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Quantitative PCR analysis of two molluscan metallothionein genes unveils differential expression and regulation

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Abstract

The mRNA levels of two components of the mussel (*Mytilus galloprovincialis*) metallothionein (MT) gene families, *MT10* and *MT20*, were evaluated using real-time quantitative-PCR and Sybr Green I chemistry in animals exposed to heavy metals in vivo and in primary cell cultures. This method was highly specific in detecting the expression of the two genes over a widely dynamic range of starting DNA amounts, showing that the basal level of MT expression is mostly due to *MT10* mRNA. Basal MT expression reflected the intracellular concentration of heavy metal as indicated by the use of the heavy metal chelator TPEN on primary cells. *MT10* was observed to be inducible by Cd, Zn, and Cu ions, and to a lesser extent by Hg. By contrast, the *MT20* expression level was very low under basal conditions, while its mRNA increased dramatically in response to Cd exposure, and to a lesser extend to Hg, leading to levels of expression similar to those of the *MT10* gene. The essential metals Cu and Zn had a very small effect on the *MT20* gene, whereas the concomitant exposure to Cu and H_2O_2 produced a rapid rise of expression. In summary, data indicate that the MT isogenes are differentially regulated by heavy metals, while hydroxyl radicals may have a role in MT20 gene activation. Also, protein expression showed metal inducibility only after Cd exposure, suggesting the occurrence of posttranscriptional control mechanisms.

Keywords: Metallothionein; Gene expression; Quantitative real-time PCR; Hydroxyl-radical; Mussels

1. Introduction

Metallothioneins (MTs) are heavy metal binding proteins displaying an increased synthesis after heavy metal tissue accumulation, hormone administration, and exposure to oxyradical-generating compounds (Hamer, 1986; Kägi, 1993). In addition, several articles have reported MT having a role in the protection of tissues and organs from oxidative stress (Sato and Bremner, 1993; Viarengo et al., 2000). MT gene expression in mammals is primarily controlled at the level of transcription (Durnam and Palmiter, 1981), through the interaction of metal sensitive factors with specific regulatory sequences of promoter regions. To date, the best characterised molecular mechanism of MT gene activation is the binding of the zinc

Abbreviations: AP-1, activator protein-1; bp, base pair; CDS, coding sequence; Ct, threshold cycle; EDTA, ethylenediamine tetraacetic acid; gsp, gene specific primer; M-MuLV H⁻ RT, point mutated RNAse H (minus) Moloney Murine Leukemia Virus reverse transcriptase; MRE, metal responsive element; MT, metallothionein; MTF-1, metal transcription factor–1; nt, nucleotide; PCR, polymerase chain reaction; Q-PCR, real time quantitative PCR; RACE, rapid amplification of cDNA ends; rRNA, ribosomal RNA; RT, reverse-transcriptase; SDS–PAGE, sodium dodecyl sulphate–polyacrilamide gel electrophoresis; TdT, terminal deoxynucleotide transferase; TPEN, N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine; UTR, untranslated region.

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finger protein Metal Transcription Factor-1 (MTF-1) to the Metal Responsive Elements (MRE) in the mouse MT-1 promoter (Saydam et al., 2002). Besides MRE sequences, various other elements have been found in MT gene promoters, including AP-1 and NF-IL6 binding sites, as well as elements responsive to oxidants (ARE) and to glucocorticoids (GRE) (Samson and Gedamu, 1998). The transcriptional regulation of the MT in *Caenorhabditis elegans* differs from vertebrates (Moilanen et al., 1999), while in *Drosophila* a homolog of the mammalian MTF-1 has recently been found (Egli et al., 2003).

In mammals MTs represent a superfamily whose components are present in multiple copies along the genome. Expression of the different isoforms seems to be differentially regulated, as different heavy metal inducers can be preferential for one isotype or another, while under basal conditions the MT-2 mRNA is more highly represented than that of MT-1 (Sadhu and Gedamu, 1988). Aquatic organisms express MTs which have often been associated with heavy metal resistance, as the metal content of the water environment may be susceptible to rapid fluctuations due to natural or anthropogenic events. The genetics of fish MT have been extensively characterised, demonstrating the presence of two differentially-regulated MT genes in the rainbow trout *Onchorhynchus mykiss*, and showing the role of MREs under basal and induced expression (Samson and Gedamu, 1998). In contrast with vertebrates, the mechanisms of MT gene expression in invertebrates have been poorly investigated. Some body of evidence indicates that invertebrate class-I (mammalian-like) MTs display peculiar biochemical features, such as a lower affinity for metal ions in both the α and β thiol cluster domains, as demonstrated for example in the crustacean *Callinectes sapidus* MT-1 (Narula et al., 1995). The common Mediterranean mussel *Mytilus galloprovincialis*, presents a tripartite structure of the MT genes, formed by three exons and two long introns, indicating that they belong to class-I MT (Ceratto et al., 2002). Furthermore, mussels seem to display a high degree of complexity in their MT gene organization, as demonstrated in the tissues of *Mytilus edulis*, in which up to nine MT isoforms were found. These have been classified into two multicomponent gene families – MT10 and MT20 (Frazier, 1986; Mackay et al., 1993; Barsyte et al., 1999) – showing differential expression due to heavy metal exposure (Lemoine et al., 2000; Lemoine and Laulier, 2003).

In order to infer more data on the mechanisms that control basal and induced gene expression in the mussel, we have conducted an expression analysis of two MT isogenes which have been herein identified from *M. galloprovincialis*. Using reverse transcription, real-time quantitative-PCR kinetics, levels of MT mRNA have been evaluated in tissue samples from the digestive gland of mussels exposed in vivo to sublethal concentrations of heavy metals (Cd, Hg, Zn, Cu), H_2O_2, and a mixture of Cu and H_2O_2 able to generate hydroxyl radicals via the Fenton reaction. Analyses have also been extended to primary cultures of digestive gland cells treated in vitro with the heavy metal chelator TPEN.

2. Methods

2.1. Animals, cell culture, and treatments

Specimens of *M. galloprovincialis* (Lam) – 4–5 cm shell length – were purchased from an aquaculture farm in La Spezia (I) and further acclimatised to aerated artificial sea water in an aquarium for 15 days at 16 °C (35‰ salinity, 1 L/animal). Mussels were then treated for 6 days with daily additions of sublethal concentrations of Cd (200 μg/L), Cu (45 μg/L), Hg (15 μg/L), and Zn (300 μg/L). Heavy metals were used in the form of chloride salts. In another experiment, Cu (45 μg/L) was administered together with 20 μM H_2O_2. After treatments, the digestive gland was rapidly dissected out, washed into artificial seawater buffered with 20 mM Hepes, pH 7.4, flash frozen into liquid nitrogen, and stored at −80 °C until further analysis.

Primary cell cultures were prepared by mechanical dissociation of digestive gland tissue as follows: freshly dissected tissue was washed extensively into ice-cold physiological saline containing 500 mM NaCl, 12.5 mM KCl, 5 mM di-sodium EDTA, 20 mM Hepes, pH 7.4. The tissue was minced using scissors and then cells were mechanically dissociated by chopping with a razor blade. The cell mixture was resuspended in 10 mL of ice-cold physiological saline and further filtered through a 100 μm sieve. The volume of the eluate was adjusted to 50 mL and then cells were washed four times into ice-cold physiological saline by centrifugation at 400 ×*g*, at 4 °C for 5 min. Cells were resuspended at $5 \cdot 10^5$ cells mL^{-1}, in 500 mM NaCl, 12.5 mM KCl, 20 mM Hepes, pH 7.4, in the presence of 100 U mL^{-1} penicillin; 100 μg mL^{-1} streptomycin, and 100 μg mL^{-1} neomycin. Finally, 3 mL of the cell preparation was plated into 30 cm^2 Petri dishes, and incubated overnight at 20 °C prior to treatment for 20 h with the intracellular heavy metal chelator N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN). The latter compound was dissolved in ethanol at 100 mM concentration and used as a 1000 X stock solution.

2.2. Molecular cloning of the full length mussel MT cDNAs

The two sequences of the *MT10* and *MT20* genes were obtained by Rapid Amplification of cDNA Ends (RACE), using primers deduced from previous works (Barsyte et al., 1999; Ceratto et al., 2002).

Total RNA was purified from 50 mg mussel digestive gland tissue by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987), using the TriReagent (Sigma-Aldrich).

For 5′ RACE, ~150 ng poly(A)$^+$ purified mRNA was reverse transcribed at 59 °C for 60 min in a 20 μl reaction,