gel was stained with Coomassie blue R-250; Lane 7, Western blotting of purified recombinant proteins using anti-CasTRAIL rabbit antiserum.

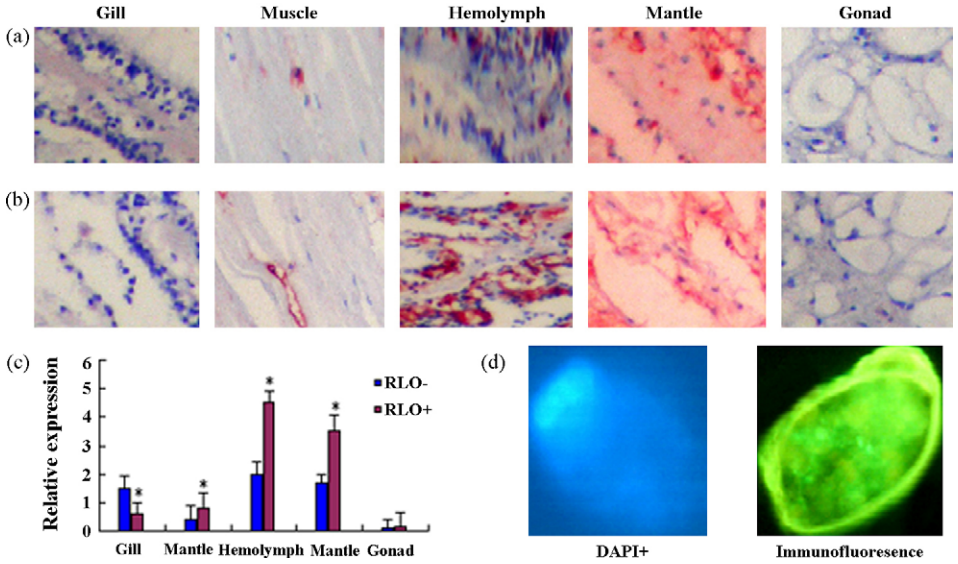

Fig. 4. Distribution, expression and subcellular localization of CasTRAIL. (a) Distribution of CasTRAIL in various tissues. (b) The expression of CasTRAIL in various tissues under RLO challenged. (c) Real-time RT-PCR analysis of the expression of CasTRAIL in various tissues under RLO challenged. The values were presented as mean ± S.E. of independent experiments done in triplicates and analyzed by Student's *t*-test; $^{*}P \leq 0.05$ when compared to control values. (d) Subcellular localization of CasTRAIL in oyster hemocyte. Anti-CasTRAIL primary antibodies and FITC-labeled secondary antibody were used in immunofluorescence staining.

of TRAIL were significantly up-regulated by RLO stimulation in tissues of hemolymph, mantle and muscle, and significantly down-regulated in gill; while no significant changes were detected in gonad (Fig. 4c). These results suggested that oyster TRAIL might be involved in anti-RLO defense mechanisms.

3.5.2. The effects of CasTRAIL on expression levels of p38-MAPK in hemocyte

To investigate the effects of CasTRAIL stimulation on the expression levels of immune-related genes, Real-time PCR was carried out using RNA from hemocytes after CasTRAIL exposure. Phosphorylation analysis of p38-MAPK was carried out as stated above. The results showed that CasTRAIL could up-regulate the expression levels of p38-MAPK both from mRNA level (at 15 min and 30 min) and protein level (at 15–60 min) (Figs. 5 and 6), which indicated that the p38-MAPK pathway was involved in CasTRAIL-mediated anti-RLO signaling pathway.

3.6. Functional analysis of CasTRAIL in inducing apoptosis

3.6.1. The observation of cytotoxicity of CasTRAIL on hemocytes

To investigate the biological activity of CasTRAIL, oyster hemocytes were screened for responsiveness to the purified protein. Flow cytometry was performed as previously described. The results showed that CasTRAIL does not cause cell apoptosis in normal hemocytes (Fig. 5a), which showed an average apoptosis rate of 7.5% in control group, and 7.5% in 24 h induction group. And no obvious DNA ladder was detected in hemocytes after 0–24 h from CasTRAIL addition (Fig. 5b). These results illustrated that CasTRAIL does not generate toxic effect to normal hemocyte.

3.6.2. Phosphorylation state of p38-, ERK, JNK MAP kinases in hemocytes

To further investigate the molecular mechanisms and signaling pathways of CasTRAIL acting on oyster hemocytes, antibodies against phospho-ERK, phospho-p38, and phospho-JNK were used to analyze the phosphorylation state of MAP kinases (p38, ERK, JNK) by Western blotting. The results revealed that CasTRAIL induced a rapid increase in the phospho-ERK levels at 30–60 min and phospho-p38 levels at 15–60 min (Fig. 5c). But the phosphorylation levels of JNK were unchanged with CasTRAIL incubation. These results clearly indicated that MAPK pathway was involved in CasTRAIL-mediated signaling, and might play the important roles in keeping a hematopoiesis in hemocytes and preventing normal hemocytes from cell death.

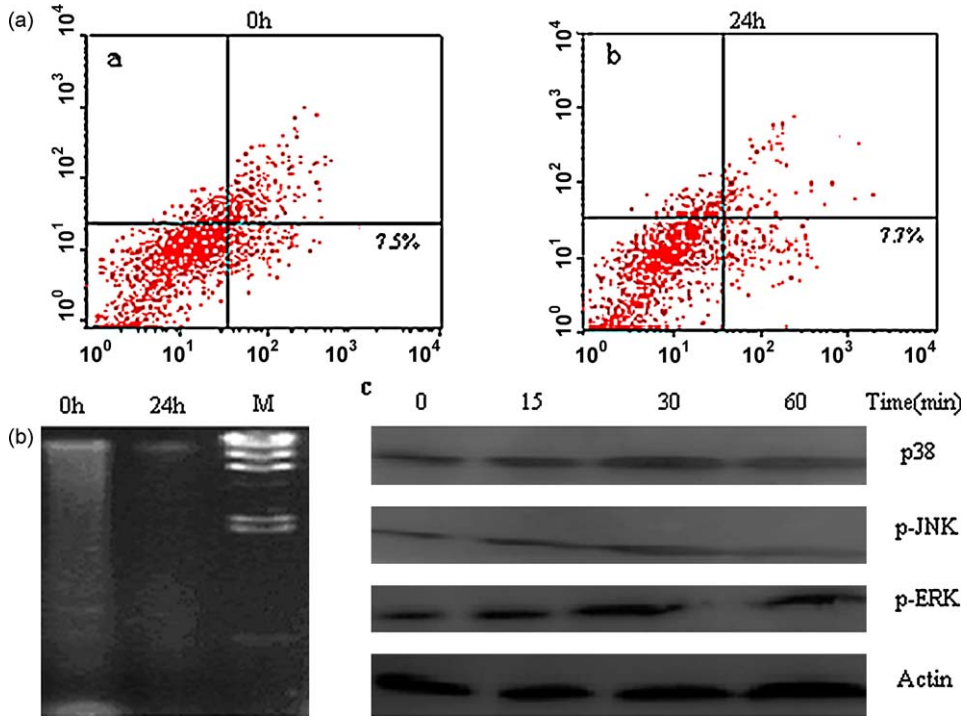

Fig. 5. Effect of CasTRAIL on oyster hemocyte and the phosphorylation state of MAPKs (p38, JNK, and ERK). Hemocytes were incubated with CasTRAIL from 0 to 60 min. Cells were labeled with the annexin V-FITC and analyzed by Flow cytometer and DNA ladder. (a) Flow cytometer analysis of hemocytes at 0 and 24 h after CasTRAIL addition. (b) DNA electrophoresis to detect the formation of DNA ladder at 0 and 24 h after CasTRAIL addition. (c) Effects of CasTRAIL on MAPKs (p38, JNK, and ERK) phosphorylation state in hemocytes. Anti-actin was used as control.

4. Discussion

Mollusca are a very diversified group of invertebrates with more than 100,000 living species, most of them are marine [40]. The oyster *C. ariakensis* is a representative species of bivalve, and it is an economically important oyster cultured in China and other areas of the world, which suffered from the mass mortality, caused by RLO, in recent years [27,29].

Here a CasTRAIL was cloned and characterized from oyster *C. ariakensis*. Interestingly, CasTRAIL showed high similarity to human sTRAIL in which it has about 99% and 98% similarity to human sTRAIL over the cDNA sequence and the amino acid sequence, respectively. In addition, it has also the similar domain structure and a 98% of identity rate over the amino acid sequence of TNF domain. But CasTRAIL has only 42% similarity with the TNF domain of grass carp, *C. idella*, and only 15–20% similarity with other TNF domain from invertebrate species including fruit fly, sea squirts, and abalone, which indicates that CasTRAIL is homologous to human sTRAIL. Furthermore, they have the same cysteine residue (C117 and C230, respectively) that is essential for the structure and pro-apoptotic activity. So they might have the similar biological functions.

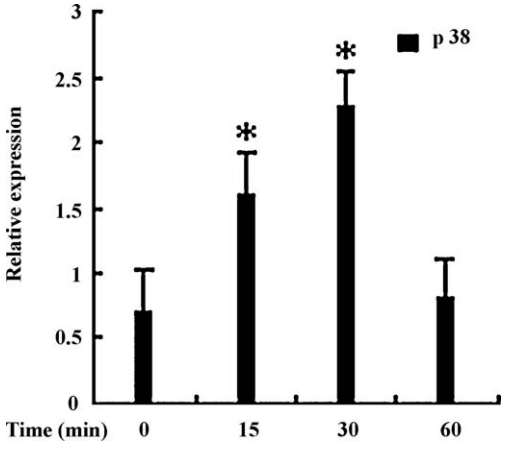

Fig. 6. Analyses of the expression levels of p38-MAPK in hemocytes. Expression levels were assessed by Real-time RT-PCR using the 28Sr DNA for normalization. The seawater-injected group was used as a control. Samples for p38 analysis were collected at 0, 15, 30 and 60 min after CasTRAIL exposure. The relative expression levels of two genes were calculated according to the $2^{-\Delta\Delta CT}$ methods. The values were presented as mean ± S.E. of independent experiments done in triplicates and analyzed by Student's *t*-test; $^{*}P \leq 0.05$ when compared to control values.

In human, sTRAIL was reported to play an important role in the induction of apoptosis of tumor cells and in the immune response [2,10], while inducing little cytotoxicity to normal cells [6]. Present studies were carried out to investigate whether CasTRAIL has similar functions as human sTRAIL. Firstly, to investigate whether CasTRAIL has cytotoxicity to normal oyster hemocytes, cells were induced with recombinant CasTRAIL protein and no obvious apoptosis was detected, which indicated that CasTRAIL has no cytotoxicity to normal oyster hemocytes. Secondly, to further investigate the molecular mechanisms and signaling pathways of CasTRAIL acting on oyster hemocytes, phosphorylation states of MAP kinases (p38, ERK, JNK) in oyster hemocytes were observed after CasTRAIL incubation. No apoptosis was detected in the treated cells, but the activation of ERK and p38-MAPK was detected, whereas no changes in the phosphorylation state of JNK were observed in oyster hemocytes. In mammals, p38-MAPK mediates the apoptotic pathway of TRAIL, while ERK suppresses TRAIL-mediated apoptosis [41]. Present results showed that MAPKs play important roles in the CasTRAIL signaling pathway through which ERK might play a key role in preventing normal hemocytes from apoptotic cell death. However, the molecular mechanism is still unknown. These findings suggested that

CasTRAIL, like human sTRAIL, has no cytotoxicity to normal cells (such as oyster hemocytes) by activation of MAPKs. Further work needs to be done to determine whether it has the ability of inducing the abnormal cell (such as tumor cell) death.

RLO is one of the major pathogens of oysters [29], results of Real-time PCR and ISH analyses showed that the expression levels of CasTRAIL in different immune-related tissues could be significantly up- or down-regulated by RLO challenge. Thus, this paper for the first time showed that oyster TRAIL was involved in anti-RLO defense responses to the challenge of rickettsia-like organism. To further investigate the possible molecular mechanisms involved in CasTRAIL anti-RLO infection, the expression levels of p38-MAPK was analyzed. It was found that CasTRAIL could induce a rapid increase in the expression level of p38-MAPK, which represents an ancient, evolutionarily conserved component of the metazoan defense against pathogen attack [42], and mediate the signaling pathway of many cytokines including TRAIL [41,43]. Recently, p38-MAPK was found with a rapid response to the challenge of out membrane protein of RLO, and the activated p38-MAPK was required for NF-κB-dependent gene expression [32]. These findings indicated that an anti-RLO pathway of oyster TRAIL might exist. It was hypothesized that oyster TRAIL might play key roles in transferring the stimuli of RLOs to activate p38-MAPK and then the transcription factor NF-κB, and NF-κB triggered the expression of subsequent cytokines and other genes to be against the RLO pathogen. The present study provided the understanding of anti-RLO processes of CasTRAIL, but the detailed molecular mechanisms still remain to be clarified.

In human, TRAIL is mostly expressed in immune cells, in which it could respond to pathogen invasion or play roles in the homeostasis of T-cells and NK cells as well as be involved in T-cell-mediated killing of virally and oncogenically transformed cells [44–46]. Tissues distribution and subcellular location study of oyster sTRAIL gene showed that it was mainly expressed in tissues of oyster defense system including hemolymph, gills and mantle, and localized at cell membrane. These results of CasTRAIL in distribution and subcellular location are similar to human sTRAIL and also well-matched its immune function concerning it showed no cytotoxicity to normal hemocytes of oyster and anti-RLO immune described above.

In summary, to the best of our knowledge, this is the first identification and characterization of a human sTRAIL homolog in invertebrates. Oyster TRAIL mostly distributes in the tissues involved in the oyster defense system, and was mainly localized at cell membranes. Similar to human sTRAIL, CasTRAIL has no cytotoxicity to normal hemocytes of oyster because of the activation of MAPKs (ERK) that might prevent hemocytes from apoptotic cell death. In addition, CasTRAIL also showed an ability of anti-RLO infection by activation of p38-MAPK pathway. Present studies provide an understanding and insight of the biological functions of CasTRAIL, but further research is required to elucidate the detailed molecular mechanisms by CasTRAIL against RLO infection.

Acknowledgements

This work was supported by research grant from the National Natural Science Foundation of China (30671619) and the Key Scientific Program of Zhejiang Province (2004C23041). We thank Dr. Patrick C. Hanington, University of New Mexico, Albuquerque, NM 87131, USA, for critical review of the manuscript.

References

[1] Shu HB, Johnson H. B cell maturation protein is a receptor for the tumor necrosis factor family member TALL-1. Proc Natl Acad Sci USA 2000;97:9156–61.
[2] Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem 1996;271:12687–90.
[3] Gruss HJ, Dower SK. The TNF ligand superfamily and its relevance for human diseases. Cytokines Mol Ther 1995;1:75–105.
[4] Schneider P, Tschopp J. Apoptosis induced by death receptors. Pharm Acta Helv 2000;74:281–6.
[5] Mariani SM, Matiba B, Armandola EA, Krammer PH. Interleukin 1 beta-converting enzyme related proteases/caspases are involved in TRAIL-induced apoptosis of myeloma and leukemia cells. J Cell Biol 1997;137:221–9.
[6] Simons MP, Leidal KG, Nauseef WM, Griffith TS. TNF-related apoptosis-inducing ligand (TRAIL) is expressed throughout myeloid development, resulting in a broad distribution among neutrophil granules. J Leukoc Biol 2008;83(3):621–9.
[7] Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. Oncogene 2003;22:8628–33.
[8] Jin H, Yang R, Fong S, Totpal K, Lawrence D, Zheng Z, et al. Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand cooperates with chemotherapy to inhibit orthotopic lung tumor growth and improve survival. Cancer Res 2004;64:4900–5.
[9] Liedtke C, Gröger N, Manns MP, Trautwein C. Interferon-alfa enhances TRAIL-mediated apoptosis by up-regulating caspase-8 transcription in human hepatoma cells. J Hepatol 2006;44:342–9.
[10] Halaas O, Vik R, Ashkenazi A, Espevik T. Lipopolysaccharide induces expression of Apo2 ligand/TRAIL in human monocytes and macrophages. Scand J Immunol 2000;51:244–50.
[11] Simons MP, Nauseef WM, Griffith TS. Neutrophils and TRAIL: insights into BCG immunotherapy for bladder cancer. Immunol Res 2007;39:79–93.
[12] Collison A, Foster PS, Mattes J. The emerging role of TRAIL as key regulator of inflammatory responses. Clin Exp Pharmacol Physiol 2009;36:1049–53.
[13] Thorburn A. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway signaling. J Thoracic Oncol 2007;2:461–5.
[14] Chang MX, Nie P, Xie HX, Wang GL, Gao Y. Characterization and expression analysis of TNF-related apoptosis inducing ligand (TRAIL) in grass carp *Ctenopharyngodon idella*. Vet Immunol Immunopathol 2006;110:51–63.
[15] Gao Y, Chang MX, Sun BJ, Nie P. TRAIL in the mandarin fish *Siniperca chuatsi*: gene and its apoptotic effect in HeLa cells. Fish Shellfish Immunol 2008;24:55–66.
[16] Igaki T, Kanda H, Yamamoto-Goto Y, Kanuka H, Kuranaga E, Aigaki T, et al. Eiger, a TNF superfamily ligand that triggers the *Drosophila* JNK pathway. EMBO J 2002;21:3009–18.
[17] Kauppila S, Maaty WSA, Chen P, Tomar RS, Eby MT, Chapo J, et al. Eiger and its receptor, Wengen, comprise a TNF-like system in *Drosophila*. Oncogene 2003;22:4860–7.
[18] Hibino T, Loza-Coll M, Messier C, Majeske AJ, Cohen AH, Terwilliger DP, et al. The immune gene repertoire encoded in the purple sea urchin genome. Dev Biol 2006;300:349–65.
[19] Robertson AJ, Croce J, Carbonneau S, Voronina E, Miranda E, McClay DR, et al. The genomic underpinnings of apoptosis in *Strongylocentrotus purpuratus*. Dev Biol 2006;300:321–34.
[20] Zhang X, Luan W, Jin S, Xiang J. A novel tumor necrosis factor ligand superfamily member (CsTL) from *Ciona savignyi*: molecular identification and expression analysis. Dev Comp Immunol 2008;32:1362–73.
[21] De Zoysa M, Jung S, Lee J. First molluscan TNF-alpha homologue of the TNF superfamily in disk abalone: molecular characterization and expression analysis. Fish Shellfish Immunol 2009;32:625–31.
[22] De Zoysa M, Nikapitiya C, Moon DO, Whang I, Kim GY, Lee J. A novel Fas ligand in mollusk abalone: molecular characterization, immune responses and biological activity of the recombinant protein. Fish Shellfish Immunol 2009;27:423–32.
[23] Yu YD, Qiu LM, Song LS, Zhao JM, Ni DJ, Zhang Y, et al. Molecular cloning and characterization of a putative lipopolysaccharide-induced TNFα factor (LITAF) gene homologue from Zhikong scallop *Chlamys farreri*. Fish Shellfish Immunol 2007;23:419–29.
[24] Park EM, Kim YO, Nam BH, Kong HJ, Kim WJ, Lee SJ, et al. Cloning, characterization and expression analysis of the gene for putative lipopolysaccharide-induced TNF-a factor of the Pacific oyster, *Crassostrea gigas*. Fish Shellfish Immunol 2008;24:11–7.
[25] Zhang D, Jiang J, Jiang S, Ma J, Su T, Qiu L, et al. Molecular characterization and expression analysis of a putative LPS-induced TNF-alpha factor (LITAF) from pearl oyster *Pinctada fucata*. Fish Shellfish Immunol 2009;27:391–6.
[26] Chen SC, Wang PC, Tung MC, Thompson K, Adams A. A *Piscirickettsia salmonis*-like organism in grouper, *Epinephelus melanostigma*, in Taiwan. J Fish Dis 2000;23:415–8.
[27] Federici BA, Hazard EI, Anthony DW. Rickettsia-like organism causing disease in a crangonid amphipod from Florida. Appl Microbiol 1974;28:885–6.
[28] Wu XZ, Pan JP. Studies on rickettsia-like organism disease of the tropical marine pearl oyster. I. The fine structure and morphogenesis of *Pinctada maxima* pathogen rickettsia-like organism. J Invertebr Pathol 1999;73:162–72.
[29] Sun JF, Wu XZ. Histology, ultrastructure and morphogenesis of a rickettsia-like organism causing disease in the oyster, *Crassostrea ariakensis* Gould. J Invertebr Pathol 2004;86:77–86.
[30] Zhu BJ, Wu XZ. Characterization and function of CREB homologue from *Crassostrea ariakensis* stimulated by rickettsia-like organism. Dev Comp Immunol 2008;32:1572–81.
[31] Yang S, Wu X. Tolliod-like gene in *Crassostrea ariakensis*: molecular cloning, structural characterization and expression by RLO stimulation. Fish Shellfish Immunol 2009;27:130–5.

[32] Zhu BJ, Wu XZ. Identification of outer membrane protein ompR from rickettsia-like organism and induction of immune response in *Crassostrea ariakensis*. Mol Immunol 2008;45:3198–204.
[33] Wu X, Sun J, Zhang W, Wen B. Purification and antigenic characteristics of a rickettsia-like organism from the oyster *Crassostrea ariakensis*. Dis Aquat Organ 2005;67:149–54.
[34] Chen L, He HX, Sun XD, Zhao J, Liu LH, Huang WQ, et al. Expression of gonadotropin-releasing hormone receptor and effect of gonadotropin-releasing hormone analogue on proliferation of cultured gastric smooth muscle cells of rats. World J Gastroenterol 2004;10:1780–4.
[35] Harlow E, Lane D. Using antibodies: a laboratory manual [M]. New York: Cold Spring Harbor Laboratory; 1999.
[36] Lacoste A, Cueff A, Poulet SA. P35-sensitive caspases, MAP kinases and Rho modulate beta-adrenergic induction of apoptosis in mollusc immune cells. J Cell Sci 2002;115:761–8.
[37] Canesi L, Gallo G, Gavioli M, Pruzzo C. Bacteria–hemocyte interactions and phagocytosis in marine bivalves. Microsc Res Tech 2002;57:469–76.
[38] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[39] Bodmer JL, Meier P, Tschopp J, Schneider P. Cysteine 230 is essential for the structure and activity of the cytotoxic ligand TRAIL. J Biol Chem 2000;275:20632–7.
[40] Jiang J. Invertebrate zoology. Beijing, China: Higher Education Press; 1982.
[41] Tran SE, Holmstrom TH, Ahonen M, Kahari VM, Eriksson JE. MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors. J Biol Chem 2001;276:16484–90.
[42] Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270:1326–31.
[43] Choi K, Song S, Choi C. Requirement of caspases and p38 MAPK for TRAIL-mediated ICAM-1 expression by human astroglial cells. Immunol Lett 2008;117:168–73.
[44] Hayakawa Y, Screpanti V, Yagita H, Grandien A, Ljunggren HG, Smyth MJ, et al. NK cell TRAIL eliminates immature dendritic cells in vivo and limits dendritic cell vaccination efficacy. J Immunol 2004;172:123–9.
[45] Janssen EM, Droin NM, Lemmens EE, Pinkoski MJ, Bensinger SJ, Ehst BD, et al. $CD4^+$ T-cell help controls $CD8^+$ T-cell memory via TRAIL-mediated activation-induced cell death. Nature 2005;434:88–93.
[46] Falschlehner C, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: decisions between life and death. Int J Biochem Cell Biol 2007;39:1462–75.