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Cu,Zn superoxide dismutase: Cloning and analysis of the *Taenia solium* gene and *Taenia crassiceps* cDNA

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ABSTRACT

Cytosolic Cu,Zn superoxide dismutase (Cu,Zn-SOD) catalyzes the dismutation of superoxide ($O_2^{\cdot-}$) to oxygen and hydrogen peroxide (H_2O_2) and plays an important role in the establishment and survival of helminthes in their hosts. In this work, we describe the *Taenia solium* Cu,Zn-SOD gene (*TsCu,Zn-SOD*) and a *Taenia crassiceps* (*TcCu,Zn-SOD*) cDNA. *TsCu,Zn-SOD* gene that spans 2.841 kb, and has three exons and two introns; the splicing junctions follow the GT-AG rule. Analysis *in silico* of the gene revealed that the 5′-flanking region has three putative TATA and CCAAT boxes, and transcription factor binding sites for NF1 and AP1. The transcription start site was a C, located at 22 nucleotides upstream of the translation start codon (ATG). Southern blot analysis showed that *TcCu,Zn-SOD* and *TsCu,Zn-SOD* genes are encoded by a single copy. The deduced amino acid sequences of *TsCu,Zn-SOD* gene and *TcCu,Zn-SOD* cDNA reveal 98.47% of identity, and the characteristic motives, including the catalytic site and β-barrel structure of the Cu,Zn-SOD.

Proteomic and immunohistochemical analysis indicated that Cu,Zn-SOD does not have isoforms, is distributed throughout the bladder wall and is concentrated in the tegument of *T. solium* and *T. crassiceps* cysticerci. Expression analysis revealed that TcCu,Zn-SOD mRNA and protein expression levels do not change in cysticerci, even upon exposure to $O_2^{\cdot-}$ (0–3.8 nmol/min) and H_2O_2 (0–2 mM), suggesting that this gene is constitutively expressed in these parasites.

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1. Introduction

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion ($O_2^{\cdot-}$) to oxygen and hydrogen peroxide (H_2O_2); therefore, it is a key enzyme in the production of reactive oxygen species (ROS) in cells (Winterbourn, 1993). Eukaryotic organisms have three genes coding for superoxide dismutase: a MnSOD located in mitochondria, one in the cytosol (Cu,Zn-SOD) and one extracellular (ECCu,Zn-SOD) (Fridovich, 1995). Knockout mice for Mn-SOD$^{-/-}$ die a few days after birth (Li et al., 1995), and knockout organisms (Cu,Zn-SOD$^{-/-}$) exhibit a decreased growth rate and shortened life span (Phillips et al., 1989; Reveillaud et al., 1994).

Analysis of Cu,Zn-SOD proximal promoters in mammals have identified regulatory regions like GC-rich, TATA box, and binding sites for NF-KB, AP1, AP-2, Sp1, NF1, GRE, HSF as well as CCAAT-enhancer binding protein (C/EBP) (Miao and St Clair, 2009). By using mammalian cell transformation systems in helminthes, potential regulatory regions such as TATA and CCAAT boxes and AP-1 sites have been found in the core promoter of the calreticulin gene (Khalife et al., 1995). Additionally, an AP-1 site and three CCAAT boxes were reported in the glutathione transferase (GST) of the 28 kDa gene promoter of *Schistosoma mansoni* (Serra et al., 1996). In the case of *Cu,Zn-SOD* promoters, a transcription start site (TSS), two CAAT boxes, and three GC-rich regions in *S. mansoni* (Mei et al., 1995), and the presence of Inr-like elements in two *Onchocerca volvulus* promoters (*Ov-sod*-1 and *Ov-sod*-2) have been described (Tawe et al., 2000).

Cu,Zn-SOD gene expression is regulated by physical, chemical, and biological stimuli, such as temperature, X-ray and UVB radiations, heavy metals, phagocytosis, $O_2^{\cdot-}$, H_2O_2, O_3, and NO_3 (Zelko et al., 2002). For example, it is known that $O_2^{\cdot-}$ increases transcription levels of Cu,Zn-SOD in *S. mansoni* miracidia, sporocyst and cercaria (Zelck and Von Janowsky, 2004); likewise, it increases mRNA levels of Mn-SOD and Cu,Zn-SODs in *Caenorhabditis elegans* larval stage (Tawe et al., 1998). Moreover, H_2O_2 augments Cu,Zn-SOD transcription expression levels in *S. mansoni* (Zelck and Von Janowsky, 2004). In addition, catalase and Cu,Zn-SOD enzymatic activity levels increase in *Heligmosomoides polygyrus* female worms isolated from infected mice with different resistance phenotypes (Ben-Smith et al., 2002).

Previously, we characterized a complementary DNA (cDNA) from *Taenia solium* superoxide dismutase (TsCu,Zn-SOD) and its recombinant product (Castellanos-Gonzalez et al., 2002). In this

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work, we describe and analyze the *TsCu,Zn-SOD* gene and compare its primary sequence with a cDNA coding for Taenia crassiceps superoxide dismutase (TcCu,Zn-SOD). Furthermore, we determined the mRNA and protein level expression in *Taenia crassiceps* cysticerci exposed to O_2^- and H_2O_2 *in vitro*.

2. Materials and methods

2.1. Biological materials

T. solium and *T. crassiceps* (WFU strain) cysticerci were obtained from infected pork muscle and from the peritoneum of BALB/cAnN female mice at 5 months of infection. Cysticerci were washed with sterile PBS and stored at −70 °C, or used for immunolocalization and *in vitro* assays.

2.2. T. solium Cu,Zn-SOD gene and T. crassiceps cDNA cloning

Preparation of the *T. solium* genomic DNA and screening of 100,000 clones from a ZAPII genomic DNA library of *T. solium* cysticerci were carried out as previously described (Campos et al., 1990), using a probe of full length cDNA encoding for TsCu,Zn-SOD. Two genomic clones of ~3000 bp were obtained and sequenced on an automated DNA sequencer ABI Prism model 373 (Perkin-Elmer, Applied Biosystems).

T. crassiceps Cu,Zn-SOD cDNA coding region was obtained by polymerase chain reaction (PCR) using 1 μg of cDNA from cysticerci and primers designed for the first six and last seven amino acids of TsCu,Zn-SOD (forward SOD-X1: 5′-ATG-AAG-GCT-GTT-TGT-GTT-3′ and reverse SOD-X2: 5′-ATT-GCT-AAG-AGC-GAG-TGA-3′) running the following cycles: 1 at 94 °C for 3 min, 30 at 94 °C for 30 s, at 55 °C for 1 min, at 72 °C for 1 min; and a final extension at 72 °C for 5 min. All PCR products were cloned into pCRII (Invitrogen) and the plasmid was prepared and sequenced as before. The sequences were analyzed using the program PROMO: (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). Only putative transcription factor binding sites with a 100% score were selected.

2.3. Transcription start site determination

T. solium and *T. crassiceps* total RNA were prepared with TRIzol (Invitrogen) and used as template for transcription start site (TSS) determination, using Smart RACE cDNA Amplification Kit (Clontech). 5′-RACE fragments amplified with reverse primer Cu,Zn-SOD3R (5′-TGT-GTC-ACC-GAA-TTC-GTG-GAC-GTG-3′) and forward primer SMARTII (5′-AAG-CAG-TGG-TAT-CAA-CGC-AGA-GTA-CGC-GGG-3′) were cloned and sequenced.

2.4. Southern blot

Southern blot was performed using 10 μg of genomic DNA (*T. solium* and *T. crassiceps*) digested with *Hind* III, *Bam* HI, *Eco* RI, resolved on a 1% agarose gel and blotted on a nylon membrane (Amersham). Prehybridization and hybridization were performed according to Sambrook et al. (1989) and the same probe was used for the screening of *T. solium* DNA library.

2.5. Western blot of 2-dimension gels (2D-WB)

Crude extracts of parasites (500 mg) were sonicated four times at 40 W (1 min) in 250 μL of buffer (8 M urea, 0.5 M CHAPS, 1 μM pepstatin, 0.6 μM leupeptin, 0.2 mM phenylmethanesulfonyl fluoride, 0.5 mM DTT), leaving 1 min on ice between each pulse. Parasite suspension (100 μL) was processed with 2D Clean-Up Kit (Amersham) following manufacturer's instructions. The supernatant (300 μg) was applied on 7-cm strips (pH 3–10 linear gradient) for 16 h at room temperature for rehydration. Focusing started at 300 V (1 h), increased to 1000 V for 30 min, and maintained at 5000 V for 2 h in an IPG-phor I unit (GE Healthcare). Strips were equilibrated 20 min in 1X Laemmli sample loading buffer (63 mM Tris–HCl, pH 6.8, 2% SDS, 30% glycerol, 1% 2-mercaptoethanol, and 0.002% bromophenol blue), run in a 12% SDS–PAGE and transferred to PVDF membrane (Millipore). Membranes were incubated first with anti-TsCu,Zn-SOD (1:500) antibodies for 1 h and then washed three times with PBS Tween-20 (0.3%). Antibodies in membranes were detected by a second antibody peroxidase-conjugated goat anti-rabbit IgG (1:2000) and a solution of diaminobenzidine and H_2O_2. Membranes that reacted with anti-TsCu,Zn-SOD were washed overnight as described before, and incubated with anti-*T. solium* triose phosphate isomerase antibodies (TTPI) (1:1000), following the procedure of detection described before. A membrane strip of each Taeniid extract was incubated with normal rabbit IgG as control (Towbin et al., 1979).

2.6. Immunofluorescence assays

T. crassiceps and *T. solium* cysticerci were embedded in Tissue-Tek (Miles Laboratories), frozen in liquid nitrogen, and stored at −70 °C. Frozen sections of 6–8 μm thick were prepared and incubated overnight with 100 μL of anti-TsCu,Zn-SOD (0.4 mg/mL) antibodies in PBS with 1% BSA, 0.05% Tween 20 (PBS-BT) overnight. Sections were rinsed three times with PBS and incubated 60 min at room temperature with FITC-conjugated goat anti-rabbit IgG (Sigma) diluted 1:50 in PBS-BT. Normal rabbit IgG was used as control at the same concentration of first antibody. Sections were rinsed, as before, mounted on a glycerol-PBS solution (9:1), and photographed in a Nikon Optiphot epifluorescence microscope.

2.7. Superoxide anion produced by xanthine-xanthine oxidase system

To produce O_2^-, xanthine (Sigma) was dissolved at concentrations from 0.001 to 0.200 mM and mixed with three different concentrations of xanthine oxidase (30, 45, and 56 mU) in 1 mL of 50 mM K_2HPO_4, 10 mM EDTA, pH 7.8, 0.019 mM cytochrome C (Sigma). The production of O_2^- in each mixture was measured by reduction of cytochrome C at OD_{550} nm for 2 min (McCord and Fridovich, 1969).

2.8. T. crassiceps viability and Cu,Zn-SOD expression under oxidative conditions with O_2^- and H_2O_2

Groups of 20 *T. crassiceps* cysticerci were incubated either in: (1) RPMI (Sigma) plus 0.5% CO_2 at 37 °C for 0, 1, 4, and 24 h; and for next assays the cysticerci were preincubated 4 h in RPMI, before being exposed to: (2) RPMI with O_2^- (0, 1.9, 2.9, and 3.8 nmol/min) for 0.5, 1, 9, and 24 h; 3) RPMI with H_2O_2 (0, 0.25, 0.5, 1, and 2 mM) for 0.5, 6, and 24 h. Parasites were further incubated for 1 h in pig bile diluted 1:3 with RPMI to measure evagination. Viability was estimated by: (1) evagination, i.e., capacity of the scolex to evaginate. (2) Contractile movements. (3) Damage in the cysticerci bladder wall, observing in an inverted microscope (Nikon Eclipse TS100).

Messenger RNA expression of Cu,Zn-SOD and TPI were determined in the same groups of cysticerci described before. To detect messenger RNA, we used the One Step RT-PCR kit (Invitrogen), with 1 μg of *T. crassiceps* total RNA as template and primers SOD-X1 and SOD-X2; for TPI, primers were TPI-10 (5′-TAC-CTG-AAG-TAT-GCT-CAG-G-3′) and TPI-12 (5′-CGC-CAA-TGC-AAG-GAA-TGA-C-3′) coding for YLKYAQD and VIPCIGE amino acids of TTPI. The program used for reverse transcriptase reaction was at 50 °C for 30 min; and for PCR the program described above. To determine Cu,Zn-SOD protein expression a western blot method was used.