Gene 519 (2013) 305–310

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Tissue-specificity and phylogenetics of *Pl*-MT mRNA during *Paracentrotus lividus* embryogenesis

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ARTICLE INFO

Article history:
Accepted 30 January 2013
Available online 26 February 2013

Keywords:
Sea urchin
Development
Gene expression
Endoderm differentiation
Metal detoxification

ABSTRACT

Metallothioneins (MTs) constitute a family of cysteine-rich, low molecular weight proteins, which generally provide protection against metal toxicity and oxidative stress counteracting the cell damage caused by essential and non-essential heavy metals. Equally important is the physiological role of MTs in the homeostasis of essential metals, which are involved in a wide variety of cellular processes. The aim of this work was to investigate the expression and the territorial localization of *Paracentrotus lividus* MT (*Pl*-MT) mRNA during sea urchin development by Quantitative Polymerase Chain Reaction (QPCR) and Whole Mount In Situ Hybridization (WMISH), as well as the phylogenetic comparison with selected MT homologs present in different phyla. We found that *Pl*-MT mRNA is accumulated in unfertilized eggs and constitutively expressed during development, with very low levels of maternal mRNA at cleavage stages, followed by a significant rise during gastrulation with a peak at the prism stage. *Pl*-MT mRNA was expressed in the vegetative plate at mesenchyme blastula, later restricted to the endoderm of gastrula embryos and finally to the gut of plutei. Indirect immunofluorescence (IF) using a specific antibody for the endoderm marker Endo1 demonstrated a co-localization with the *Pl*-MT transcripts in the midgut and hindgut after the intestine differentiation occurs and when larval feeding begins. Our results show for the first time the constitutive temporal and tissue-specific expression of MT in *P. lividus* embryos, providing new information for studies on the mechanisms controlling basal and induced MT gene expression.
The analysis of the phylogenetic relationship of *Pl*-MT with homologs from different phyla, ranging from yeast to vertebrates, suggests the evolutionary process of these proteins, which could have been selected not only on the basis of their ability to bind metals but also by their tissue-specificity.

1. Introduction

Metallothioneins (MTs) form a large family of cysteine (Cys)-rich, low molecular weight, proteins having a size ranging from 7 to 14 kDa. These proteins have a great affinity for both essential (zinc, copper, selenium) and xenobiotic (cadmium, mercury, silver and arsenic) metals, binding them through a number of Cys residues, which represent nearly 30% of their amino acid composition (Ryvolova et al., 2011). The metal binding capability of MTs may account for most of their physiological features in the organism. In fact, MTs are involved in physiological homeostasis of essential metals as well as in mechanisms of detoxification. In general, most of the studies have been focused on the MT detoxification role. Indeed, it has been demonstrated that an increase in heavy metal concentration inside the cells stimulates the "de novo" synthesis of MT, which binds metal cations, sequestering them away from critical macromolecules, thus protecting cells from toxicity (Nordberg, 1998). A role in protection against radiation and oxidative damages has also been suggested (Cai et al., 2000; Ebadi et al., 1996).

At the same time, extensive research has been carried out on the biological function of MTs in physiological conditions. It has been shown that these proteins are involved in a wide variety of cellular processes. Definitely, MTs are involved in the uptake, distribution, storage and release of essential metals, as zinc and copper, necessary for a number of vital cellular functions. In addition, some studies suggest that MTs may contribute in regulating cellular proliferation and apoptosis, as well as in neuro-protection and neuro-degeneration mechanisms (Rana, 2008; Santos et al., 2012; Shimoda et al., 2003).

MTs are ubiquitous proteins present in many organisms, ranging from plants to humans (Capdevila and Atrian, 2011). Due to the extreme heterogeneity among MTs, a complex classification of these

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine; Cys, Cysteine; Cd, Cadmium; cDNA, DNA complementary to RNA; DIG, digoxigenin; dNTP, deoxyribonucleoside triphosphate; FITC, Fluorescein isothiocyanate; IF, Indirect immunofluorescence; Ig, Immunoglobulin; kDa, kilodalton(s); MFSW, Millipore filtered sea water; nt, nucleotide; NBT, 4-nitro blue tetrazolium chloride; oligo, oligodeoxyribonucleotide; ORF, open reading frame; PCR, Polymerase Chain Reaction; RNase, ribonuclease; RT-PCR, Reverse transcriptase PCR; TBST, Tris-Buffered Saline Tween 20; TRITC, Tetramethylrhodamine isothiocyanate; WGA, wheat germ agglutinin; WMISH, Whole Mount In Situ Hybridization; UTR, unstranslated region(s); ′ (prime), denotes a truncated gene at the indicated side.

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http://dx.doi.org/10.1016/j.gene.2013.01.063

proteins has been developed by Binz and Kagi (1999), who grouped all aminoacidic MT sequences into 15 families and a number of subfamilies, subgroups and isoforms, on the basis of phylogenetically related and alignable sequences. The most extensively studied MTs belong to the mammalian subfamily, including 4 isoforms, each encoded by a cluster of at least 7 functional genes (MT-1) or by a single gene (MT-2, MT-3 and MT-4) and showing different tissue specificities (for a review see Vasak and Meloni, 2011). MT-1 and MT-2 were found in many organs, while MT-3 and MT-4 were specifically found in nervous system and in stratified epithelia tissues, respectively (Bell and Vallee, 2009).

The MT heterogeneity is observed in both invertebrates and vertebrates. In particular, new insights on MTs role have been gained from marine invertebrates, such as sponges, bivalves, crustaceans, and echinoderms (Capdevila and Atrian, 2011; Schroder et al., 2000; Tanguy and Moraga, 2001; Vergani et al., 2005). The increasing interest towards marine organisms comes mostly from monitoring programs, in which MTs are accounted as useful biomarkers for the assessment of environmental pollution (Canesi et al., 2010; Marrone et al., 2012; Sanchez and Porcher, 2009). For example, the induction of MT gene expression after exposure to Cd has been well documented in a variety of marine organisms including sponges (Schroder et al., 2000), bivalve mussels (Wang and Rainbow, 2010; Wang et al., 2010) and sea urchins (Russo et al., 2003; Filosto et al., 2008, for review see Amiard et al., 2006; Roccheri and Matranga, 2009).

In the sea urchin, some MT genes have been isolated from different species: *Sp*MTA, *Sp*MTB1, and *Sp*MTB2 from *Strongylocentrotus purpuratus* (Goldstone et al., 2006; Nemer et al., 1991), *Lp*MT1 from *Lytechinus pictus* (Cserjesi et al., 1997), and *Sn*MT and *Sg*MT from *Sterechinus neumayeri* and *Sphaerechinus granularis* (Scudiero et al., 1997). In *Paracentrotus lividus* embryos, the first complete MT cDNA had been obtained in our lab and referred to as *Pl*-MT (Russo et al., 2003). Very recently, Ragusa et al. (2012) reported the isolation of five different MT cDNAs from *P. lividus* embryo. Two of the five MT genes (*Pl*MT7 and *Pl*MT8) are constitutively expressed and upregulated in response to cadmium treatment, whereas the other three genes (*Pl*MT4, *Pl*MT5 and *Pl*MT6) appear to be specifically switched-on after cadmium treatment (Ragusa et al., 2012). Moreover, by nucleotide sequence alignment performed by BLAST, the authors showed a high identity between *Pl*-MT (Russo et al., 2003) and *Pl*MT8, one of the constitutively expressed genes.

Although the involvement of MTs in the detoxification processes after heavy metal exposure has been greatly described in the sea urchin embryo, their role in physiological conditions has not received adequate attention.

The aim of this study was to analyze the temporal and spatial expression of *Pl*-MT mRNA during normal development by Quantitative Polymerase Chain Reaction (QPCR) and Whole Mount In Situ Hybridization (WMISH). A phylogenetic analysis was also performed using the *Pl*-MT deduced amino acid sequence for multiple alignment with MT homologs from different phyla, ranging from vertebrate and marine invertebrate organisms to plant and yeast.

Results showed an interesting temporal regulation of *Pl*-MT expression and a distinct tissue specificity, providing useful information for the understanding of its physiological function during embryo development.

2. Materials and methods

2.1. Embryo culture and Quantitative real-time PCR

Gametes were collected from gonads of the sea urchin *P. lividus* harvested along the Northwestern coast of Sicily, Italy. Eggs were fertilized and embryos were reared at 18 °C in Millipore filtered sea water (MFSW) containing antibiotics (50 mg/l streptomycin sulfate and 30 mg/l penicillin), at the dilution of 4000/ml in glass beakers, with gentle stirring. Total RNA from embryos was extracted according to Russo et al. (2003), precipitated with 2 M LiCl (f.c.) overnight at 4 °C and quantified by readings at 260 nm using an Eppendorf bio-photometer. cDNAs were synthesized according to the Invitrogen Superscript II RNase H reverse transcriptase protocol. Quantification measurements of mRNA transcripts were performed following the manual of Applied Biosystems Step One Plus Real Time PCR, a Comparative Threshold Cycle Method, using SYBR Green chemistry (Livak and Schmittgen, 2001). *Pl*-Z12-1 mRNA was used as the internal endogenous reference gene which was found to be constant during development (Costa et al., 2012). The QPCR was run as follows: 1× cycle denaturing 95 °C for 10′ for DNA polymerase activation; and 38× cycles: melting 95 °C for 15″, annealing/extension 60 °C for 60″. The oligonucleotides used to amplify the 108nt *Pl*-MT cDNA were: *Pl*-MT F, 5′ TGCCTGACACCAAGTGCGTATGT 3′ and *Pl*-MT R 5′ AGCATACTGATGCACCATCCT3′. Statistical analysis of values was performed on three independent experiments, by one-way ANOVA analysis of variance test, followed by the multiple comparison test of Tukey's, using the Origin 8.1 statistical program, and level of significance was set to $P<0.05$.

2.2. Whole Mount In Situ Hybridization and indirect immunofluorescence

Whole-mount in situ hybridization was performed as previously described (Russo et al., 2010). All the pre-hybridization and hybridization steps were carried out in 96-well plates, using 30–40 embryos per well. Hybridizations were carried out with 1 μg/ml of a 303 nt long *Pl*-MT RNA probe (full-length, consisting of the entire ORF and the 3′ UTR) at 62 °C, for 18 h. Anti-sense and sense probes were synthesized by run-off transcription and digoxigenin-labeled (Roche). The DIG-labeled probe was detected by staining with NBT/BCIP solutions. Immunofluorescence (IF) experiments were performed on embryos after the WMISH procedure, including NBT/BCIP staining. Briefly, embryos were incubated with a monoclonal antibody specific for the endoderm marker Endo 1 (a kind gift from Prof. D.R. McClay), diluted 1:5 in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween20) overnight at 4 °C. After extensive washes, embryos were incubated for 1 h with TRITC-conjugated rabbit anti-mouse IgG (Sigma Chemical Co., St. Louis, MO, USA), diluted 1:200 in TBST. Moreover, primary mesenchyme cells were specifically labeled incubating the embryos with FITC-conjugated wheat germ agglutinin (FITC-WGA; Sigma Chemical Co., St. Louis, MO, USA), diluted 1:400 for 10′. Embryos were observed under a Zeiss Axioscop-2plus microscope, equipped for epifluorescence and images recorded by a digital camera.

2.3. Bioinformatic and phylogenetic analysis

Multiple alignment was performed with Clustal W2 and Boxshade programs (Thompson et al., 1994). Alignment was examined and adjusted manually. A phylogenetic tree was generated by using the Gene Bee by Tree-Top program choosing a Phylip format—Blosum62 matrix, with bootstrap values, in agreement with alignment (Saitou and Nei, 1987). Accession numbers relative to the protein sequences are summarized in Table 1.

3. Results and discussion

3.1. Temporal expression of Pl-MT

To determine the temporal expression of *Pl*-MT transcripts in unfertilized eggs and during the development from cleavage to pluteus stages, we carried out quantitative measurements of mRNA levels by QPCR experiments. The PCR product from unfertilized egg was used as a reference sample and assumed as 1 in arbitrary units. We found that *Pl*-MT mRNA was expressed at very low levels in embryos at cleavage stages, namely 8-cell and morula, as compared to the unfertilized egg (Fig. 1). A 3.4-fold increase of the transcription levels of *Pl*-MT was