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Characterization and metal-induced gene transcription of two new copper zinc superoxide dismutases in the solitary ascidian *Ciona intestinalis*

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ABSTRACT

Antioxidant enzymes are known to protect living organisms against the oxidative stress risk, also induced by metals. In the present study, we describe the purification and molecular characterization of two Cu,Zn superoxide dismutases (SODs), referred to as Ci-SODa and Ci-SODb, from *Ciona intestinalis*, a basal chordate widely distributed in temperate shallow seawater. The putative amino acid sequences were compared with Cu,Zn SODs from other metazoans and phylogenetic analyses indicate that the two putative Ci-SODs are more related to invertebrate SODs than vertebrate ones. Both phylogenetic and preliminary homology modeling analyses suggest that Ci-SODa and Ci-SODb are extracellular and intracellular isoform, respectively. The mRNA of the two Cu,Zn SODs was localized in hemocytes and in ovarian follicular cells, as revealed by in situ hybridization. The time course of SOD mRNA levels in the presence of three different metals showed upregulation of *ci-soda* and inhibition of *ci-sodb*. Spectrophotometric analysis confirms the presence of SOD activity in *Ciona* tissues. Our in silico analyses of the *ci-soda* promoter region revealed putative consensus sequences similar to mammalian metal-responsive elements (MRE), suggesting that the transcription of these genes directly depends on metals. These data emphasize the importance of complex metal regulation of *ci-soda* and *ci-sodb* transcription, as components of an efficient detoxification pathway allowing the survival of *C. intestinalis* in continued, elevated presence of metals in the environment.

1. Introduction

All oxygen-utilizing organisms maintain constitutive antioxidant defences, able to detoxify and scavenging reactive oxygen species (ROS) that are continuously produced as a by-product of aerobic metabolism (Acworth et al., 1997; Halliwell and Gutteridge, 1999).

These compounds may produce structural and functional changes in lipids, proteins and nucleic acids. They are intermediates of the molecular oxygen reduction and consist of both radical and non-radical species, including superoxide anions ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), peroxyl radicals ($^{\bullet}RO_2$) and hydroxyl radicals ($^{\bullet}OH$), the reactivity and properties of which vary considerably. Neither $^{\bullet}O_2^-$ nor H_2O_2 are particularly reactive in aqueous solutions, but they can be involved in Haber–Weiss and Fenton reactions producing $^{\bullet}OH$ (Ternay and Sorokin, 1997): this radical reacts instantaneously with all organic molecules, and it is considered the most reactive and destructive species.

About 4% of the oxygen utilized by cells is converted in ROS. The controlled production of ROS has great effect on innate immune defense against invading microorganisms, can activate signal transduction pathways like those involving NF-kB or MAPK, and mediate various biological responses such as cell growth and apoptosis (De la Funte and Victor, 2000; Lesser, 2006; Hooper et al., 2007). However, when the rate of formation is excessive, ROS production can overwhelm the antioxidant capacity of organism, inducing oxidative stress (Sies, 1985; Acworth et al., 1997). The latter is a state of unbalanced cell and tissue oxidation, involving enhanced intra- and extracellular ROS production, lipid and protein peroxidation, DNA cleavage; it often causes a general disturbance

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of the cellular redox balance, i.e. the ratio of reduced to oxidized glutathione (GSH/GSSG) and the NADH/NAD ratio. Oxidative stress has been related to many pathophysiological states, e.g. ischemia–reperfusion injury, hyperoxia, but also to hypoxia, iron overload and intoxication (Di Giulio et al., 1989; Staniek and Nohl, 1999; Irato et al., 2007).

Organisms are able to respond to a strong ROS formation by increasing the expression of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and the concentration of many other molecules (e.g. vitamin C, carotenoids, ubiquinone, glutathione) that take parts in the defense against ROS and repair of oxidative damages (Demple et al., 1999; Halliwell and Gutteridge, 1999; Santovito et al., 2005, 2012).

Superoxide dismutase (SOD EC 1.15.1.1) is a family of metalloenzymes that catalyze the dismutation of the $^{\bullet}O_2^-$ into dioxygen and hydrogen peroxide (McCord and Fridovich, 1969; Fridovich, 1986). The physiological role of these proteins is to protect cells by scavenging superoxide anions produced under oxidative conditions by energy-generating processes (McCord and Fridovich, 1969; Keele et al., 1971). There are four known types of SODs, characterized by the specific metals present in the active sites: copper/zinc SOD (Cu,Zn SOD), manganese SOD (Mn SOD), iron SOD (Fe SOD) and nickel SOD (Ni SOD). Mn SOD appears to be localized mostly in the mitochondrial matrix and is also present in prokaryotes (Kawaguchi et al., 1989; Fridovich, 1985). Fe SOD is found in prokaryotes and in a few plants families, and the Ni SOD was isolated only from prokaryotes (Barondeau et al., 2004). Fe SODs and Mn SODs have a dimeric structure and the residues for metals coordination in the same position (Barra et al., 1985).

Two types of Cu,Zn SOD have been described in eukaryotes, encoded by two different genes, namely an intracellular (IC) SOD (SOD1) and an extracellular (EC) one (SOD3). SOD1 is located in the cytosol, nucleus, peroxisomes and chloroplasts, whereas SOD3 can anchor to the plasma membrane or circulate in the extracellular fluids (Marklund, 1982). The SOD3 primary structure includes a N-terminal signal cleavage peptide for secretion which is absent in SOD1 (Fattman et al., 2003).

Various gene sequences of Cu,Zn SODs from aquatic animals have been reported (Santovito et al., 2006; Lin et al., 2000; Bao et al., 2009; Kim et al., 2011; Chakravarthy et al., 2012), although only few Cu,Zn SOD cDNAs have been sequenced from invertebrates chordate (Abe et al., 1999).

A comparative study of Cu,Zn SOD amino acid sequences, including bacteria and mammals, revealed that the Cu,Zn SOD sequence contains both invariant and variable regions (Bannister et al., 1991). The former are those directly involved in metal ligand binding or are responsible for maintaining the structure and function of the active site, for the interaction between members of the typical dimer, and for β-barrel folding. The presence of numerous invariant residues, even in alignments from very distant species, makes this enzyme one of the most evolutionary stable globular proteins characterized to date (Getzoff et al., 1989). Nonetheless, the variable regions allow the evaluation of divergences between various groups within the animal kingdom.

In the present study, we report on the molecular characterization and gene transcription of two SODs from the solitary tunicate *Ciona intestinalis*, indicated as Ci-SODa and Ci-SODb. With the aim of evaluating differences at molecular level in relation to their evolutionary history, we also compared the sequence of SODs from *C. intestinalis* with those from other eukaryote species. In this respect, the evolution of Cu,Zn SODs is particularly interesting since, in contrast to Mn and Fe SODs, which appear to have evolved at a relatively constant rate over the entire history of eukaryotes, they evolved rapidly in relatively recent times (Lee et al., 1985).

We also demonstrated the presence of SOD activity in *C. intestinalis* tissues by spectrophotometric analysis.

2. Materials and methods

2.1. Animals and treatment

Specimens of *C. intestinalis* were collected in the southern area of the Lagoon of Venice, near Chioggia, kept in aerated aquaria in filtered seawater (FSW) at the constant temperature of 19 °C, and fed with Liquifry Marine (Liquifry Co., Dorking, UK). After 5-day acclimatization, the animals were randomly divided in four aquaria: 3 groups of 25 specimens each were exposed to $CdCl_2$, $CuCl_2$ or $ZnCl_2$, at the sub-lethal concentrations of 10 μM for each metal; 25 untreated animals, were maintained in FSW and used as controls. After 6, 24, 48, 72, 96 and 120 h, four specimens from each of four aquaria were dissected; their intestine, stomach and ovary were collected, and used for in situ hybridization (ISH). Intestine was also used in all bio-molecular analyses, in this case it was frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Primers design, RNA extraction, DNA synthesis, cloning and sequencing

The NCBI database contains the mRNA sequences of two *C. intestinalis* SODs (Ci-SODs), named Ci-SODa and Ci-SODb (GenBank accession numbers: XM_002121100.1 and XM_002122526.1, respectively). We cloned partial cDNA sequences of these transcripts to confirm their expression, design functional primers for the gene transcription study, and build the antisense probes for ISH.

For the primer design, amino acid and nucleotide sequences of SODs from eukaryotic organisms were obtained from NCBI database and aligned by ClustalW. Figs. 1 and 2 report the alignments of amino acid sequences of type 3 and type 1 SODs, respectively. The considered species include and SOD1 *Strongylocentrotus purpuratus* (purple sea urchin), *Drosophila melanogaster* (fruit fly), *Salmo salar* (Atlantic salmon), *Danio rerio* (zebrafish), *Bos taurus* (cattle) and *Homo sapiens*, and SOD3 of *Schistosoma japonicum*, *Dictyocaulus viviparus* (bovine lungworm), *Rattus norvegicus* (Norway rat), *Oryctolagus cuniculus* (rabbit), *Danio rerio* and *Argopecten irradians* (bay scallop).

The primers were designed in the conserved domains and the primer sequences were analyzed with IDT Oligo analyzer (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/). Used primers are shown in Table 1.

Total RNA was purified from the intestine of *C. intestinalis* by the SV Total RNA Isolation System (Promega, Madison, WI) and its purity was verified spectrophotometrically by the A260/280

Table 1
PCR primers used in this study.

Name	Sequences 5′–3′	Tm (°C)
SOF	CAGTGGCAGGATCTAAATACGCCC	60
SOR	GGGCAAACTGGGGGGTGAAC	61.5
SODF	GGSMACANTCYATYATTGG	56
CiS154Fw1	GTGGATCGCTTACAGGGTTG	60
CiS154Re1	TGCACAACAACAGCTCTTCC	60
CiS154Fw2	CTGGAGGAAGATTGGCGTGTGGG	60
CiS154Re2	CCCACACGCCAATCTTCCTCCAG	60
CiS154Fw3	GCACTGGGGGTCATTTCAACCC	60
CiS154Re3	GGGTTGAAATGACCCCCAGTGC	60
Oligo-dT adaptor primer	GTTTTCCCAGTCACGACT18	75.4
Anchor primer	GTTTTCCCAGTCACGAC	56.2
AcFw	GAGGTTATTCCTTCACCACCAC	58
AcRe	GGATGGGCCAGACTCG	58

S = G or C; M = A or C; N = A, G or C; Y = C or T.

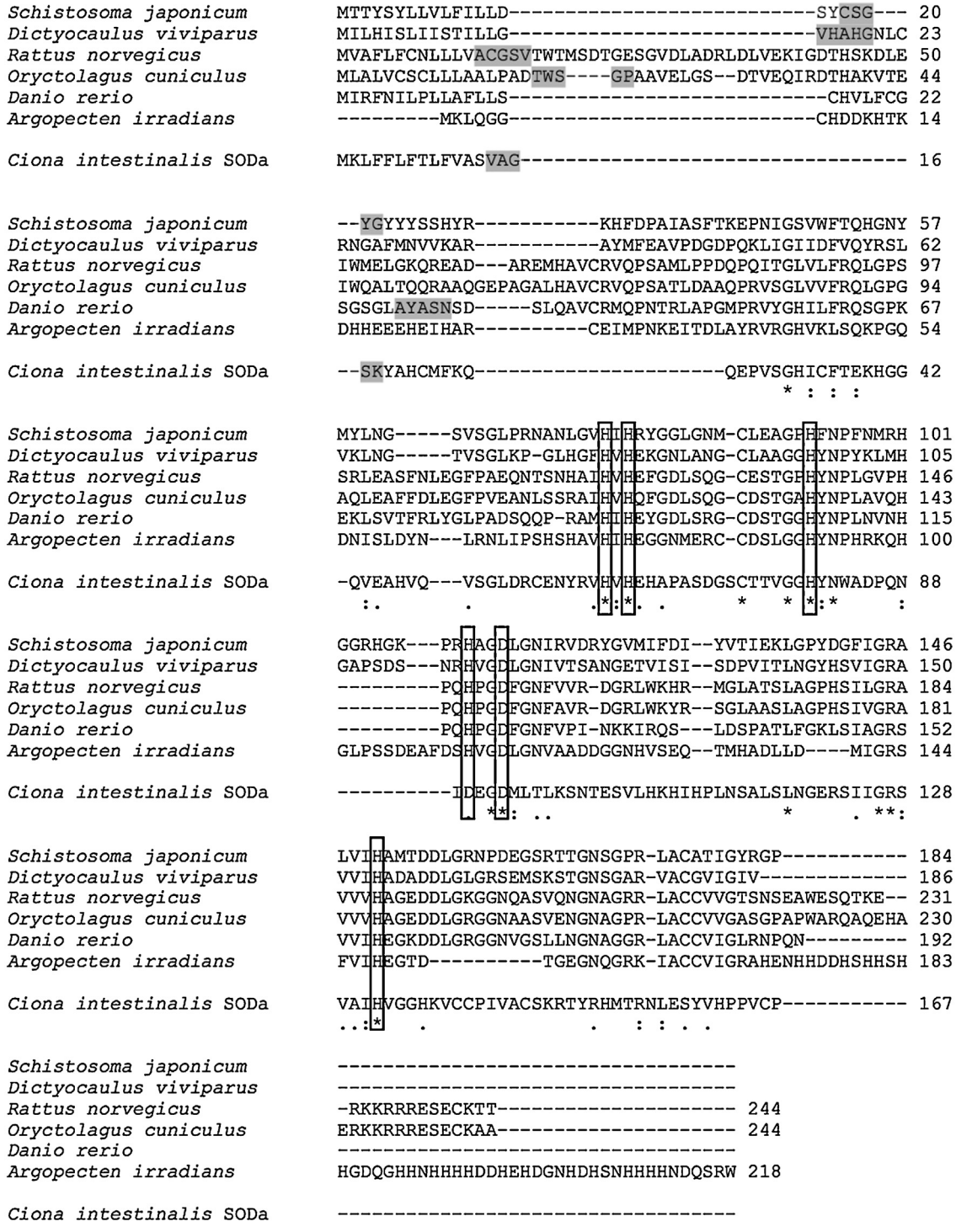

Fig. 1. Multiple alignment of Ci-SODa and other metazoan type 3 SOD amino acid sequences. Residues important for catalytic functionality are boxed. Residues for the cleavage are gray highlighted. The symbols at the bottom of the sequences correspond to the definitions of the CLUSTAL program: (*) fully conserved; (:) highly conserved; (.) conserved substitution.

ratio. The integrity of RNA preparation was checked by visualization of rRNA in ethidium bromide-stained agarose gels. Reverse transcription was performed with ImProm-II™ Reverse Transcription System (Promega) with 1 μg of RNA and Oligo-dT adaptor primer (Table 1).

Reactions were performed with Go-Taq Polymerase (Promega) (5 U/μL) with the following cycling parameters: 94 °C for 2 min, then 30 cycles of 94 °C for 30 s, melting temperature (Tm) for 30 s (Tms were indicated in Table 1), 72 °C for 1 min, and a last step at 72 °C for 10 min.

Amplicons were purified from the gel with a NucleSpin Extract kit (Macherey–Nagel), ligated in pGEM T-Easy Vector (Promega) and cloned in XL1-Blue *Escherichia coli* cells (Invitrogen). Positively screened clones were sequenced at BMR Genomics (University of Padova) on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Carlsbad, CA).

Fig. 2. Multiple alignment of Ci-SODb and other metazoan type 1 SOD amino acid sequences. Residues important for catalytic functionality are boxed. The symbols at the bottom of the sequences correspond to the definitions of the CLUSTAL program: (*) fully conserved; (:) highly conserved; (.) conserved substitution.

2.3. sqRT-PCR

cDNAs were amplified with the specific primers reported in Table 1. *C. intestinalis* β-actin (GenBank accession number: DQ369967.1) was used as housekeeping gene and amplified with specific primers (Table 1). PCR amplifications were carried out with the following program: 94 °C for 2 min, then a variable number of cycles of 94 °C for 30 s, 30 s at specific melting temperature (Table 1), 72 °C for 1 min. For all the genes, the number of amplification cycles was optimized (32 for *ci-soda*, 30 for *ci-sodb*, 28 for *ci-β-actin*) to ensure that PCR products were quantified during the exponential phase of the amplification.

The amplification products were separated by electrophoresis on 1% agarose GelRed-stained gel (Biotium) and the relative intensities were quantified with the software Quantity-one through a quantitative ladder Gene Ruler™ (Fermentas).

2.4. In situ hybridization

For tissue location of mRNAs, sense and antisense probes for Ci-SODa and Ci-SODb were obtained by PCR amplification of a specific insert and T7 RNA or SP6 polymerase promoter regions with specific primers. PCR products were separated by electrophoresis and gel-purified with the UltraPrep kit (AHN Biotechnologie GmbH). The fragments were used as templates for probe synthesis with SP6 Polymerase (Promega) or T7 Polymerase kits (Promega). The sense and antisense probes were further purified with mini-Quick Spin Columns™ (Roche).

Intestine, stomach and ovary from control and Cd-treated animals (for 3 days) were used for ISH. Tissues were fixed overnight in freshly prepared 4% paraformaldehyde in MOPS buffer (0.1 M MOPS, 1 mM $MgSO_4$, 2 mM EGTA, 0.5 M NaCl), dehydrated in ethanol, and embedded in Paraplast Plus (Sigma, St. Louis, MO). Serial sections (7 μm thick) were obtained with a Jung microtome and left to adhere to microscope Superfrost Plus (Menzel-Glaser, Braunschweig, Germany) slides, dewaxed for 15 min with xylene, rehydrated, and used immediately. Sections were incubated for 6 min in 10 mg/ml proteinase K (Promega) in phosphate-buffered saline (PBS; 1.37 M NaCl, 0.03 M KCl, 0.015 M KH_2PO_4, 0.065 M Na_2HPO_4, pH 7.2). The enzymatic reaction was stopped by dipping slides in a solution of 0.2% glycine in PBS and slides were washed in PBS. Samples were prehybridized in Hybridization Cocktail 50% formamide (Amresco, Solon, OH) for 1 h at 65 °C and then hybridized in the same solution overnight at 65 °C with 1–2 μg/ml of biotin-labeled riboprobe. Sections were washed twice in 2× SSC pH 4.5 (0.3 M NaCl, 40 mM sodium citrate), three times in formamide 50% in 2× SSC pH 4.5 for 30 min at 65 °C, and twice in PBS containing 0.1% Tween-20 (PBS–T). Subsequently, slides were incubated in 5% H_2O_2 in methanol for 30 min, washed with PBS–T, treated with Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) for 30 min at RT, washed with PBS–T, then stained with 3,3′-diaminobenzidine for 1 min. Lastly, sections were counterstained with hematoxylin and then dehydrated and mounted in Eukitt (Electron Microscopy Sciences, Hatfield, PA).

Table 2
SOD sequences used for phylogeny and their GenBank accession numbers.

	GenBank ID	Species
IC-Cu,Zn SOD	NP_476735.1	*Drosophila melanogaster*
	NP_001171498.1	*Apis mellifera*
	AFQ00704.1	*Bubalus bubalis*
	XM_779481.3	*Strongylocentrotus purpuratus*
	ABL96616.1	*Homo sapiens*
	AAC47734.1	*Tritrichomonas foetus*
	ACJ73933.1	*Ctenopharyngodon idella*
	AAK84037.1	*Sus scrofa*
	AER68087.1	*Cryptococcus gattii*
	BAF42028.1	*Cryptococcus liquefaciens*
	CAA29121.1	*Rattus norvegicus*
	AAR90328.1	*Anopheles gambiae*
	AAC96613.1	*Paramecium bursaria*
	ADG85264.1	*Equus caballus*
	NP_571369.1	*Danio rerio*
	CAX76412.1	*Schistosoma japonicum*
	CAQ68509.1	*Mytilus galloprovincialis*
	ABQ40391.1	*Haliotis diversicolor supertexta*
	ACI28282.1	*Cristaria plicata*
	AFO64940.1	*Ruditapes philippinarum*
	ACM48346.1	*Argopecten irradians*
	ABD60754.1	*Biomphalaria glabrata*
EC-Cu,Zn SOD	AET83769.1	*Leptopilina boulardi*
	NP_001106630.1	*Xenopus tropicalis*
	AES07191.1	*Mustela putorius furo*
	AFM90430.1	*Callorhinchus milii*
	EAW92823.1	*Homo sapiens*
	EDL99891.1	*Rattus norvegicus*
	XP_002192051.1	*Taeniopygia guttata*
	NP_001181261.1	*Macaca mulatta*
	ACK38046.1	*Argopecten irradians*
	XP_002660566.1	*Danio rerio*
	AAY60161.1	*Crassostrea gigas*

2.5. Molecular phylogeny, in silico promoter studies and sequence analyses

All considered sequences (Table 2) were aligned using T-Coffee multiple sequence alignment software package (Notredame et al., 2000). ProtTest 3 was used for selection of the best-fit model of protein evolution (Darriba et al., 2011). 122 candidate models and three types of criteria (Akaike Information Criterion-AIC, Corrected Akaike Information Criterion-cAIC and Bayesian Information Criterion-BIC) were used in these statistical analyses. The SOD amino acid sequences phylogenetic tree was built using the amino acid sequences and Bayesian inference (BI) method implemented in Mr. Bayes 3.2 (Ronquist et al., 2012). Four independent runs, each one with four simultaneous Markov Chain Monte Carlo (MCMC) chains, were performed for 1,000,000 generations sampled every 1000 generations. FigTree v1.3.1 software was used to display the annotated phylogenetic trees.

To identify the genomic scaffolds containing the examined genes, complete coding sequences were blasted against the JGI *C. intestinalis* database v. 2.0 (http://genome.jgi-psf.org/Cioin2/Cioin2.home.html; Dehal et al., 2002). The 2000 bp upstream of coding sequences were analyzed with the Primer Premier 5.00 software package (Premier Biosoft International, Palo Alto, CA) to identify putative transcription factor binding sites such as antioxidant responsive element (ARE), xenobiotic responsive element (XRE) and metal responsive element (MRE). Open Reading Frame tool by NCBI (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used to detect the open reading frame (ORF) The 3′- and 5′-UTR regions were analyzed in silico to find the domains of rapid degradation, polyadenylation sequence and tail signals. We used SignalP 4.0 program (http://www.cbs.dtu.dk/services/SignalP/; Petersen et al., 2011) to search for potentially cleavage site.

2.6. Molecular modeling

Considering that some IC-SODs have been structurally characterized via X-ray crystallography, we employed a comparative modeling approaches to study Ci-SODa and Ci-SODb folding (for which no experimental structure is currently available). In this work preliminary homology protein modeling was carried out with SwissModel bioinformatic tools (http://swissmodel.expasy.org) (Arnold et al., 2006).

2.7. Superoxide dismutase activity

To assess the SOD activity, intestine from control animals were homogenized with a Polytron in lysis buffer (20 mM Tris–HCl, 1 mM EDTA, 1 mM DTT, 0.5 M saccharose, 0.15 M KCl, pH 7.6), centrifuged at 20,000 × *g* for 50 min at 4 °C and the supernatants were collected and used in subsequent analyses.

Spectrophotometric assay for SOD activity of intestine homogenate was performed according to Beauchamp and Fridovich (1971) using the reduction of nitro blue tetrazolium (NBT) as detector of $^{\bullet}O_2^-$ produced via the riboflavin photoreduction. One SOD unit was defined as the amount needed to cause half-maximal inhibition of this reaction. Measurements were carried out at 560 nm on a Perkin-Elmer Lambda EZ201 spectrophotometer. Data where normalized to total protein concentrations, estimated with the Folin phenol reagent method (Lowry et al., 1951) using bovine serum albumin as standard.

2.8. Statistical analysis

Data were expressed as average of 3 independent experiments ± SD. Statistical analyses were performed with the PRIMER statistical program. Student's *t*-test for comparison of pairs or ANOVA for different groups was followed by the Student–Newman–Keuls test to assess significant differences ($p < 0.05$). Linear regression analyses were also performed to correlate the transcription of genes for the examined enzymes.

3. Results

3.1. Organization of C. intestinalis Cu,Zn SOD genes.

In the *C. intestinalis* genome (scaffold n. 1012) we found a putative Cu,Zn SOD sequence. To test if this nucleotide sequence was transcribed, RT-PCR analysis was performed using mRNA from the intestine of *C. intestinalis* specimens. The obtained amplicon, cloned and sequenced, consisted in a sequence of 487 nt corresponding to the expected partial coding sequence (Fig. S1). We named this gene *ci-soda*. The in silico analysis of *Ci-SODa* indicates that the open reading frame (ORF) includes 504 nt and encodes for a putative protein of 167 aa, with a deduced molecular weight of 18,486 Da. The comparison between the genome scaffold and the *C. intestinalis* EST database suggests that the 5′- and 3′-UTR regions consist of 301 nt and 91 nt, respectively. The 3′-UTR region does not include the usual consensus polyadenylation signal AATAAA. However, an alternative putative signal (Caron et al., 2001) may be identified: TATAAA, at nt 578–583, 326 nt from the polyadenylation site (Fig. S1). In the N-terminal portion of deduced amino acid sequence there is a potential cleavage site (Fig. 1). This gene is organized in 6 exons and 5 introns, the first intron being completely included in the 5′-UTR region. The genomic portion upstream the 5′-UTR region did not show the typical TATA and CAAT boxes. The expected Antioxidant Response Element (ARE) are missing in the 4563 nt upstream the 5′-UTR but three putative Metals Response Element (MRE) were present (two reverse and one forward) at −2802, −2256 and −1615 nt, respectively.

In the NCBI database we have identified the mRNA sequence for a second putative Cu,Zn SOD, that we named Ci-SODb that was not reported in the *C. intestinalis* genome database, and was localized in chromosome 1, LOC100181799. The in silico analysis of this gene sequence indicates that it is organized in a single exon and the ORF is 464 nt, encoding for a putative protein 154 aa with a calculated molecular weight of 15,714 Da (Fig. S2). The comparison between the scaffold and the NCBI *C. intestinalis* EST database suggests that the 5′- and 3′-UTR regions consist of 1164 nt and 247 nt, respectively. The 3′-UTR region includes the usual consensus polyadenylation signal AATAAA at 595 nt downstream the start codon (Fig. S2). In the N-terminal region of deduced amino acid sequence we did not find any potential cleavage site (Fig. 2) that is present in Ci-SODa (Fig. 1).

The absence of this sequence in the genome database of *C. intestinalis* (version 2.0) did not allow us to extrapolate the sequence upstream the 5′-UTR region to detect putative regulatory sequences.

3.2. Sequence analyses and multiple alignments

The putative amino acid sequences of Ci-SODa and Ci-SODb were compared with other eukaryotic Cu,Zn SODs available in GenBank (Tables S1–S2). Ci-SODa showed only limited identity/similarity with SODs from other species, the highest identity being with *Danio rerio* EC-Cu,Zn SOD (24.8%; Table S1). Ci-SODb, that has a large number of conserved residues, demonstrates the highest identity with *Cristaria plicata* IC-Cu,Zn SOD (62.8%; Table S2). Multiple alignment revealed that Ci-SODa does not share a common consensus pattern with other Cu,Zn SODs (Fig. 1), including the potential cleavage site. However, the amino acids important for the enzyme activity are generally conserved, the only exception being represented by the residues for Zn^{2+} coordination. Ci-SODb sequence share a common consensus pattern with other type 1 SODs (Fig. 2).

3.3. Evolutionary relationships and molecular modeling

The SOD amino acid sequences were aligned using T-Coffee in combined libraries of local and multiple alignments, which are known to induce high accuracy and performance in sequence alignments. ProtTest3 software determined the WAG+G model as the best-fit model of SOD amino acid sequence evolution with a gamma shape value (four rate categories) of 1.0 using all statistical criterion: AIC, cAIC and BIC ($-\ln L = -9416.90$). Phylogenetic relationships of all these different SOD isoforms were determined using the most powerful statistical method of Bayesian Inference (BI). The best phylogeny (arithmetic mean: −9462.36; harmonic mean: −9487.92) generated by the BI method under strict clock model is depicted in Fig. 3. Although the tree is not completely resolved, in our phylogenetic reconstruction Ci-SODa, that clusterizes with EC-SODs, appears phylogenetically unrelated to vertebrate sequences as this group is clearly separated from all other sequences (posterior probability 55%). Likewise, Ci-SODb is similar to IC-SODs and emerges with sequences from invertebrates (posterior probability 98%).

SwissModel identified in *Criptococcus liquefaciens* SOD1 (PDB code 3CE1, 1.20 Å resolution, X-ray method) a 3D structure similar to Ci-SODa, with an identity value of 25.85% (Fig. 4A). The software identified in *Alvinella pompejana* SOD1 (PDB code 3F7K, 1.35 Å resolution, X-ray method) a 3D structure similar to Ci-SODb, with a 63.82% of identity, confirming in the model the presence of the ligands for Cu and Zn. The predicted quaternary structure is a dimer (Fig. 4B).

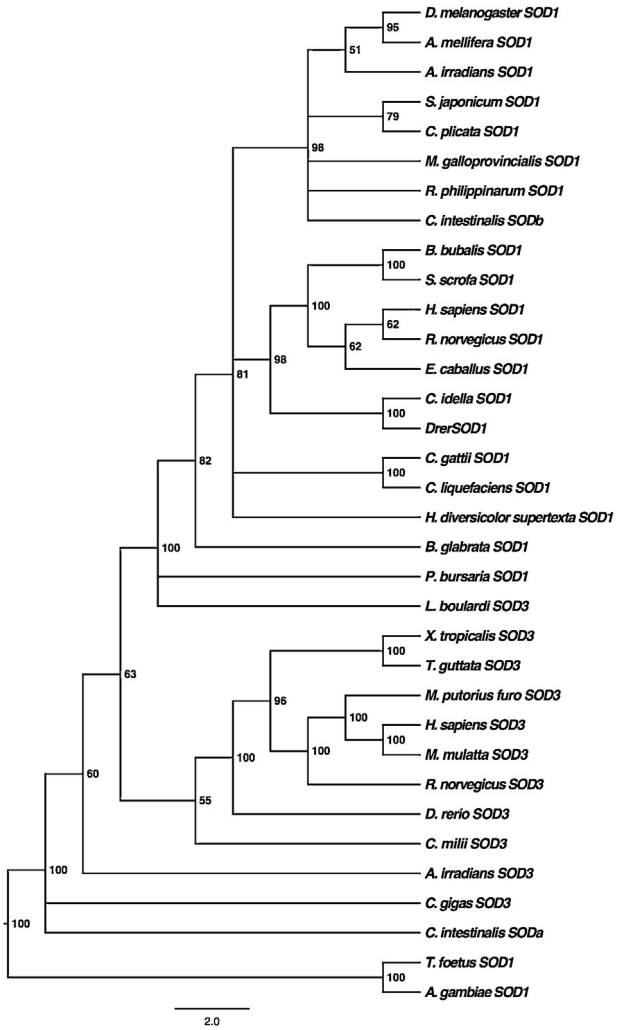

Fig. 3. Phylogenetic relationships among different SOD isoforms cDNA sequences using Bayesian Inference method (arithmetic mean = −9,462,355; harmonic mean = −9,487,919). Posterior probability values higher than 50% are indicated on each node. The scale for branch length (2.0 substitution/site) is shown below the tree.

3.4. Ci-SODa and Ci-SODb mRNA transcription

Fig. 5 shows the level of Ci-SODa mRNA transcription during the exposure of *C. intestinalis* to Cd, Zn and Cu. The time-course of the gene transcription in presence of Cd was of similar to controls up to 24 h and 2–3 folds higher after 48 h (Fig. 5A). Zn treatment elicited the most effective induction, the mRNA level being significantly (3–5 folds; $p < 0.01$) higher with respect to controls during the entire experiments (Fig. 5B). During Cu exposure, the Ci-SODa mRNA level reached a maximum at 48 h, followed by a significant ($p < 0.05$) decrease at 72 and 96 h (Fig. 5C). At 120 h there was a new, significant ($p < 0.05$) increase, with mRNA level reaching the maximum value. The differences with respect to the controls were always statistically significant ($p < 0.001$) after 24 h of exposure.

All the three metals induced a strong reduction of Ci-SODb mRNA transcription in the first phase of the experiment (Fig. 6). Zn and Cu elicited a slow increase of mRNA accumulation after 48 h, which reached, at the end of experiments, levels similar to the controls (Fig. 6B and C). This increase is present and more evident also in specimens treated with Cd, with values that remained significantly ($p < 0.05$) higher than controls up to 120 h (Fig. 6A).

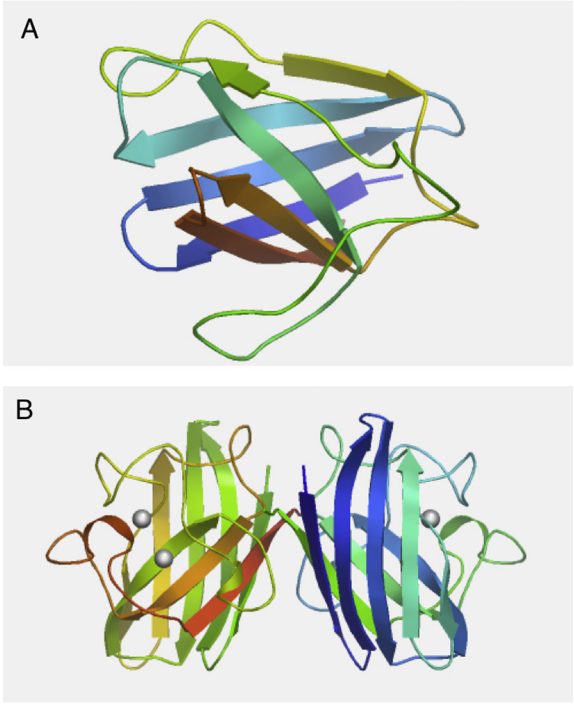

Fig. 4. 3D structures of *Ciona intestinalis* Cu,Zn SODs. (A) Tertiary structure of modeled Ci-SODa. (B) Quaternary structure of modeled Ci-SODb. The spheres represent the Cu and Zn ions.

3.5. Tissue-specific mRNA localization

ISH analyses of stomach, intestine and ovary sections from animals exposed to $CdCl_2$ for 3 days showed that hemocytes in blood lacunae and follicular cells around the oocytes contained Ci-SODa Ci-SODb mRNAs. For Ci-SODa, a considerable difference in stain intensity of hemocytes in blood lacuna is present between control and Cd-treated specimens (Fig. 7).

3.6. SOD activity in cell-free extract

The presence of active Cu,Zn SOD in the intestine was demonstrated by spectrophotometric analysis. In cell-free extract from intestine Cu,Zn SODs activity amounts to 7.89 ± 0.65 U/mg of total proteins.

4. Discussion

Our results indicate that genes for Cu,Zn-SODs are expressed in *C. intestinalis*, as demonstrated by the presence of both mRNA and active protein in tissues. One enzyme (Ci-SODb) shows similar features to other cytoplasmic Cu,Zn-SODs, such as the size of 154 aa, the conserved residues forming the coordination sphere of copper (His^{47}, His^{49}, His^{64} and His^{121}) and zinc ions (His^{64}, His^{72}, His^{81} and Asp^{84}), the presence of Thr^{138} which narrows the active site channel and, together with the positively charged guanidinium group of Arg^{144}, guides the superoxide anion toward the catalytic Cu^{2+} coordination site, stabilizing its bonding.

The other SOD (Ci-SODa) results longer (167 aa) and some amino acids important for the catalytic activity are not conserved, such as the two His and the Asp coordinating zinc that are substituted by Asp^{90}, Asp^{93} and His^{131}, respectively. In the deduced amino acid sequence, a putative cleavage site is present, although its sequence is different from that found in the majority of metazoan EC-SODs. In the promoter region typical TATA and CAAT box sequences are absent, a feature common to EC-SODs (Zelko et al., 2002). For all

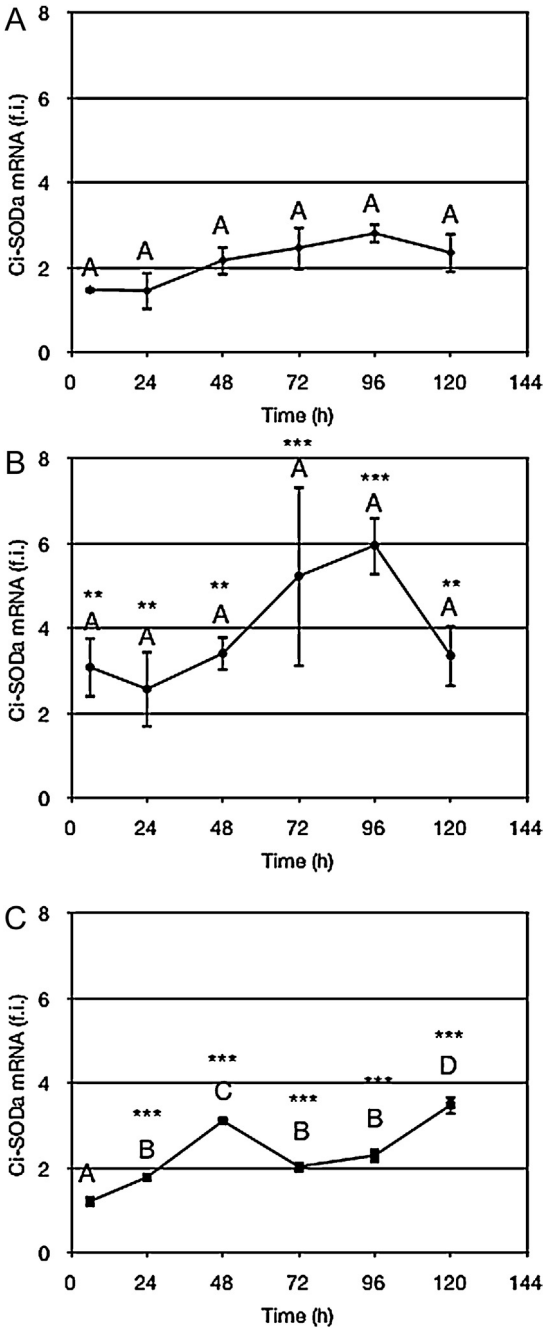

Fig. 5. Relative expression levels of Ci-SODa after treatment with 10 μM $CdCl_2$ (A), 10 μM $ZnCl_2$ (B) and 10 μM $CuCl_2$ (C). Gene expression levels shown relative to untreated control (dashed line at 1 ± 0.0). Asterisks: significant differences with respect to controls (***$p < 0.001$, **$p < 0.005$, *$p < 0.05$): letters: Student–Newman–Keuls *t*-test with respect to treatment.

these reasons we suppose that the ci-soda gene codifies for an EC-Cu,Zn SOD (SOD3). As partial confirmation of our hypotheses, the phylogenetic analysis made with amino acid sequences of eukaryotic Cu,Zn SODs, shows that Ci-SODb clusterizes with type 1 SODs and Ci-SODa emerges as sister group of type 3 SODs. The phylogenetic reconstructions do not match the phylogenesis, placing the sequence of *C. intestinalis* far from the enzymes of Chordata. It is therefore possible that Ci-SODs differentiation did not follow a linear evolutionary process, similar to the one that leaded to vertebrate and urochordate diversification, but evolved in a peculiar way, perhaps involving the absence of a common ancestor and the possibility of adaptive convergence at functional level. This result

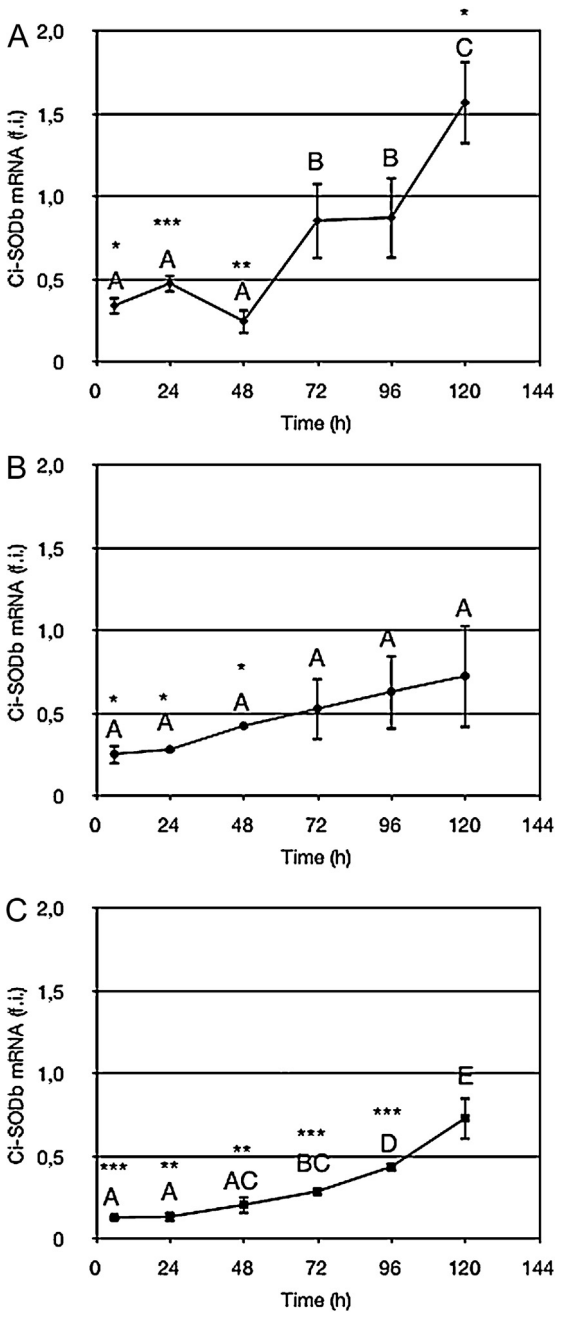

Fig. 6. Relative expression levels of Ci-SODb after treatment with 10 μM $CdCl_2$ (A), 10 μM $ZnCl_2$ (B) and 10 μM $CuCl_2$ (C). Gene expression levels shown relative to untreated control (dashed line at 1 ± 0.0). Asterisks: significant differences with respect to controls (***$p < 0.001$, **$p < 0.005$, *$p < 0.05$): letters: Student–Newman–Keuls *t*-test with respect to treatment.

is comparable with what was previously observed for other genes from *C. intestinalis*, such as glutathione synthase (GS) and glutamate cysteine ligase catalytic subunits (GCLC), two genes involved in GSH biosynthesis, for which phylogeny did not position the sequences from *C. intestinalis* as sister group of Vertebrata (Franchi et al., 2011; Nava et al., 2009; Stach et al., 2010). Moreover, our results indicate that SOD1 did not constitute a monophyletic group, and this is inconsistent with the hypotheses of some authors that EC-SODs diverged from cytosolic forms at early stages of evolution, before the differentiation of fungi, plants and metazoans (Bordo et al., 1994; Zelko et al., 2002). Therefore, we cannot exclude the possibility that the EC-SODs may have appeared at different times during the evolution, and this would, at least in part, justify the separation of Ci-SODa from other vertebrate SODs.

The homology modeling analyses identified in *Alvinella pompejana* Cu,Zn SOD the protein with the most similar 3D structure with respect to Ci-SODb, thus confirming, in the model, the presence of the ligands for Cu^{2+} and Zn^{2+}. The predicted quaternary structure is a dimer. These data confirm both the possible cellular localization (IC-SOD) and the evolutionary relationship with invertebrate SODs.

The situation is different for the Ci-SODa. The homology modeling results indicated that the higher similarity is with the SOD of the yeast *Cryptococcus liquefaciens*. However, it is to note that the homology percentage is very low (25.85%), but higher than that calculated with other SODs, both intracellular and extracellular. On the basis of these results, it is difficult to insert Ci-SODa in the EC-SOD group, also considering that *C. liquefaciens* is a unicellular organism. The obtained results indicate that Ci-SODa differ more than expected from those of mammals, amphibians, and insects, whereas some early-diverged enzymes, such as those of fungi and plants, exhibit unexpectedly slow rates of change (Smith and Doolittle, 1992).

Our results clearly show that both SOD genes are transcribed in *C. intestinalis*, although Ci-SODb sequence is not present in the published genome sequence (v. 2.0). The data relative to the time-course of mRNA accumulation seems to indicate that both genes may be involved in the physiological response against the presence of metals in the aquatic environment. The gene *ci-soda* seems to be particularly transcribed in specimens exposed to Cu or Zn, whereas *ci-sodb* is underexpressed with respect to controls in the first phases of the experiment, and increases its transcription in specimens exposed to Cd for 120 h. These results seem to depict a picture in which the intracellular isoform (Ci-SODb) may have an important role in the protection against the presence of non-essential metals, which are potentially toxic already at low concentrations, such as Cd. The other isoform (Ci-SODa) should play an important role against an excess of essential metals such as Cu and Zn.

The induction of SOD genes can be directly stimulated by metals or ROS, the rate of formation of the latter can be increased by the presence of metals. The potential inducibility of SOD genes by metals is supported by the presence of putative metal response elements (MRE) in their promoter regions: it is known that these sequences can be present in multiple copies and can drive gene transcription in the presence of a metal transcription factor (MTF-1; Imbert et al., 1990; Thiele, 1992; Miles et al., 2000). Analogously, gene activation by ROS is mediated by specific antioxidant response elements (ARE) that are involved in the gene transcription with a specific transcription factor (Nrf-2; Rushmore et al., 1991; Venugopal and Jaiswal, 1996).

As for *ci-sodb*, since the promoter sequence of this gene is still unknown, we cannot make any hypothesis on the metal or ROS regulation of its transcription. Conversely, for *ci-soda* we have verified the presence of three putative MRE sequences in its promoter region, together with the absence of putative AREs. This result seems to indicate that the increased gene transcription, observed in specimens exposed to Cu or Zn, is related to a direct effect of these metals. The absence of ARE sequences in the promoter region of *ci-soda* is an unusual feature for a gene codifying for a SOD and may indicate that the *ci-soda* gene is constitutively transcribed in the cells, and the presence of active protein can be regulated by ROS at post-transcriptional level, analogously to what reported by Gordeeva et al. (2006) in mice. However, the effective absence of ARE in the promoter of *ci-soda* need to be confirmed with site specific mutagenesis experiments, because it is always possible that the sequence of this regulatory element is not conserved in *C. intestinalis*. If this datum will be confirmed, the feedback loop of ci-soda regulation could also involve only MREs. In fact, we can hypothesize a mechanism where $^{\bullet}O_2^-$ oxidize thiol groups of metal

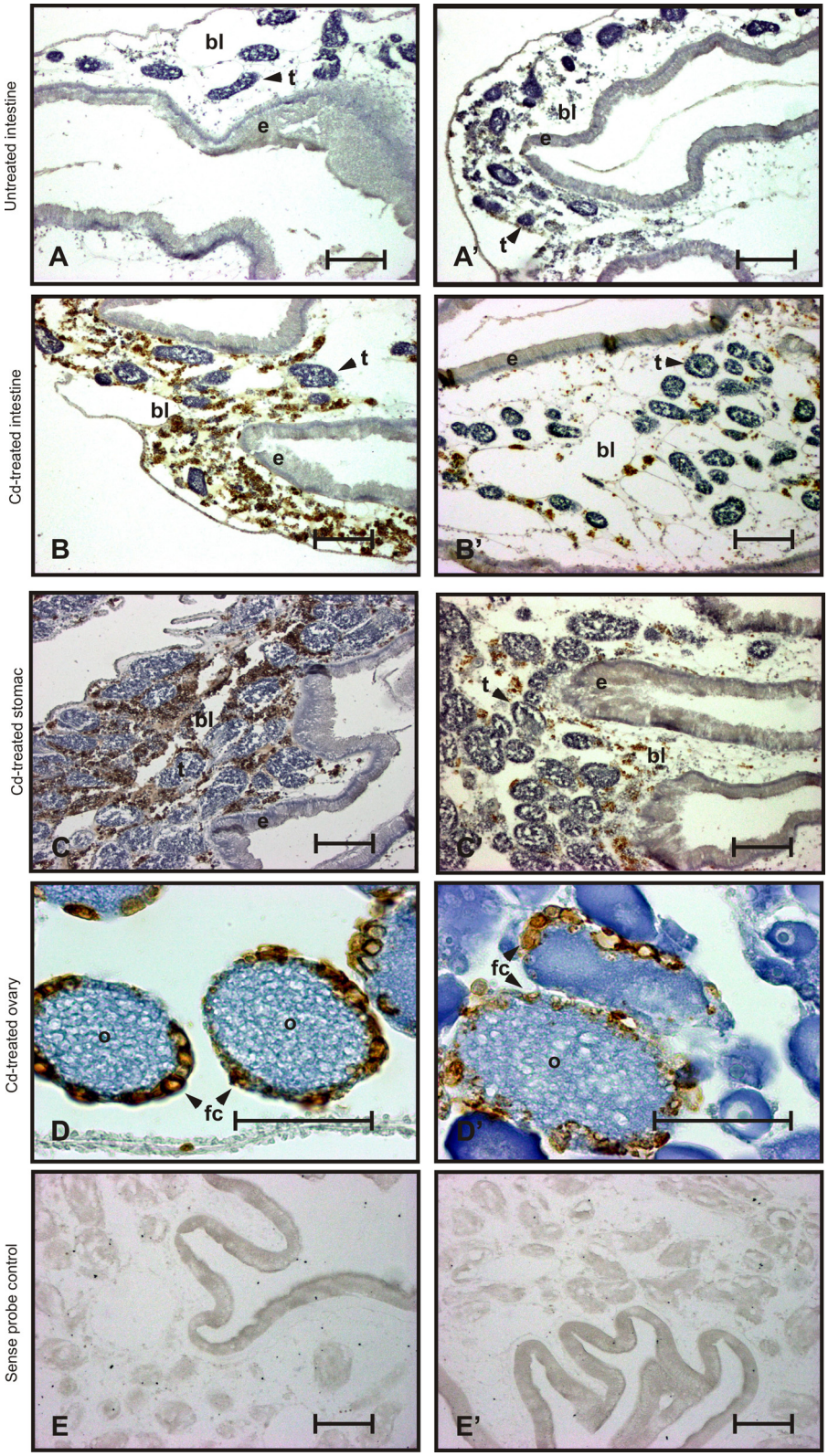

Fig. 7. In situ hybridization with riboprobes for Ci-SODa and Ci-SODb mRNAs on paraffin sections. (A–D) Ci-SODa antisense riboprobe. (A′–D′) Ci-SODb antisense riboprobe. (E, E′) Ci-SODa and Ci-SODb sense probe, respectively. (A, A′) Paraffin sections of intestine from untreated animals. (B, B′) Paraffin section of intestine from Cd-treated specimens; (C, C′) paraffin section of stomach from Cd-treated specimens; (D, D′) paraffin section of ovary from Cd-treated specimens. bl: blood lacunae; t: testis; e: visceral epidermis; fc: follicular cells; o: oocyte. Brown: riboprobe staining. Blue: counter-staining with hematoxylin. Scale bar: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

chelating proteins, such as metallothioneins, producing the release of Zn^{2+} which can be bound by MTF-1 and activate this transcription factor. The subsequent increase of SOD biosynthesis will eliminate the excess of $^{\bullet}O_2^-$, reducing the activation of gene transcription via MREs.

Another hypothesis is that, in *C. intestinalis*, the first line of defence against ROS could be constituted by non-enzymatic component of the antioxidant system, such as GSH and MTs. In previous studies conducted on *C. intestinalis* we identified AREs and half-AREs into promoter regions of genes involved in biosynthesis of these thiol-rich peptides and, using the same experimental conditions, we demonstrated the direct correlation between GSH or MT expression and both metal accumulation and ROS formation (Franchi et al., 2011, 2012). The responses stimulated by the different metals seem to be in relation to their redox properties and to the presence in the cells of GSH and mainly MTs (Irato et al., 2003). In fact, among the used metals only the Cu is redox-active and may directly favor the production of ROS by Fenton and Haber–Weiss reactions (Ternay and Sorokin, 1997).

Zn and Cd are not redox-active metals and their action suggests the involvement of the regulation of gene transcription by MREs. As said above, Zn can directly interact with these sequences by binding to MTF-1 (Boldrin et al., 2008). Cd can be bound by Zn-MTs, with consequent release of Zn which can activate the MTF-1, as demonstrated by Günther et al. (2012) in *Drosophila melanogaster*. Therefore, the activation of MTF-1 may be at the basis of the mechanism that leads the gene transcription of *ci-soda* (and, probably, also of *ci-sodb*), with a different temporal response for the two metals, from the temporal point of view. In particular, this response could be rapid for Zn, and slower for Cd.

Although, Cd is not a redox-active metal, we cannot exclude that it favors the ROS production, for example interfering with cellular redox reactions. In fact, specimens of *C. intestinalis* exposed to Cd in our experimental conditions showed an increment of ROS production in hemocytes (Franchi et al., 2012). The present results showed that the same cells actively transcribe SOD genes. This is in agreement with Shida et al. (2003), who proposed that *C. intestinalis* hemocytes may be considered functionally equivalent to vertebrate liver cells, while maintaining a main role related to the immune and inflammation responses. In addition, ISH revealed the presence of the transcript for SODs also in ovarian follicular cells. Previous ISH results, indicated that such cells express also mRNA for glutathione synthase and the catalytic subunit of glutathione cysteine ligase (Franchi et al., 2012). This suggests that follicle cells exert an active role in protecting oocytes and young germinative cells against the risk to be damaged by toxic molecules such as metal ions or ROS, analogously to what observed in humans (Matos et al., 2009).

In conclusion, in *C. intestinalis* exposed to metals, the cells respond increasing the transcription of the Ci-SODa gene, belonging to the SOD3 family. Ci-SODa could be secreted into extracellular environment to protect tissues against the $^{\bullet}O_2^-$ generated outside the cells, for example during the oxidative burst. Recently, it has been proposed that peroxinectin involved in the PO activating system from *C. intestinalis* (Cammarata et al., 2008; Parrinello et al., 2003) could also binds Ci-SOD3 at the surface of circulating hemocytes (Cammarata and Parrinello, 2009), and this interaction facilitated by the close localization, can modulate both the enzyme activities. The hydrogen peroxide, produced by the superoxide dismutases could be the substrate for peroxinectin and antimicrobial substances (Johansson, 1999; Cerenius et al., 2008; Vizzini et al., 2013). Also Ci-SODb, belonging to the SOD1 family, is highly transcribed in this animal, acting against the intracellular ROS.

Collectively, our data on Cu,Zn SOD gene transcription, together with those previously obtained for MT, GS and GCL, confirm the complex interactions among various components of the antioxidant system in *C. intestinalis* in relation to environmental changing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2013.06.020.

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