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Methionine sulfoxide reduction in ciliates: Characterization of the ready-to-use methionine sulfoxide-*R*-reductase genes in *Euplotes*

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ABSTRACT

Genes encoding the enzyme methionine sulfoxide reductase type B, specific to the reduction of the oxidized methionine-R form, were characterized from the expressed (macronuclear) genome of two ecologically separate marine species of *Euplotes*, i.e. temperate water *E. raikovi* and polar water *E. nobilii*. Both species were found to contain a single *msrB* gene with a very simple structural organization encoding a protein of 127 (*E. raikovi*) or 126 (*E. nobilii*) amino acid residues that belongs to the group of zinc-containing enzymes. Both *msrB* genes are constitutively expressed, suggesting that the MsrB enzyme plays an essential role in repairing oxidative damages that appear to be primarily caused by physiological cell aging in *E. raikovi* and by interactions with an O_2 saturated environment in *E. nobilii*.

1. Introduction

Reactive oxygen species are physiologically produced by every aerobic cell and oxidize a vast array of cellular constituents, which need to be continuously repaired by anti-oxidative enzymes in order to prevent loss of activity and accumulation within cells that would otherwise cause deleterious effects on the organisms' lifespan (Friguet, 2006; Stadtman, 2006). Elective targets of protein oxidation are methionine residues; in particular, those residues that are exposed on the molecular surface (Friguet, 2006; Vogt, 1995). Their modification into hydrophilic sulfoxides may cause effective changes of the protein polarity with consequent alterations of the molecule functions (Petropoulos and Friguet, 2006). Reversible oxidation of Met residues may also serve as a regulatory mechanism of protein activity and cellular signaling (Bigelow and Squier, 2005).

Consequently, methionine sulfoxide reductases (Msr) that catalyze the reduction of methionine sulfoxide back to methionine are usually regarded as a family of essential enzymes with a virtually universal distribution; only organisms living in anoxic environments or within host cells lack them due to secondary, adaptive loss (Delaye et al., 2007; Moskovitz, 2005).

Two functionally distinct and structurally unrelated Msr subfamilies, designated MsrA and MsrB, are commonly recognized. The MsrA subfamily is specific to repair the methionine-sulfoxide S-enantiomer, while the MsrB subfamily repairs the methionine-sulfoxide R-enantiomer (Sharov et al., 1999; Weissbach et al., 2002). Although different in sequence and structure, the MsrA and MsrB subfamilies work with a similar three-step reaction mechanism based on the catalytic and recycling activities of two cysteins. It involves (i) the attack of the catalytic Cys residue on oxidized-methionine with the production of a sulfenic acid intermediate, (ii) the attack of the recycling Cys residue on the sulfenic acid intermediate with the formation of a disulfide bond between the catalytic and recycling cysteines, and (iii) the reduction of the disulfide bond preferentially operated by thioredoxin (Boschi-Muller et al., 2008; Kim and Gladyshev, 2007; Neiers et al., 2004; Weissbach et al., 2002).

Ciliates represent one of the major eukaryotic components of every aquatic microbiota (Fenchel, 1987) and are very common experimental material (Nanney, 1980). However, virtually nothing is known about the structure and activity of their Msrs. Only annotations of genes encoding Msrs of both the A and B types have been reported in the genome databases of *Paramecium* (Aury et al., 2006) and *Tetrahymena* (Stover et al., 2012).

Working on two species of the ciliate *Euplotes*, namely *E. raikovi* and *E. nobilii*, which are phylogenetically close yet ecologically separated (Jiang et al., 2010; Vallesi et al., 2008), significant increases in methionine-oxidized protein concentrations have recently been recorded in correlation with distinct causes. In *E. raikovi*, a species dwelling in temperate seawater, the oxidative damages have been observed to ensue in correlation with cell aging (Alimenti et al., 2012); in *E. nobilii*, which inhabits polar seawater (Valbonesi and

Abbreviations: Msr, methionine sulfoxide reductase; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sec, selenocysteine; SECIS, selenocysteine-insertion sequence element.

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Luporini, 1990), the oxidative damages are likely the result of the cell adaptation to cope with a unique environment characterized by unusually high (saturated) O_2 concentrations (unpublished results).

In light of these observations, we studied the molecular mechanisms on which *E. raikovi* and *E. nobilii* rely to repair the oxidative damage of their proteins, and identified the genes encoding the enzyme Msr of type B. These genes, designated *Er-msrB* in *E. raikovi* and *En-msrB* in *E. nobilii*, were cloned from the transcriptionally active genome of the cell macronucleus, that in *Euplotes* (and ciliates in general) consists exclusively of free, linear DNA molecules amplified to hundreds or thousands of copies, each capped with two telomeres that are uniformly characterized by repetitions of the C_4A_4 motif in position 5′ and the G_4T_4 motif in position 3′ (Jahn and Klobutcher, 2002; Klobutcher et al., 1998). Coherent with their unusual organization characterized by an apparent lack of any structural information for the regulation of transcription, the *Er-msrB* and *En-msrB* genes equally show a constitutive expression and behave as housekeeping genes to ensure cell basal functions with their constant activity.

2. Materials and methods

2.1. Cell cultures

Cell cultures of the *E. raikovi* strain 13 (deposited at the American Type Culture Collection (ATCC), reference number "PRA-327") and the *E. nobilii* strain Far (Di Giuseppe et al., 2011) were maintained under a cycle of 12 h of moderate light and 16 h of darkness at 22 °C (*E. raikovi*), or 4–6 °C (*E. nobilii*). They were grown in natural seawater (salinity, 30–33‰; pH, 8.1–8.2), using the green alga *Dunaniella tertiolecta* as nutrient. Cells were deprived of food for 2–3 days before being used in experiments.

2.2. DNA and RNA purification

DNA was purified from cells according to standard protocols (Vallesi et al., 2010). Total RNA was extracted from cells using Trizol reagent (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) following the procedure described by the manufacturer, and purified from contaminating DNA by treatment with RNAse-free DNAse I (1 U/μg of RNA) (Fermentas International Inc., Thermo Fisher Scientific Inc., Maryland), at 37 °C for 1 h, in the presence of 40 U RiboLock (Fermentas International Inc.). After solubilization in RNase-free water, RNA was quantified by absorbance at 260 nm and its quality was verified in 1.2% agarose gel.

2.3. DNA amplification by polymerase chain reaction (PCR)

DNA amplifications were performed by PCR in the Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany), using oligonucleotides synthesized by Invitrogen (Life Technologies Corporation) and listed in Table 1. DNA aliquots of 0.5 μg were used as template in 50 μl-reaction mixtures containing 0.5 μM of each primer, 0.3 mM dNTP, 1 U of Platinum Taq polymerase High Fidelity (Invitrogen, Life Technologies Corporation), 60 mM Tris–SO_4 (pH 8.9), 2 mM $MgSO_4$, and 18 mM ammonium sulfate. In general, 35 PCR cycles were carried out, each consisting of a 30 s 94 °C denaturation step, a 30 s annealing step, and a 30 s 72 °C elongation step. The temperature of the annealing step varied from 55 to 65 °C, depending on the G + C content of the primers. A final incubation step, at 72 °C for 5 min, was added to the last cycle.

The resulting PCR products were run on 1.7% agarose gel, visualized with ethidium bromide, purified and cloned into the pCR TOPO 2.1 vector using TOPO-TA Cloning kit (Invitrogen, Carlsbad, CA USA), according to the manufacturer's recommendations. Sequencing was performed at the BMR Genomics Center of the University of Padua (Italy).

Table 1
Primers used to amplify *msrB* genes: designations and sequences.

Name	Nucleotide sequence (5′–3′)
Common primers	
msrB-fw1[a]	AAGTTCAATWCAGGATGHGGNTGGC
msrB-rv1[a]	GTTGCCTTTGGTCCATCATTGAANACRTGNCC
Tel	CCCCAAAACCCCAAAACCCC
Er-msrB specific primers	
fw2	CTTCACATGGCATGATTAGGACTG
rv2	GCTCCTGCTTTATCATTGAAAGCT
5′-fw	CCCCTACTTTTTAGAACTTCATAGA
3′-rv	CCCCTTCACAATATCCAATAATTGA
fwA	ATGAGTGAAGAGACGAAAGATGAC
rvA	TCAGTCCTAATCATGCCATGTGAAG
En-msrB specific primers	
fw2	GTGGAGGTGCAATGTGATAAA
rv2	CTTAATAGCACCTGCCTTATCATT
5′-fw	TTCATAAGCAAATTTGGTAGATACATT
3′-rv	TATTCATATCTTATGCATTGAAAATG
fwA	ATGAGTGAAGAGACTAAAGATGAC
rvA	CCACCCGAGTCATACCATGTGTTGT

[a] In the degenerate primers, W and R represent alternatives between A and T, and A and G; H, alternatives among A, C and T; N, alternatives among the four nucleotides.

2.4. Reverse transcriptase (RT)-PCR

The 3′-end of the *Er-msrB* and *En-msrB* transcripts was obtained by converting 1 μg-aliquots of total RNA into cDNA by reverse transcription with oligo$(dT)_{20}$ and 200 U SuperScript III reverse transcriptase in 10 μl-volume, following the protocol provided by the SuperScript III First-Strand Synthesis System (Invitrogen, Life Technologies Corporation). 2 μl-aliquots of cDNA were next amplified using the gene-specific primer "fwA" (Table 1) and oligo$(dT)_{20}$ under the conditions described in the previous section (Section 2.3).

Variations in the expression of the *En-msrB* and *Er-msrB* genes in cells treated with H_2O_2 were assessed by converting 1 μg-aliquots of total RNA into cDNA by reverse transcription with random hexamers and 200 U SuperScript III reverse transcriptase in 10 μl-volume (as above), and using 2 μl-aliquots of cDNA in PCR amplifications with the *msrB* gene-specific primers, "fwA" and "rvA" (Table 1). The template concentration and external control for PCR amplifications were obtained by amplifying 2 μl-aliquots of cDNA with the 18S-rRNA specific primers, 5′-CGCAAGGTCTACTGAGRTGATTC-3′ and 5′-CCATAGCC RCCCTCCTGTCT-3′. The amplification conditions were the following: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s, for 30 cycles. Absence of DNA contaminations was verified by running PCR with total RNA preparations not incubated with reverse transcriptase as template. Amplified products were separated by electrophoresis in 1.7% agarose gels and visualized by ethidium bromide staining. The *msrB* gene expression levels were quantified using the ImageJ software (NIH, USA) and normalized to the 18S internal control gene.

2.5. Southern and Northern blot analysis

Aliquots of DNA and total RNA (15 μg and 40 μg, respectively) were separated by electrophoresis on agarose gels and blotted onto Hybond-N + membranes (GE Healthcare, Life Sciences, Piscataway, NJ, USA) according to standard procedures (Sambrook et al., 1989). The products of 156-bp obtained by PCR amplifications with the "msrB-fw1" and "msrB-rv1" primers (Table 1) were labeled with ^{32}P using the random hexamer priming method (Feinberg and Vogelstein, 1983) and used as specific probes of the *Er-msrB* and *En-msrB* genes. Hybridizations were carried out overnight at 65 °C. Blotted membranes were washed at the hybridization temperature, dried, and exposed for autoradiography using a PhosphorImager system (BioRad).