The identification, cloning and characterization of earthworm metallothionein

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Abstract Combining standard gel chromatographic techniques and novel molecular methodologies (Directed Differential Display and quantitative PCR), it has been possible to isolate and sequence two isoforms of the first true earthworm metallothionein. Both proteins are characteristically high in cysteine residues and possess no significant aromatic residues. Metal responsiveness was confirmed by determining metallothionein specific expression profiles in earthworms exposed to soils of differing heavy metal concentrations. Analysis of the derived amino acid sequence of isoform 2 identified two putative *N*-glycosylation signal sequences, suggesting that the two isoforms may have different subcellular distributions and functions. Possible implications for intracellular metal trafficking are discussed.

Key words: Metallothionein; Earthworm; *Lumbricus rubellus*; Heavy metal pollution; Quantitative PCR

1. Introduction

In most organisms, the physiological tolerance mechanisms to heavy metals have been attributed, at least in part, to the induction of metal chelating gene products [1–6]. This leads to the assumption that the genes responsible for this mechanism will be up-regulated or differentially expressed within the heavy metal exposed population. These metal chelating gene products include low molecular weight, cysteine rich proteins, or metallothioneins (MTs), known to be involved in heavy metal detoxification and homeostasis. In contrast to the well-characterized vertebrate metal binding proteins, the information on metal associated proteins from invertebrates is sparse.

Earthworms have been shown to accumulate high concentrations of heavy metals within their tissues from a variety of metal contaminated soils, mainly from anthropogenic sources [7–12]. There is evidence that an excess of heavy metals is dealt with by at least two coexisting intracellular pathways [13,14]. The first pathway involves the retention of certain metals (predominantly lead and zinc) in insoluble calcium phosphate granules, the chloragosomes [11]. By doing so, earthworms are able to render them insoluble such that they do not interfere with essential biochemical reactions in the cytoplasm. The second pathway is characterized by the ability of sulphur donating ligands to bind cadmium within morphologically difficult to discern vacuoles, or cadmosomes [14]. Although functionally and morphologically different, both the chloragosome and the cadmosome are thought to be lysosome homologues, but representing different lysosomal subpopulations [15].

In summary, extensive effort has been devoted to identify the cellular mechanism(s) of heavy metal detoxification in earthworms; however, until now no genetic sequence has been forthcoming of the major cadmium binding protein, the earthworm metallothionein homologue. The identification of MT from the abundant soil inhabiting earthworm is considered important as these organisms hold a prominent status in terrestrial ecotoxicology, a fact recognized in the two acute toxicity tests approved by the EU, OECD and US-EPA, both of which utilize the related compost earthworm *Eisenia foetida* [16,17].

In the present report, we describe the identification of two MT isoforms in the earthworm *Lumbricus rubellus* (HOFFM.) and provide an initial characterization of its significant up-regulatory potential when exposed to cadmium.

2. Materials and methods

Initial purification of the cadmium binding proteins was performed on 12 earthworms (total wet weight: 6.5 g) native to a heavy metal contaminated mine site (Rudry, O.S. Grid Reference: ST 200 855) by gel chromatography as described previously [18]. Subsequently, the Cd pool was desalted on a G-25 Sephadex (Pharmacia) column (0.75×85 cm) and applied to a Q-Sepharose (Pharmacia) ion exchange column (0.5×8.5 cm). All samples were allowed to enter the column matrix by gravitation; the eluent was fractionated, analyzed for metal content by atomic absorption spectrophotometry using a Varian SpectrAA-100 (Varian Instruments) and spectral absorbance by diode ray spectrophotometry (Hewlett Packard, Model 8452A). In addition, nitric acid extractable metals were quantified in soils and earthworms by atomic absorption spectrophotometry as described above.

N-terminal blockage of the purified protein sample was removed by exposure to a non-specific proteinase, Proteinase K. The digest was performed by incubating 1.5 nmoles protein (eluted from a Q-Sepharose column) and 15 pmole Proteinase K in HPLC pure water (both Sigma) for 2 h at 37°C. Subsequent size separation was applied to remove the proteolytic enzyme and any small peptide fragments. Size separation was performed with Microcon concentrators (Amicon) with a molecular weight cut-off of 30 kDa and 3 kDa respectively. Following the removal of the blockage, N-terminal analysis was performed by automated Edman degradation as non-reduced and alkylated liquid samples applied to a Biobrenetm coated filter using an Applied Biosystems 476A protein sequencer with 610 data analysis software. Amino acid separation was performed on a Pharmacia Bio-

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Abbreviations: MT, metallothionein; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region; CDS, coding sequence

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tec Biochrom20 amino acid analyzer linked to EZ-chrome software as recommended by the manufacturer.

The earthworm MT cDNA sequence originated from a previously unidentified clone isolated by means of Directed Differential Display as described earlier [19]. Sequence identity was established by designing an inner anti-sense primer to the available partial sequence within the 3′ untranslated region of the gene and performing a RACE PCR (Clontech) utilizing a cDNA mini-library synthesized from earthworms native to Rudry Pb/Zn/Cd mine. All amplified products were cloned into the pGEM-T Vector system (Promega) according to the manufacturer's protocol, and sequenced using the PCR based Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP. The resulting amplified DNA fragments were separated on a gel matrix using a LI-COR DNA sequencer model 4000L and analyzed with the BASE ImagIR Image Analysis data collection package (Version: 02.21). The final sequences of isoform 1 and 2 were determined on both strands. Expression profiles of metallothionein were quantified by fluorescent based quantitative PCR using the Light-Cycler (Idaho Technology) as described earlier [20], however, with metallothionein specific primers designed to amplify both identified isoforms. Every PCR product was assessed for its amplification specificity by real time melting curve analysis, ensuring that only true amplification products were included in the quantitation.

3. Results

The work described encompasses a bi-directional approach: first a protein based biochemical investigation and second a molecular genetic analysis of the major Cd binding protein in the earthworm *L. rubellus*.

3.1. Biochemical protein analysis

3.1.1. Characteristics of the metalliferous soil and its native earthworm population. Earthworms utilized for protein extraction were collected from Rudry mine site exhibiting exceptional levels of lead (2337 μg Pb/g dry wt), zinc (5902 μg Zn/g dry wt) and cadmium (604 μg Cd/g dry wt). Whilst earthworms native to the mine site, accumulated lead and zinc to less than half of the soil metal concentration (892 μg Pb/g dry wt and 2470 μg Zn/g dry wt, respectively), cadmium was bioconcentrated by at least a factor of two (1213 μg Cd/g dry wt).

3.1.2. Purification of cadmium binding proteins. A cytosolic cell extract was produced by homogenization of earthworm tissue under isotopic conditions and subsequent ultracentrifugation. Proteins associated with cadmium were separated from the Pb and Zn binding components by means of standard gel chromatographic (Fig. 1, panel A) and desalting procedures (Fig. 1, panel B). A final purification step was performed by application to a Q-Sepharose ion exchange column over a linear gradient of 10 to 300 mM Tris-HCl (pH 8.1). Zinc impurities, coinciding with an elevated A_{280} value eluted prior to the major cadmium peak, which was followed by a minor cadmium peak (Fig. 1, panel C). However, only the major cadmium peak contained elevated A_{254} values (which are indicative of metal thiolate bonds), hence this peak was chosen for further investigation.

3.1.3. Characterization of the major cadmium binding protein. The amino acid analysis performed on the Q-Sepharose purified protein showed that it contained a high proportion of cysteine (24.5%), but lacked significant amounts of aromatic residues. Furthermore, an absence of histidine and methionine was observed (Table 1). This analysis was distinctly different to an analysis performed on the zinc binding moiety which was separated from the cadmium binding proteins in the early stages of the purification protocol (Fig. 1, panel A), where the cysteine and glycine residues were lower and the proportion of

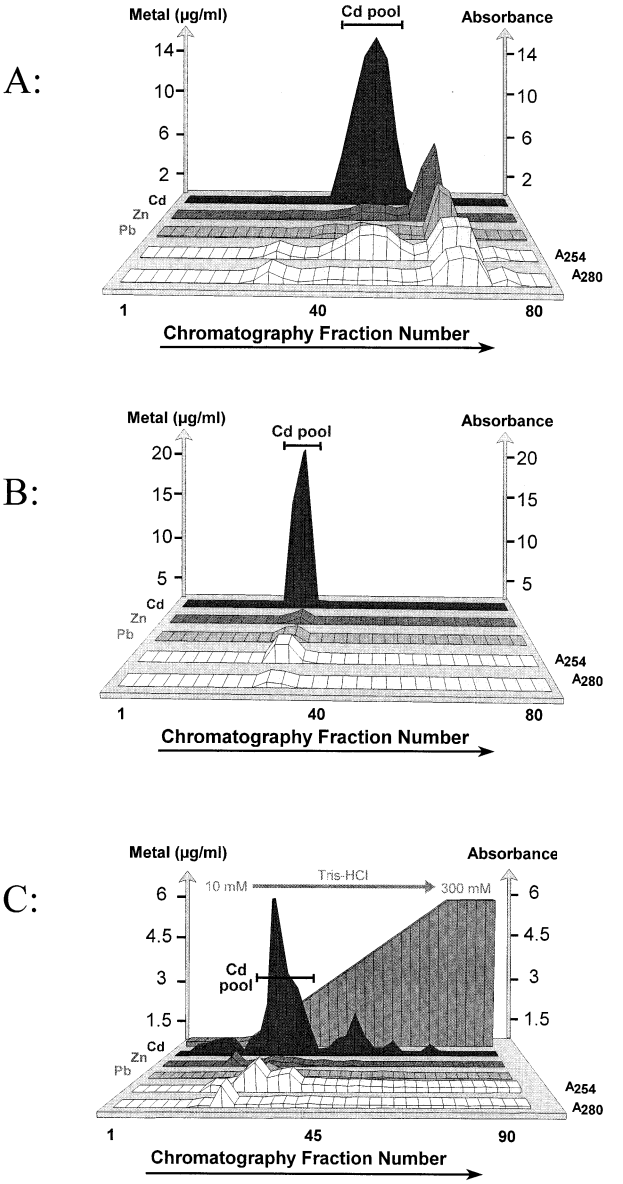

Fig. 1. Chromatographic purification of cadmium bound proteins present in the cytosolic supernatant of the earthworm *L. rubellus*. Panel A shows the elution profile after fractionation on a Sephadex G-75 column. The Cd containing fractions (horizontal bar) were pooled, concentrated and applied to a Sephadex G-25 column (panel B) and subsequently applied to an anion exchange Q-Sepharose column (panel C). Every third fraction (fraction volume: 2 ml (size exclusion) and 2.5 ml (ion exchange)) was analyzed for metal concentration and spectral absorbance.

glutamic acid, valine and leucine were higher (data not shown).

This initial indicator of the presence of a metallothionein like protein (i.e. marked by the high cysteine content and few aromatic residues) was substantiated by spectrophotometric analysis which identified acid labile absorbance at 254 nm indicative of cadmium thiolate bonds within the protein (Fig. 2).

Following the amino acid analyses, a further aliquot of the cysteine rich protein eluted from the Q-Sepharose resin was rendered up for N-terminal sequencing. Initial sequence analysis yielded non-data, suggesting that N-terminal blocking

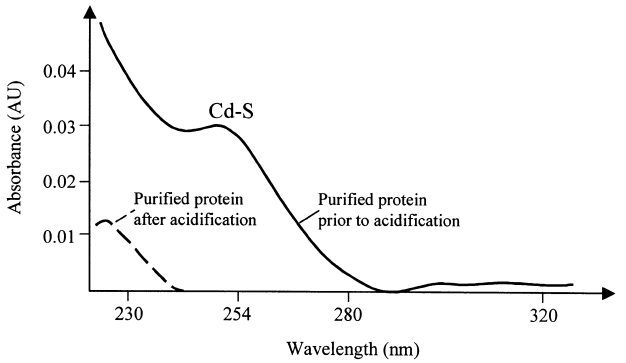

Fig. 2. Spectral absorbance of purified metallothionein protein before and after acidification with 6 M HCl. Note the characteristic acid labile absorbance at 254 nm, indicative of Cd-S bonds.

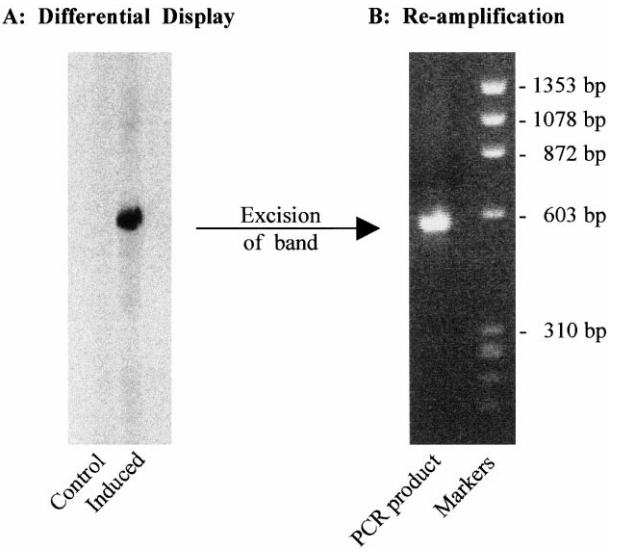

Fig. 3. Differential display and re-amplification of earthworm metallothionein. Panel A shows the band that was excised from the differential display gel of mRNA derived from unpolluted control (Dinas Powys) and Pb/Zn/Cd induced (native Rudry) earthworm populations using a specific Cys-Lys-Cys primer and an anchored poly-T-XG primer (see [19,20]). The product was re-amplified utilizing the same poly-T primer and specific primer. The band shown is the cloned product, subjected to electrophoresis on a 2% agarose gel, containing ethidium bromide (0.5 μg/ml). Nucleic acids were visualized under ultraviolet light (panel B). Marker = nucleic acid molecular weight markers (*Hae*III digested ϕX 174 DNA, Promega).

was preventing Edman degradation of the protein. In order to remove this barrier, a further aliquot of protein was exposed to the broad range proteolytic enzyme, Proteinase K, that cleaves at the carboxyl side of hydrophobic, aliphatic and aromatic amino acids. It has been observed that this protease is incapable of digesting the metal associated protein segments but will readily cleave any leader sequence not involved in metal binding [21,22]. Following the enzymatic digestion, size separation was used to remove the proteolytic enzyme and any small peptide fragments generated. The remaining protein fragment was sequenced by Edman degradation over nine cycles, resulting in the identification of six clear signals, one unidentifiable overlapping signal and two clear blank cycles. As the cysteine side-chains had not been chemically modified these residues were not expected to be detected, and with cysteine being a substantial component of this protein it was assumed that the blank positions could be assigned to cysteine residues. Using this assumption the protein sequence determined was N-T-?-C-C-G-F-D-A- (where ? equals an unresolved cycle).

3.2. Molecular genetic analysis

3.2.1. Isolation and identification of earthworm metallothionein. A Directed Differential Display protocol performed on two ecologically dissimilar earthworm populations designed to identify metal responsive genes containing Cys-Lys-Cys motifs [19] resulted in the isolation of an unidentified product specific to a population of earthworms native to Rudry Pb/Zn/Cd mine (Fig. 3, panel A). The partial sequence (211 bp) obtained from this clone started with the polyadenylation signal within the 3′ untranslated region of the gene, but did not extend into the coding region. In order to obtain the coding region, and thus facilitate the identification of the fragment, numerous RACE PCR amplifications were performed utilizing a Marathon RACE (Clontech) cDNA library produced from earthworms native to Rudry mine (see Fig. 4 for sequencing strategy). All resultant products were applied to agarose gel

Table 1
Derived amino acid composition of the major Cd binding protein isolated by Q-Sepharose ion exchange chromatography (see Fig. 1, panel C) and the theoretical amino acid compositions calculated from the known amino acid sequence of the two metallothionein isoforms

	Q-Sepharose purified Cd-MT (protein)	MT isoform 1 (cDNA)	MT isoform 2 (cDNA)
Aspartic acid	11.7	10.2	11.7
Threonine	3.7	2.5	3.9
Serine	7.3	6.3	7.8
Glutamic acid	5.1	3.7	3.9
Proline	1.1	2.5	3.9
Glycine	12.1	12.7	10.4
Alanine	14.0	17.7	15.6
Cysteine	24.5	25.3	26
Valine	0.4	0	0
Methionine	0.0	1.3	1.3
Isoleucine	0.2	0	0
Leucine	3.1	2.5	1.3
Tyrosine	0.3	0	0
Phenylalanine	2.7	1.3	1.3
Histidine	0.1	0	0
Lysine	8.5	8.9	10.4
Arginine	5.3	5.1	2.6

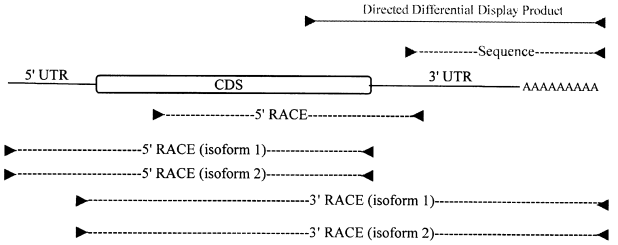

Fig. 4. Schematic diagram of the amplification strategy to obtain the gene encoding for metallothionein in the earthworm *L. rubellus*, indicating the approximate position of the differential display amplification and the total sequence obtained after 5′ and 3′ RACE PCR.

electrophoresis, purified, cloned and sequenced. Overall, it was possible to identify two separate sequences, with a nucleotide and amino acid similarity of both coding regions being 80.9% and 74.7%, respectively. Although distinctly different from each other, the coding regions are relatively small, show a conserved arrangement of the cysteine residues and, with the exception of one phenylalanine, lack aromatic amino acids (Fig. 5). These arguments provide strong, if not conclusive, evidence of the identification of two earthworm metallothionein isoforms. In conclusion, it was possible to illustrate that the actual amino acid sequence and composition of isoform 1 closely matches the equivalent data obtained from protein analysis (Fig. 5 and Table 1), thereby indicating that the majority of cadmium is chelated by metallothionein.

3.2.2. Quantification of earthworm metallothionein. To confirm that both isoforms are true members of the metallothionein family, it was considered important to establish their identity as truly metal responsive. Expression profiles of metallothionein were quantified by a fully quantitative fluorescent based PCR technique (Light Cycler, Idoho Technology) in earthworms native or exposed to soils with different heavy metal matrices. Initially, the metallothionein specific expression profile was assessed in three natural earthworm populations. Namely an unpolluted reference site (Dinas Powys, O.S. Grid Reference: ST 149 723) characteristically low in contaminating heavy metals, Rudry mine, a mixed metal (Pb/Zn/Cd) contaminated site (see above), and South Caradon mine (O.S. Grid Reference: SX 265 700), a soil with copper as the sole heavy metal pollutant ([Cu] > 800 μg/g dry wt). In comparison, metallothionein levels, which are low in the unpolluted control population, increase 5-fold in the resident to South Caradon copper mine. Highest levels of metallothionein were observed in the population of earthworms native to the heavily Cd polluted Pb/Zn mine (Rudry) where, compared to Dinas Powys (control), levels rose by a factor of over 1750 (Table 2).

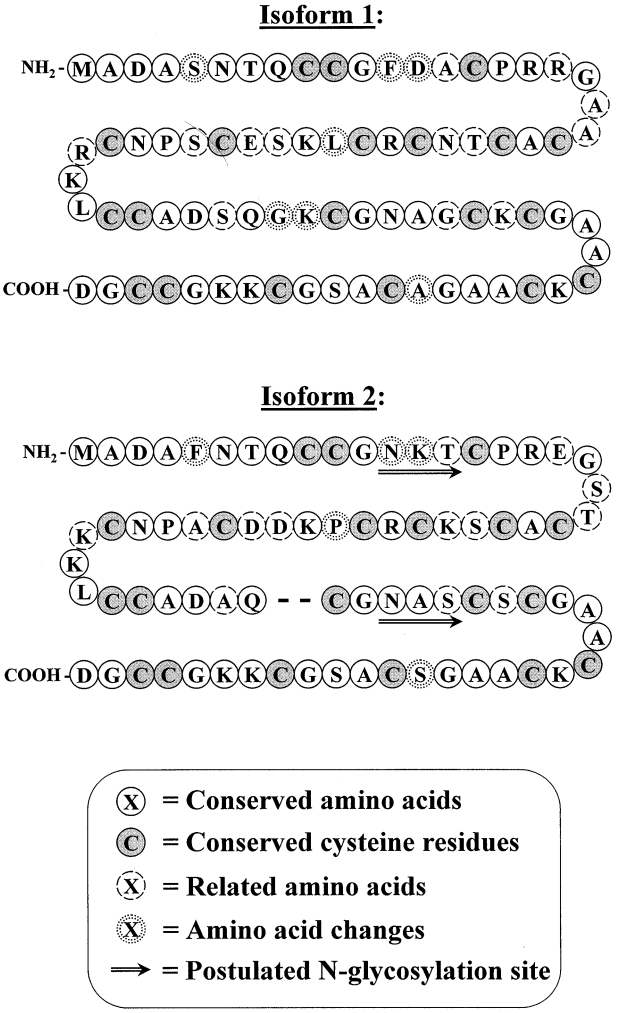

Fig. 5. Schematic diagrams depicting the amino acid sequence of earthworm metallothionein isoforms 1 and 2.

Furthermore, control earthworms were acclimatized for 2 weeks to OECD standard artificial soil under laboratory conditions [17], thereafter either killed for analysis or transferred for a further 2 weeks to artificial soil spiked with 86 μg $CdCl_2$/g dry soil. In contrast to earthworms maintained on artificial soil only, metallothionein transcripts increased 30-fold in earthworms maintained on artificial soil supplemented with the cadmium salt (Table 2). As demonstrated in this experimental setup, the exclusion of inter-population and micro-climatic variability showed that earthworms have the intrinsic ability to up-regulate metallothionein expression when exposed to sublethal quantities of cadmium.

Table 2
The relative expression of metallothionein quantified within different populations of earthworms (all *L. rubellus*) exposed to different heavy metal regimes

Native populations		Transplant experiment	
Dinas Powys (unpolluted control)	7.5 (±0.2)	Dinas Powys earthworms maintained on artificial soil for 4 weeks	2.6 (±0.1)
Rudry mine (Pb, Zn and Cd polluted)	13 376.7 (±838.7)	Dinas Powys earthworms maintained on artificial soil for 2 weeks, then transferred to artificial soil (+86 μg/g $CdCl_2$) for a further 2 weeks	78.6 (±3.8)
South Caradon mine (Cu polluted)	37.6 (±6.9)		

All values (±standard error, $n = 3$) were normalized with actin as an invariable control and are expressed as numbers of metallothionein per actin molecule.

Fig. 6. Amino acid alignment of the purified metallothionein protein (after an extensive purification protocol and proteolytic digestion) with the amino acid sequence deduced from metallothionein, isoform 1 cDNA.

4. Discussion

The methods initially designed for the purification of metal binding proteins in vertebrates are equally relevant and effective in the isolation and purification of invertebrate proteins [23]. Current methodologies involve three purification steps: gel filtration (size exclusion), buffer desalting and ion exchange. These purification steps have facilitated the identification of the amino acid compositions of invertebrate metalloproteins, including sample species from oligochaetes [24], arthropods [25], molluscs [26] and nematodes [27].

In oligochaetes, the isolation of a cadmium induced metal binding protein has been reported for at least three different species, namely from *E. foetida* [2], *L. rubellus* and *Dendrobaena rubida* (both [24]). But even these close phylogenic relatives exhibit remarkably diverse sequence compositions. Unfortunately, all data are limited to amino acid compositions only, which provide no indication of the actual protein sequence. Although an effective tool for most proteins, N-terminal analysis faces a number of specific problems in the case of MT family. The plethora of cysteine residues, whose prevalence classifies proteins into this family, and N-terminal modifications which are synonymous with the majority of vertebrate MT are features which have made MT protein sequence derivation time intensive and requiring large quantities of material [28]. The cadmium binding protein from *L. rubellus* proved no exception and initial attempts at protein sequencing failed. However, by applying functional knowledge concerning the proteolytic protection afforded to metal binding clusters in other MTs [21,22] a small amount of internal amino acid sequence could be derived (Fig. 6). These protein based hindrances promoted the development of genetic based approaches in order to determine MT sequence.

Molecular genetic approaches have been markedly successful in isolating and sequencing vertebrate or Class I MTs [29,30] where a high degree of overall sequence identity exists between species. However, in invertebrates, from the amino acid sequences available, little overall similarity can be observed between the invertebrate Class II MTs and their vertebrate analogues [31]. Consequently, a novel molecular genetic approach had to be developed in order to identify the earthworm MT sequence. The known conserved features of invertebrate MTs include the existence of short cysteine motifs (e.g. Cys-Lys-Cys), the small size of the predicted gene product and the up-regulation of MTs in response to metal ions (specifically cadmium). The method used incorporated Directed Differential Display using degenerate primers encoding conserved cysteine motifs, a process known to amplify small DNA fragments (<1 kb) and compared gene expression in normal earthworms with those exposed to high cadmium levels. By designing the practical approach appropriately the limited information available about earthworm MT has enabled us to identify full length coding sequences for two metallothionein isoforms from the earthworm *L. rubellus*.

Nucleotide (Genbank/EMBL) and protein (SWISSPROT) database searches with both isoform sequences revealed numerous low similarity hits to metallothioneins, albeit none significant, thus strengthening the hypothesis that invertebrate metalloproteins are less conserved than the vertebrate equivalents [31]. Furthermore, MTs have been historically split into two classes, Class I which are those with sequence homology to equine MT (this includes most vertebrate MTs) and Class II proteins which are those unrelated cysteine rich sequences that include invertebrate MT from molluscs to yeast [28]. On a purely statistical basis, it would seem that these novel earthworm proteins would be classified as Class II MTs. However, a structural description of Class I MTs would include 20 cysteine residues divided into two domains linked by a three amino acid link region (Lys-Lys-X or Arg-Lys-X), an N-terminal domain containing nine cysteine residues and a C-terminal domain containing 11 cysteines with cysteines evenly distributed within the protein except for doublets at the beginning and end of the C-terminal domain [32]. Analyses of the two earthworm isoforms reveal that, although slightly longer than the archetypal Class I MTs, they conform to all these structural features of a Class I MT, notwithstanding the fact that the existence of two functional domains must be confirmed. The conservation of these features could well be a function of common ancestry, although if this were the case should not these features be more prevalent in other invertebrate MTs? An alternative explanation would be the parallel evolution of these features as a common solution to the same functional challenges, that of metal sequestration and binding. If the latter is true these proteins provide a fascinating insight into the functionally important features of a family of ubiquitous proteins.

Apart from their structural importance these two earthworm genes raise intriguing functional questions. Indeed, Dallinger et al. [33] identified distinct metallothionein isoforms in the snail *Helix pomatia*, which, although similar in molecular weight and composition, preferentially bind either cadmium or copper in discrete tissues. It will be intriguing to discover if a similar mechanism exists within the earthworm, a differentiation not possible under the present biochemical and genetic analysis. Furthermore, the earthworm provides extensively characterized organelle system for cadmium sequestration. These so called 'cadmosomes' exhibiting metal thiolate densities which under electron probe X-ray emission analysis display a metal:sulfur ratio of 1:3, identical to that found in the isolated MT protein (unpublished data).

Although, the majority (90%) of the cadmium present in the worms appears in the cytosolic fraction used for protein purification the remaining 10% of the cadmium and 40% of the zinc is removed in the microsomal pellet. Furthermore, the small region of protein sequence determined is characteristic of only isoform 1, could isoform 2 be 'cadmosome' specific? Due to the complex procedures needed to determine the protein sequence, we cannot discount the possibility that isoform 2 is present within the soluble MT pool. However, analysis of the derived amino acid sequence of isoform 2 identified two putative *N*-glycosylation signal sequences present only in isoform 2 (Fig. 3) these may facilitate post-translational modification which in turn may be influential in targeting this specific isoform into intracellular organelles. Therefore, circumstantial evidence points to the intriguing and unique possibility that we have MT isoforms that have specific subcel-

lular locations. Future work will have to identify whether these proteins are involved in organelle sequestration of heavy metals and/or as metal shuttles delivering metal to these compartments, or whether they act as metal chaperones with a function to protect the biochemistry of the cytosol against potential toxic free metal ions. The earthworm system provides a fascinating and powerful biological model system in which to address these questions.

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