Cynoglossus semilaevis thioredoxin: a reductase and an antioxidant with immunostimulatory property

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Abstract Thioredoxin (Trx) is a small redox protein existing ubiquitously in all living organisms and plays an important role in multiple cellular processes, including transcriptional regulation and immune response. To date very few studies have been carried out to examine the function of piscine Trx. In this study, we identified and analyzed the function of a Trx homologue, CsTrx1, from half-smooth tongue sole (*Cynoglossus semilaevis*). The deduced amino acid sequence of CsTrx1 is composed of 107 residues and shares 54.1–60.8% overall identities with the Trx of other teleosts. CsTrx1 contains the highly conserved CXXC motif, which in mammals is known to be the active site, in the form of CQPC. Expression of *CsTrx1* as determined by quantitative real-time reverse transcriptase PCR was highest in liver and upregulated in time-dependent manners by bacterial infection and by exposure to iron, copper, and hydrogen peroxide. Purified recombinant CsTrx1 (rCsTrx1) exhibited insulin disulfide reductase activity and antioxidant activity, both which, however, were lost when the two cysteine residues in the CQPC motif were mutated to serine. Further analysis showed that rCsTrx1 was able to

stimulate the proliferation of head kidney leukocytes, upregulate the expression of immune relevant genes, and enhance the resistance of leukocytes against bacterial infection. Taken together, these results indicate that CsTrx1 is a biologically active reductase and an antioxidant that requires the CXXC motif for activity and that CsTrx1 possesses cytokine-like immunoregulatory property. These results suggest a role for CsTrx1 in protecting cells against oxidative stress caused by oxidant exposure and pathogen infection.

Keywords Thioredoxin · *Cynoglossus semilaevis* · Redox · Antioxidant · Oxidative stress · Immunoregulatory

Introduction

Thioredoxin (Trx) is a class of small redox proteins that was first isolated in *Escherichia coli* as the hydrogen donor for ribonucleotide reductase, an enzyme that catalyzes the formation of deoxyribonucleotides from ribonucleotides (Laurent et al. 1964). Subsequently, Trx was found to exist in all kingdoms of living organisms covering both prokaryotes and eukaryotes (Holmgren 1985). Structurally, Trx is approximately 12 kDa in size and possesses a highly conserved active site that contains a redox-active dithiol group in the form of CXXC (Powis and Montfort 2001). Trx interacts with a large number of thiol proteins through a redox process, during which Trx binds via the active site to the disulfide of the target protein, and the first cysteine thiolate of the CXXC motif, a strong nucleophile with an low pK_a value, then attacks the disulfide of the target protein and forms a disulfide intermediate with the target thiol group. The disulfide intermediate is subsequently reduced by the second thiol group in the CXXC, resulting in the formation of a disulfide in the CXXC motif of Trx and a dithiol in the target protein (Chivers et al. 1997;

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Schultz et al. 1999; Mössner et al. 2000; Fomenko and Gladyshev 2003; Lillig and Holmgren 2007). The oxidized Trx is reduced by thioredoxin reductase using reducing agents from NADPH or ferredoxins; the reduced Trx can then act again as a reductase (Arnér and Holmgren 2000). In this manner, Trx regulates the function of many proteins involved in diverse cellular processes including redox homeostasis, gene transcription, and immune response (Nakamura et al. 1997; Lillig and Holmgren 2007).

In mammals, two types of thioredoxin have been identified, i.e., Trx1 and Trx2, which are located in, respectively, the cytoplasm and the mitochondria. However, Trx1 can be actively secreted into the extracellular by various normal and transformed cells including fibroblasts, epithelial cells, and activated B and T leukocytes (Rubartelli et al. 1992; Masutani et al. 1996). Since Trx1 is leaderless, its secretion is thought to be different from that via the classical ER-Golgi route. Some secreted Trxs are known to act as cytokines in transformed T cells and possess chemotactic activity for monocytes and leukocytes (Teshigawara et al. 1985; Tagaya et al. 1989; Schenk et al. 1996; Bertini et al. 1999).

In teleost, Trx sequences have been identified in a number of species; however, except for the Trx of rock bream (*Oplegnathus fasciatus*) (Kim et al. 2011), it is not clear whether these piscine Trx possess redox activity. In this report, we identified and analyzed the activity and biological property of a Trx homologue, CsTrx1, from half-smooth tongue sole (*Cynoglossus semilaevis*), an important economic species cultured widely in northern China. We found that CsTrx1 possesses CXXC-dependent reductase activity and antioxidant activity and that recombinant CsTrx1 exhibits cytokine-like immunomodulator property. From these results, we concluded that CsTrx1 very likely plays a role in cellular defense against oxidative stress and pathogen infection.

Materials and methods

Fish

Half-smooth tongue sole (*C. semilaevis*) were purchased from a commercial fish farm in Shandong Province, China and maintained at 20°C in aerated seawater. Fish were acclimatized in the laboratory for 2 weeks before experimental manipulation and fed daily with commercial dry pellets (purchased from Shandong Sheng-Suo Fish Feed Research Center, Shandong, China). Before each experiment, fish were randomly sampled for the examination of bacterial recovery from blood, liver, kidney, and spleen, and no bacteria were detected from any of the examined fish. Fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, MO, USA) before tissue collection.

Cloning of *CsTrx1*

A complementary DNA (cDNA) library of half-smooth tongue sole head kidney (HK) and spleen was constructed as described previously (Wang et al. 2011). One thousand five hundred clones of the library were randomly selected and subjected to DNA sequence analysis; one clone was found to contain the cDNA of *CsTrx1* with 5'- and 3'-untranslated regions (UTRs). The nucleotide sequence of *CsTrx1* has been deposited in GenBank database under the accession number JN862929.

Sequence analysis

cDNA and amino acid sequence analyses were performed with the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI. The molecular mass and theoretical isoelectric point were predicted using EditSeq in DNASTAR software package (DNASTAR Inc. Madison, WI, USA). Multiple sequence alignment was created with the ClustalX program. Signal peptide search was performed with SignalP 3.0. Phylogenetic analysis was performed as described previously with ClustalX and the neighbor-joining algorithm of MEGA 4.0 (Li et al. 2011).

Plasmid construction and mutation of CsTrx1

To construct pEtCsTrx1, which expresses a His-tagged CsTrx1, the coding sequence of CsTrx1 was amplified by PCR with primers F1 (5'- GATATCATGGTTTACGAAGT GAAAG -3'; underlined sequence, *EcoRV* site) and R1 (5'- GATATCTTCTTTTTCATACTGCTTC -3'; underlined sequence, *EcoRV* site); the PCR products were ligated with the T-A cloning vector pBS-T (Tiangen, Beijing, China), and the recombinant plasmid was digested with *EcoRV* to retrieve the *CsTrx1*-containing fragment, which was inserted into pET259 (Hu et al. 2010) at the *EcoRV* site. To construct pEtCsTrx1M, which expresses the mutant CsTrx1 (CsTrx1M) bearing serine substitutions at C35 and C38, a two-step overlap PCR was carried out as follows: the N-terminal fragment of *CsTrx1* bearing C35S and C38S mutations was generated by PCR with primers F2 (5'- GCGCGCGATATCATGGTTTACGAAGTGAAAG -3'; underlined sequence, *EcoRV* site) and MR1 (5' - TTTGGAGGGTTGGGACCATGTCGCTGTGAAG -3'), and the C-terminal fragment of *CsTrx1* bearing the same mutations was generated by PCR with primers MF1 (5'- ATGGTCCCAACCCTCCAAAAACATATCTCCCGT -3') and R2 (5'- GGCGCGCGATATCTTCTTTTTCATACTGCTTC -3'; underlined sequence, *EcoRV* site); the PCR

products were mixed at an equal volume and diluted 100×, and the dilution was used as a template to PCR the complete sequence encoding CsTrx1M with primers F1 and R1. The PCR products were inserted into pET259 at the *EcoRV* site as described above, resulting in pEtCsTrx1M.

Purification of recombinant protein

Escherichia coli BL21(DE3) (Tiangen, Beijing, China) was transformed with pEtCsTrx1 and pEtCsTrx1M respectively, and the transformants were cultured in Luria–Bertani broth (LB) medium at 37°C to mid-log phase, and isopropyl-β-D-thiogalactopyranoside was then added to the culture to a final concentration of 0.4 mM. After growth at 30°C for an additional 5 h, recombinant protein was purified using nickel–nitrilotriacetic acid columns (GE Healthcare, USA) as recommended by the manufacturer. The purified protein was dialyzed for 24 h against phosphate-buffered saline (PBS) and concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. The concentration of the purified protein was determined using the Bradford method (Bradford 1976) with bovine serum albumin as a standard.

qRT-PCR analysis of *CsTrx1* expression under different conditions

CsTrx1 expression in fish tissues under normal physiological conditions

Brain, muscle, heart, gut, head kidney (HK), spleen, gill, and liver were taken aseptically from five fish and used for total RNA extraction with the RNAprep Tissue Kit (Tiangen, Beijing, China). One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real-time reverse transcriptase-PCR (qRT-PCR) was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described previously (Zheng et al. 2010). PCR efficiency (99.9%) was determined as described previously (Zheng and Sun 2011). Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The expression level of *CsTrx1* was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with β-actin as the control. The primers used to PCR β-actin gene were reported previously (Sun et al. 2011). The primers used to PCR *CsTrx1* were RTF1 (5'- ATGGTTTACGAAGTGAAAGATTACAATG -3') and RTR1 (5'- TTCTTTTTCATACTGCTTCACTGCA -3'). All data are given in terms of messenger RNA levels relative to

that of β-actin and expressed as means plus or minus standard errors of the means (SE).

CsTrx1 expression in response to bacterial challenge

The fish bacterial pathogen *Vibrio anguillarum* C312 (Zheng et al. 2010) was cultured in LB medium and resuspended in PBS to 10⁷ CFU/ml. Half-smooth tongue sole (~12.6 g) were divided randomly into two groups (*N*=30) and injected intraperitoneally with 100 μl of *V. anguillarum* and PBS, respectively. Fish (five for each time point) were killed at 1, 4, 12, 24, and 48 h post-infection, and tissues were excised under aseptic conditions and used for qRT-PCR analysis as described above.

CsTrx1 expression in response to metal ions and H₂O₂

Tongue sole primary hepatocyte culture was established as follows. Liver was removed from three tongue sole (average 760 g) under aseptic conditions and washed three times with PBS containing 100 U of penicillin and streptomycin (Thermo Scientific HyClone, Beijing, China). The liver was cut into small pieces and digested with trypsin (Sigma, St. Louis, MO, USA). The digested solution was centrifuged at 500×*g* for 10 min, and cell pellet was resuspended in RPMI 1640 (Thermo Scientific HyClone, Beijing, China) containing 15% fetal bovine serum (Thermo Scientific HyClone, Beijing, China) and 100 U of penicillin and streptomycin. The cells were seeded in monolayers in 96-well culture plates with RPMI 1640 and cultivated at 25°C.

To examine *CsTrx1* expression, FeCl₂, CuSO₄, and H₂O₂ (all were purchased from Sangon, Shanghai, China) were added into the above hepatocyte cell culture at the final concentrations of 20, 20, and 100 μM, respectively. After incubation at 25°C for various hours, the cells (10⁵) were collected and used for RNA extraction with the Total RNA Kit I of Omega Bio-tek (Beijing, China). *CsTrx1* expression was then determined by qRT-PCR as described above.

Reducing activity of rCsTrx1

The ability of rCsTrx1 to catalyze reduction of insulin disulfide was determined with the turbidimetric assay (Holmgren 1979). Briefly, rCsTrx1 or rCsTrx1M (4 μM) was added to the assay buffer containing 0.1 M PBS, 2 mM DTT, 2 mM EDTA-Na₂ and 1.25 mg/ml insulin. The mixture was incubated at 25°C for 30 min, and generation of reduced insulin polypeptides, which formed a white precipitate and hence increased the turbidity of the solution, was monitored by measuring absorbance at 650 nm. The assay was performed in triplicate.

Preparation of HK leukocytes

To prepare HK leukocytes, HK was removed from three tongue sole (~680 g) as described above under aseptic conditions and washed 3× with PBS containing 100 U of penicillin and streptomycin. The tissue was passed through a metal mesh, and the cell suspension was overlaid on a 1.070 and 1.077 discontinuous density of Percoll solution (Solarbio, Beijing, China). After centrifugation at 300×g for 30 min at 4°C, the white interface fraction was collected and washed 3× with PBS. The leukocytes were resuspended in L-15 medium (Thermo Scientific HyClone, Beijing, China), and the viability of the cells was examined by trypan blue dye exclusion method. The cells were adjusted to 5×10^5 viable cells/ml in L-15 and distributed into 96-well tissue culture plates (10^5 cells/well).

Protective effect of rCsTrx1 against oxidative damage

Fifteen microliters of rCsTrx1 and rCsTrx1M (1.5 mg/ml) or PBS (control) were mixed with 15 µl 20 mM DTT and incubated at 25°C for 0.5 h. The solution was diluted in L-15 medium to various concentrations, and the diluted solutions were added to leukocytes cultured in a 96-well tissue culture plate (100 µl/well). H_2O_2 was added into the plate at the final concentration of 200 µM. The plate was incubated at 22°C for 1 h and added with 20 µl of 5 mg/ml MTT {3-(4,5)-dimethylthiazol-2-yl-4-methyl-5-phenyltetrazolium bromide} (Sangon, Shanghai, China). After incubation at 22°C for 4 h, 200 µl dimethyl sulfoxide was added to the plate to dissolve the reduced formazan. The plate was read at 490 nm with a microplate reader. Results were expressed as protection index, which is the fold difference between rCsTrx1-treated cells and control cells. The assay was performed independently for four times.

Effect of rCsTrx1 on HK leukocytes

Effect on cellular proliferation

To determine the effect of rCsTrx1 on leukocyte proliferation, L-15 containing or not containing (control) rCsTrx1 or rCsTrx1M at various concentrations was added to leukocytes cultured in a 96-well tissue culture plate (100 µl/well). The plate was incubated at 22°C for 2 days and added with 20 µl of 5 mg/ml MTT. After incubation at 22°C for 4 h, 200 µl dimethyl sulfoxide was added to the plate to dissolve the reduced formazan. The plate was read at 490 nm with a microplate reader. Results were expressed as proliferation index, which is the fold difference between rCsTrx1-treated cells and control cells. The assay was performed independently for four times.

Effect on the expression of immune genes

rCsTrx1 (final concentration, 1.5 µg/ml) or PBS (control) was added to HK leukocytes in a 96-well culture plate as described above. The plate was incubated at 22°C for 2 h, and total RNA was prepared from the cells as described above and used for qRT-PCR analysis of the expression of the genes encoding toll-like receptor 9 (TLR9), myeloid differentiation primary response gene 88 (Myd88), interleukin (IL) 1β, IL-1β, CsIL8 (a homologue of IL-8) (Sun et al. 2011), CsCCK1 (a homologue of CC chemokine) (Li et al. 2011), and major histocompatibility complex (MHC) class Iα and IIα as described above. These genes were selected because there are the major immune relevant genes that are available in sequence. The primers used to PCR TLR9, Myd88, IL-1β, CsIL8, and MHC I and II have been reported by Zhang et al. (2012); the primers used to PCR CsCCK1 has been reported by Li et al. (2011).

Effect on cellular resistance against bacterial infection

V. anguillarum C312 was cultured to mid-logarithmic phase, washed with PBS, and resuspended in L-15 to 5×10^6 CFU/ml. HK leukocytes prepared above in a 96-well culture plate (10^5 cells/well) were added with or without rCsTrx1 (1.5 µg/ml) diluted in L-15 and incubated at 22°C for 0.5 h. After the incubation, the cells were mixed with *V. anguillarum* suspension (100 µl/well). The plate was incubated at 22°C for 2 h and washed 3× with PBS. The cells were lysed by adding 50 µl of 0.2% Tween 20 to each well. Fifty microliters of the lysate was plated in triplicate on LB agar plates containing ampicillin (selection marker for *V. anguillarum* C312). The plates were incubated at 28°C for 32 h, and the colonies that emerged on the plates were counted. The assay was performed three times independently.

Statistical analysis

All statistical analyses were performed with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Data were analyzed with analysis of variance (ANOVA), and statistical significance was defined as $P < 0.05$.

Results and discussion

Characterization of the sequence of CsTrx1

The cDNA of CsTrx1 is 555 bp, which contains a 5'-UTR of 25 bp, an open reading frame of 324 bp, and a 3'-UTR of 206 bp with a poly-A tail formed by 25 adenines (Fig. 1). A putative polyadenylation signal, AATAAA, is located at 10 bp upstream the poly-A tail. The translation initiation


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-25                               ggacgagatttcacccaccgtcacg
 1  atggtttacgaagtgaagattacaatgacttcactcagcagctgaagagagctggaac
    1  M  V  Y  E  V  K  D  Y  N  D  F  T  Q  Q  L  K  R  A  G  N
61  aaactgggtgggtgggtgacttcacagcgacatgggtgccaaacctgcaaaaacatatctccc
    21  K  L  V  V  V  D  F  T  A  T  W  C  Q  P  C  K  N  I  S  P
121  gtgtttgaagaactggcaaactcagtataagaacgtgggttttcctgaaggtggatgtagat
    41  V  F  E  E  L  A  N  Q  Y  K  N  V  V  F  L  K  V  D  V  D
181  gaggtgacgacgtcagtcagacagaatgtgaaatcaactgcatgcccacattcctcttttat
    61  E  A  D  D  V  S  T  E  C  E  I  N  C  M  P  T  F  L  F  Y
241  aggaacgaaaagcgggtgcactccttttcaggtgcaaactcggacacactgagggctgca
    81  R  N  E  K  R  V  H  S  F  S  G  A  N  S  D  T  L  R  A  A
301  gtgaagcagtatgaaaaagaataaatacccccaatgggtccacgttctacagcaaacctgt
   101  V  K  Q  Y  E  K  E  *
361  tagtctctgtccataactcatctagtcctttcattatgacatacgtcctgagtcctatgtca
421  gtgcagactgggttatcaacatcatgtaatcatcaacttttaactcaaaagacaaggtgga
481  atgaaataaaataaaggaaaaccttaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

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Fig. 1 The nucleotide and deduced amino acid sequences of CsTrx1. The nucleotides and amino acids are numbered along the left margin. The translation start and stop codons are in **bold**, the polyadenylation signal is underlined, and the CXXC motif is **boxed**

codon of CsTrx1 (ATG) is immediately preceded by the nucleotides GTCACG and followed by a guanine, which conform well to the Kozak consensus sequence for translation initiation (Kozak 1987). The deduced amino acid sequence of CsTrx1 is composed of 107 residues and has a molecular mass of 12.4 kDa and an isoelectric point of 4.9. Like all known Trx, CsTrx1 contains no apparent signal peptide sequence. Blast analysis showed that CsTrx1 shares 54.1–60.8% overall sequence identities with the Trx of *Oreochromis niloticus*, *Oplegnathus fasciatus*, *Sebastes schlegelii*, *Salmo salar*, *Oncorhynchus mykiss*, *Osmerus mordax*, *Anoplopoma fimbria*, and *Ictalurus punctatus* (GenBank accession numbers XP_003447575, BAK38716, BAK82164, ACI69512, ACO08388, ACO09971, ACQ58191, and NP_001187021, respectively) (Supplemental data Fig. 1). The overall sequence identity between CsTrx1 and human Trx1 is 48.6%. Phylogenetic analysis indicated that mammalian and teleost Trx, which include CsTrx1, formed a group separate from that formed by invertebrate Trx; however, within the vertebrate group, CsTrx1 constituted a branch that fell outside the cluster formed by all other Trx (Fig. 2). In silico analysis indicated that CsTrx1 contains the conserved CXXC motif; however, unlike known fish Trx and mammalian Trx1, in which the CXXC motif is in the form of CGPC (Supplemental data Fig. 1), the CXXC motif of CsTrx1 is composed of CQPC. In humans, Trx1 contains, besides the two Cys residues in the CXXC motif, three additional Cys residues in the C terminus, i.e., C62, C69, and C73, which are known to be involved in regulation of Trx activity (Lillig and Holmgren 2007). In the case of CsTrx1, in addition to the two cysteine residues that form the putative catalytic site, CsTrx1 possesses two more cysteine residues, i.e., C69 and C73, which are counterparts of the C69 and C73 in human Trx1 and highly conserved among

fish Trx (Supplemental data Fig. 1). Taken together, these results indicate that CsTrx1 is a Trx homologue and possesses structural features that are conserved among lower and higher vertebrate Trx.

Expression of *CsTrx1* under normal physiological conditions and during bacterial infection

qRT-PCR analysis showed that under normal physiological conditions, *CsTrx1* expression was detected, in increasing order, in heart, brain, kidney, spleen, muscle, gut, gill, and liver (Supplemental data Fig. 2). Compared to the expression level in heart, the expression levels in brain, kidney, spleen, muscle, gut, gill, and liver were, respectively, 1-, 1.8-, 2.1-, 2.6-, 3.2-, 3.2-, and 13.8-fold higher. These results are similar to those reported by Kim et al. (2011), who observed that the expression of the rock bream thioredoxin *RbTRx1* is highest in liver and abundant in muscle and gill. In humans, Trx1 expression is known to be stimulated by bacterial components and viral infection (Holmgren 1985; Ericson et al. 1992; Ejima et al. 1999). Likewise, in rock bream, *RbTRx1* expression was upregulated by bacterial and viral challenge. Similar to these studies, in our study, we found that when tongue sole were infected experimentally with the bacterial pathogen *V. anguillarum*, *CsTrx1* expression in kidney, spleen, and liver was significantly induced at 1, 4, 12, 24, and 48 h post-infection (Fig. 3). In kidney and liver, the *CsTrx1* expression patterns were similar, with the expression level increasing with time and reaching maximum (25.2- and 48-fold, respectively) at 12 h post-infection (Fig. 3a, b). In spleen, *CsTrx1* expression was highest (35.8-fold) at 1 h post-infection and then dropped with time until 12 h post-infection, from which time point to 48 h post-infection *CsTrx1* expression remained

Fig. 2 Phylogenetic analysis of CsTrx1. The phylogenetic tree was constructed using the neighbor-joining method of MEGA 4.0 based on the thioredoxin sequences of vertebrates, invertebrates, and bacteria. Numbers on the nodes indicate percentage frequencies in 1,000 bootstrap replications. The accession numbers of the analyzed sequences are as follows: *Anoplopoma fimbria*, ACQ58191; *Salmo salar*, AC169512; *Esox lucius*, ACO13599; *Oncorhynchus mykiss*, ACO08388; *Oplegnathus fasciatus*, BAK38716; *Danio rerio*, NP_001002461; *Sebastes schlegelii*, BAK82164; *Ictalurus punctatus*,

NP_001187021; *Oreochromis niloticus*, XP_003447575; *Osmerus mordax*, ACO09971; *Litopenaeus vannamei*, ACA60746; *Eriocheir sinensis*, ACQ59118; *Maconellicoccus hirsutus*, ABM55528; *Apis mellifera*, XP_392963; *Gallus gallus domesticus*, P08629; *Melopsittacus undulatus*, AAO72714; *Taeniopygia guttata*, XP_002189076; *Rattus norvegicus*, NP_446252; *Mus musculus*, NP_035790; *Homo sapiens*, NP_003320; *Escherichia coli*, P0AA25; *Edwardsiella tarda*, ACY82946

stable (Fig. 3c). These results indicate that, like what has been observed in other fish and mammalian Trx, *CsTrx1* expression is positively regulated by bacterial challenge, which suggests a potential involvement of *CsTrx1* in host immune defense against pathogen infection.

CsTrx1 expression in response to iron, copper, and H₂O₂

It is known that in mammals, expression of Trx1 is induced by various stress signals that promote production of reactive oxygen species (ROS), which are detrimental to cells (Spector et al. 1988; Sachi et al. 1995; Nakamura et al. 1997; Yegorova et al. 2003). In our study, we examined *CsTrx1* expression under stress conditions caused by iron, copper, and H₂O₂. Of these agents, H₂O₂ is a strong oxidizer and a producer of reactive hydroxyl free radicals, while iron and copper are essential to almost all living organisms on account of their involvement in many fundamental cellular processes (Touati 2000). Both iron and copper have a strong catalytic power to generate ROS through Fenton's reaction and therefore are potential inducers of oxidative pressure. In our study, to

examine whether *CsTrx1* expression was affected by oxidative stress, primary hepatocytes of tongue sole were exposed to Fe (II), Cu, and H₂O₂ treatment respectively, and *CsTrx1* expression was determined by qRT-PCR at 0.5, 1, 2, 4, 12, 24, and 48 h post-treatment. The results showed that both metal ions and H₂O₂ induced significant expression of *CsTrx1* in time-dependent fashions (Fig. 4). Copper enhanced *CsTrx1* expression to significant levels at 0.5, 1, 2, 4, 12, and 24 h post-treatment, with peak expression (93.1 fold) occurring at 2 h post-treatment. Similarly, significant elevation of *CsTrx1* expression was induced by H₂O₂ at 1, 2, 4, 12, and 24 h post-treatment, with maximum induction (13-fold) occurring at 4 h post-treatment. The induction levels caused by Fe(II) were significant at 4 and 12 h post-treatment, which were, respectively, 6.1- and 32.2-fold of that of the control. Since the amounts of copper and iron used in the treatment are excessive and probably beyond the detoxification capacity of the cells, it is likely that the stimulating effect observed with these metals on *CsTrx1* expression is due to ROS production catalyzed by the metal ions. Together, these results support the hypothesis that *CsTrx1* is involved in anti-oxidative stress, and enhanced

Fig. 3 *CsTrx1* expression in fish tissues in response to bacterial infection. Tongue sole were infected with *Vibrio anguillarum* or PBS (control), and *CsTrx1* expression in kidney (a), liver (b), and spleen (c) was determined by quantitative real-time RT-PCR. Data are presented as means $\pm$ SE ($N=5$). Significances between *V. anguillarum*-infected fish and control fish are indicated with asterisks. * $P<0.05$; ** $P<0.01$

expression of *CsTrx1* may serve as a protective mechanism to detoxify the reactive intermediates generated under stress conditions.

Biological activity of recombinant *CsTrx1* (r*CsTrx1*) and its dependence on the CXXC motif

To examine the activity of *CsTrx1*, r*CsTrx1* was purified from *E. coli* as a His-tagged protein. SDS-PAGE analysis showed that the purified protein exhibited a molecular mass comparable to that predicted for recombinant *CsTrx1* (13.6 kDa) (Supplemental data Fig. 3). In addition, since although the CXXC sequence is preserved in teleost Trx, it is not clear whether it is an essential active site as in mammalian Trx, we also examined the functional importance of the CXXC motif. For this purpose, the C35 and C38 residues that constitute the CXXC site of *CsTrx1* were mutated to S35 and S38, respectively. The resulting mutant protein, *CsTrx1M*, was purified under the same condition as that under which r*CsTrx1* was purified (Supplemental data Fig. 3). Subsequent activity analysis showed that r*CsTrx1* was able to reduce the insulin disulfide, whereas no reductase activity was observed with r*CsTrx1M* (Fig. 5). These results indicate that r*CsTrx1* is a biologically active Trx that depends on the conserved CXXC motif for activity. The functional essentialness of the C35 and C38 residues suggests the possibility that *CsTrx1* may utilize a reduction mechanism similar to that observed in mammalian Trx1, in which the CXXC motif acts as a catalytic site and interacts directly with target protein.

Protective effect of r*CsTrx1* against oxidative damage

Since, as shown above, r*CsTrx1* possesses reductase activity, we examined whether it was protective against oxidative damage. For this purpose, tongue sole HK leukocytes were exposed to H_2O_2 challenge in the presence or absence of r*CsTrx1* or *CsTrx1M*, and the viability of the cells was subsequently examined. The results showed that the presence of r*CsTrx1*, but not *CsTrx1M*, significantly increased the viability of leukocytes (Fig. 6a), suggesting that r*CsTrx1* was able to protect leukocytes against the toxic effect of H_2O_2 . These results are in agreement with the observations in human Trx1, which showed that human Trx1 possesses direct antioxidant property and is able to remove H_2O_2 (Spector et al. 1988). Similar antioxidant capacity has also been found in shrimp Trx and rock bream Trx (Aispuero-Hernandez et al. 2008; Kim et al. 2011). In our study, since the CXXC-defective r*CsTrx1M* failed to have any effect on cell survival, which suggests that the protection exerted by *CsTrx1* requires the integrity of the active site, it is very likely that the protective effect of *CsTrx1* is the result of the redox activity of *CsTrx1* that reduces H_2O_2 and thus

Fig. 4 *CsTrx1* expression in response to iron, copper, and H_2O_2 . Tongue sole hepatocytes were treated with Fe(II), copper, and H_2O_2 , respectively, and *CsTrx1* expression was determined by quantitative real-time RT-PCR at various time points. Values are shown as means $\pm$ SE ($N=5$). Significances between control (untreated cells) and metal ion/ H_2O_2 -treated cells are indicated with asterisks. * $P<0.05$; ** $P<0.01$

eliminates the potential damage caused by ROS production. These results are in line with those of the expressional study described above, which showed that H_2O_2 stimulated *CsTrx1* expression, and suggest further that *CsTrx1* may play a direct role in protecting cells against oxidative stress.

Effect of rCsTrx1 on leukocytes

Effect on cellular proliferation

In mammals, Trx1 is a growth factor that can stimulate the growth of leukocytes, fibroblasts, and some tumor cells (Wakasugi et al. 1990; Oblong et al. 1994; Powis and Montfort 2001). Similarly, in teleost, the Trx of channel catfish and rock bream are able to induce proliferative response of peripheral blood B cells and kidney leukocytes, respectively (Khayat et al. 2001; Kim et al. 2011). In our study, to examine whether *CsTrx1* had any growth-promoting effect, tongue sole

HK leukocytes were cultured in the presence of rCsTrx1 or rCsTrx1M, and cellular proliferation was subsequently determined by MTT assay. The results showed that rCsTrx1 stimulated leukocyte proliferation in a manner that depended on the dose of the protein, whereas no stimulation was observed with rCsTrx1M regardless of the protein concentration (Fig. 6b). These results indicate that, like human and other teleost Trx, *CsTrx1* possesses a growth factor-like property that is probably mediated by the redox activity of the protein.

Effect on immune response

Studies have shown that human Trx1 can act as a cytokine on different cell types (Teshigawara et al. 1985; Tagaya et al. 1989). For example, treatment of monocytes, fibrosarcoma, and endothelial cells with Trx1 induced the expression of TNF, IL-1, IL-6, IL-2, and IL-8 (Schenk et al. 1996; Padmini and Rani 2010). In teleost, immune-regulatory property has only been found in the Trx of flathead mullet, which modulates the activity of apoptosis signal-regulating kinase 1 and c-Jun NH2-terminal kinase 1/2 (Takeda et al. 2003; Padmini and Vijaya Geetha 2009). In our study, we examined whether rCsTrx1 had any effect on the expression of the immune genes that so far have been identified in tongue sole. For this purpose, tongue sole HK leukocytes were treated with or without rCsTrx1 or rCsTrx1M, and the expression of TLR9, Myd88, IL-1 β , CsIL8 (a homologue of IL-8), CsCCK1 (a CC chemokine), MHCII α , and MHCII β were determined by qRT-PCR. The results showed that, compared to untreated cells, cells treated with rCsTrx1 exhibited significant induction of all the examined genes (Fig. 7). In contrast, cells treated with rCsTrx1M exhibited induction profiles similar to those of the untreated cells. These results indicate that, like human Trx1, rCsTrx1 possesses immunoregulatory activity.

Fig. 5 Reduction of insulin catalyzed by rCsTrx1 and rCsTrx1M. rCsTrx1 and rCsTrx1M were added to the assay buffer containing 1.25 mg/ml insulin. Reduction of insulin was determined by measuring absorbance at OD₆₅₀, which was plotted against the reaction time. Data are presented as means $\pm$ SE ($N=3$)

Fig. 6 Effect of rCsTrx1 against oxidative damage (a) and on lymphocyte proliferation (b). **a** Tongue sole head kidney leukocytes were exposed to H_2O_2 challenge in the presence or absence (control) of different concentrations of rCsTrx1 or rCsTrx1M, and the viability of the cells was

subsequently examined by MTT assay. **b** Tongue sole leukocytes were treated with or without (control) different concentrations of rCsTrx1 or rCsTrx1M, and proliferation of the cells was determined by MTT assay. Data are presented as means $\pm$ SE ($N=4$). * $P<0.05$

Effect on cellular resistance against bacterial infection

With the above results, we wondered whether the immunoregulatory effect observed with rCsTrx1 would affect the capacity of leukocytes to resist pathogen infection. To investigate this question, tongue sole HK leukocytes were treated with or without rCsTrx1 before being incubated with the fish pathogen *V. anguillarum*. Cellular infection was subsequently analyzed by bacterial recovery analysis, which determined the number of *V. anguillarum* that had infected the leukocytes. The results showed that the number of *V. anguillarum* recovered from rCsTrx1-treated leukocytes (1564 ± 87 CFU) was significantly ($P<0.01$) lower than that recovered from untreated leukocytes (3953 ± 195 CFU). Hence, pre-treatment with rCsTrx1 significantly enhanced cellular resistance against *V. anguillarum* infection. Given that, as shown above, rCsTrx1 treatment stimulated the expression of TLR9 and Myd88, which mediate the signal transduction pathway that facilitates elimination of invading pathogens (Hu et al. 2011), and the expression of IL-8 and

CC chemokine, which have been shown to induce protection of lymphocytes against bacterial infection (Li et al. 2011; Sun et al. 2011), it is possible that the enhanced cellular resistance against *V. anguillarum* infection effected by rCsTrx1 is due at least in part to pre-stimulation of leukocytes and to the induction of the immune genes that contribute to host defense against pathogen invasion.

In conclusion, in this study, we showed that CsTrx1 is a functional Trx with reductase activity and antioxidant activity, both which depend on the conserved CXXC motif. Consistent with its antioxidant property, *CsTrx1* expression is upregulated by iron, copper, hydrogen peroxide, and bacterial infection, all which induce oxidative stress. Purified recombinant CsTrx1 exhibits cytokine-like immunostimulatory properties and is able to promote leukocyte proliferation and upregulate the immune response of target cells, which, as a result, enhance cellular resistance against bacterial invasion. Take together, these results support a role for CsTrx1 in the protection of cells against stress caused by oxidant exposure and pathogen infection.

Fig. 7 Immune response induced by rCsTrx1. Tongue sole leukocytes were treated with or without (control) rCsTrx1 or rCsTrx1M, and the expression of immune relevant genes was determined by quantitative real-time RT-PCR. Values are shown as means $\pm$ SE ($N=4$). * $P<0.05$; ** $P<0.01$

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