Identification and characterization of a recombinant metallothionein protein from a marine alga, *Fucus vesiculosus*

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A cDNA library was constructed from macroalgae adapted to prolonged elevated environmental copper levels. To investigate the possible existence of a metallothionein (MT) gene, the library was screened with degenerate probes designed using plant MT cysteine-rich motifs. A gene was identified (1229 bp) with a putative open reading frame (204 bp) encoding a 67-amino-acid protein exhibiting several characteristic features of MT proteins, including 16 cysteine residues (24%) and only one aromatic residue. Although the protein sequence showed high identity with plant and invertebrate MTs, it contained a unique 'linker' region (14 amino acid residues) between the two putative metal-binding domains which contained no cysteine residues. This extended linker is larger than the tripeptide found in archetypal vertebrate MTs, but does not conform either with the 40-amino-

acid linkers commonly found in plant MT sequences. An S-peptide *Fucus* MT fusion protein expressed in *Escherichia coli* exhibited a relative molecular mass of ~14 kDa. The recombinant fusion bound seven Cd ions, of which 50% were dissociated at pH 4.1. Under anaerobic conditions, the Cd ions were displaced by Cu(I), which associated with the protein at a ratio of 13:1. Laboratory exposure of *F. vesiculosus* to elevated copper resulted in induction of the MT gene. Thus this paper describes, for the first time, an MT gene identified from macroalgae which is induced by copper exposure and whose encoded protein product binds cadmium and copper.

Key words: copper, heavy metal, marine macroalgae.

INTRODUCTION

The metallothioneins (MTs) are a family of small, cysteine-rich proteins with the unique ability to form stable metal–thiolate clusters surrounded by a protein backbone [1–5]. The true role of MTs has not yet been fully determined, but involvement in the homeostasis of essential metal ions and/or the detoxification of heavy metals, such as cadmium or mercury, is suggested by the exceptional metal-binding properties of these proteins. The highly conserved nature of MTs suggests an important function which is essential to biological systems. MTs have been identified in a range of species, including invertebrates, plants, fungi and yeast [1,6]. However, no protein or genetic data have been presented on MTs from algal sources, although a large number of studies have focused on the ability of macroalgae to sequester metal from the environment [7–14] and on the cellular changes induced by the presence of metals, for example lipid composition [15].

Historically, tolerant macroalgae such as *Fucus* spp. have been used as biological indicator organisms in the marine ecosystems due to their resistance to heavy metals and their ability to accumulate metals, so that their intracellular composition reflects average pollutant loads in the sea over extended periods [7–9,16]. *Fucus* spp. have been shown to be resistant to heavy-metal pollution, accumulating high levels of copper and zinc [9], and their widespread distribution makes them an ideal model system for investigating the genetic mechanisms of tolerance and toxicity that are induced during exposure to pollution.

The predictable relationship between MT expression and metal concentration in certain organisms (e.g. teleosts and mussels [17,18]) suggests that MTs would be an appropriate environmental biomonitor. The isolation and characterization of MTs from model bioindicator organisms should have implications for

understanding the biological response to pollutants and for environmental biomonitoring.

Two distinct types of MTs might be involved in the macroalgae response to heavy metals, the gene-encoded class I and class II MTs or the enzymically synthesized class III MTs (phytochelatins). In vertebrate systems, gene-encoded MTs predominate, whereas in lower organisms, such as yeast, both systems are observed, working in a metal-ion-specific manner [19]. Initial analysis of plant responses to heavy metals identified only phytochelatins, but recently an increasing number of genes encoding MTs have been isolated from a range of higher-plant species [6,20,21]. However, there is only limited information concerning the protein products of these genes from the plant kingdom, and it is not understood how the different types of MT work in harmony to achieve metal homeostasis.

Phytochelatins have been identified from a number of macroalgae species [22], but the presence of gene-encoded MTs has not been reported. The present study describes the isolation and characterization of the first MT gene from macroalgae and, as such, will increase our understanding of the mechanisms that these biological systems use to handle heavy metals. The detection of MT genes may also offer an important monitoring tool for heavy-metal pollution in the marine environment.

MATERIALS AND METHODS

Algal material

Macroalgae (*Fucus serratus* and *Fucus vesiculosus*) were collected from the metal-polluted estuary of the Restronguet river at the highest point of seaweed occupation, at Devoran, Devon, U.K. (SW 803386) [9], and from Oxwich Bay, Wales, U.K. (SS 503868), which has low levels of metal pollution. The tissue of both species

Abbreviations used: CTAB, cetyltrimethylammonium bromide; IPTG, isopropyl β -D-thiogalactoside; MT, metallothionein.

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The nucleotide sequence data reported will appear in EMBL/GenBank/DBJ Nucleotide Sequence Databases under the accession no. AJ005834.

was washed carefully with distilled water to remove attached micro-organisms and debris, then pieces of 1–2 cm were cut from the frond tips and either processed directly or frozen in liquid nitrogen for storage.

Intact specimens of *Fucus vesiculosus* were collected from Sully, Wales, U.K. (ST 152683) and washed with distilled water prior to performing laboratory metal exposures. Two samples of *F. vesiculosus* were immersed in aerated sterile seawater (pH 8.3); copper chloride was added to one sample to achieve a final concentration of 2.5 mg/l. Both samples were subsequently incubated at 14 °C for 72 h with a 12 h light/dark cycle, after which frond tips were removed and frozen in liquid nitrogen for further analysis.

Metal analysis

Atomic absorption spectrophotometry was used to quantify cadmium, copper and zinc content, at wavelengths of 228.8, 213.9 and 324.8 nm respectively, using a Varian SpectrAA-100 instrument fitted with a background corrector (Varian Instruments, Walton-on-Thames, U.K.).

Metal measurements in *Fucus* spp. were determined in two independent sets of experiments. Samples of 0.5–0.8 g fresh weight of tissue were taken, dried and subjected to acid extraction by boiling the samples in conc. HNO_3 and H_2SO_4 . The solution was oxidized with hydrogen peroxide, followed by dilution with 1 M HNO_3 and centrifugation (12000 g; 30 min). The clear supernatant was used for the measurement of metal concentrations by comparison with standard curves obtained with known concentrations of metal salts (1.5, 1.0 and 0.5 $\mu\text{g/ml}$).

RNA preparation

Brown algae are known to have a high polysaccharide and polyphenol content combined with a rigid cell wall. Therefore a cetyltrimethylammonium bromide (CTAB) extraction method was used to isolate total RNA. Frozen material was ground to a fine powder in liquid nitrogen using a mortar and pestle and resuspended in extraction buffer (10 ml/g) consisting of 0.1 M Tris/HCl, pH 7.5, 1.5 M NaCl, 0.05 M EDTA, 0.05 M dithiothreitol and 2% (w/v) CTAB. After incubation for 15 min with occasional shaking at room temperature, the viscous solution was extracted with chloroform/3-methylbutan-1-ol (24:1, v/v) and centrifuged (20 min at 10000 g), and the remaining supernatant was subjected to ethanol precipitation [20% (v/v) final concentration]. The precipitated polysaccharides were removed by chloroform extraction followed by a centrifugation step (20 min at 10000 g). RNA in the remaining aqueous top layer was selectively precipitated overnight with 2.4 M LiCl at -80°C . A phenol/chloroform (1:1, v/v) extraction was performed to further purify the total RNA. A total of 50–70 g (fresh weight) of macroalgae was required to extract 0.5–1.0 mg of total RNA.

Poly(A)⁺ RNA was prepared using oligo(dT) columns (Pharmacia) according to the manufacturer's instructions. The quality of the poly(A)⁺ RNA was confirmed by *in vitro* translation using rabbit reticulocyte lysate (Promega) and [³⁵S]methionine (37 TBq/mmol; Amersham International) according to the Promega protocol.

cDNA library construction

Poly(A)⁺ RNA (2–5 μg) was reverse-transcribed and second-strand cDNA synthesis was carried out as detailed in the ZAP express cDNA Gigapack III Gold Cloning kit (Stratagene) protocol. The introduction of *Xho*I and *Eco*RI restriction sites at the 5' and 3' ends of the cDNA allowed site-directed cloning. The

cDNA fragments were size-fractionated on Sepharose CL-4B columns, and inserts larger than 500 bp were ligated into the ZAP Express vector arms. Packaging, titrating and amplification were carried out as described in the manufacturer's protocol using the host strain XL1 Blue MRF'.

cDNA library screening

Approximately 80000 recombinants from the cDNA library were plated out in duplicate and transferred on to neutral nylon membranes (Nytrap membrane; Schleicher and Schuell). The membranes were baked at 100 °C for 1 h to fix the DNA. Prehybridization of the membrane was at 42 °C for at least 1 h [0.5 M KH_2PO_4 , pH 7.0, 5% SDS and 2% Rad-Free blocking reagent (Schleicher and Schuell)]. Oligonucleotide probes were purchased from Pharmacia (Pharmacia Biotech Ltd) and were labelled at their 5' ends with biotin. The final concentration of the probe was 30 ng/ml. The membrane was hybridized at 65 °C in $0.1 \times \text{SSC}$ ($1 \times \text{SSC}$ consists of 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for 16 h. The membrane was washed in $2 \times \text{SSC}/0.1\%$ SDS for 2×5 min at room temperature, and then under the high-stringency conditions of $0.1 \times \text{SSC}/0.1\%$ SDS at 65 °C for 2×5 min. The hybridization signal was developed as described in the manufacturer's protocol. Positive clones were isolated and *in vivo* excision was performed in order to extract plasmid pBK-CMV containing the cDNA insert (Stratagene).

Sequencing

DNA was prepared for sequencing using the Wizard Plus SV purification kit (Promega). Sequencing reactions were performed using the ThermoFluorescent Sequenase kit (Amersham International) with appropriate IR labelled primers (MWG Biotech). A LI-COR DNA 4000L automated sequencer was used to obtain DNA sequences, and analysis was performed using the Genetics Computer Group package (Human Genome Project, Cambridge, U.K.).

Quantification of MT gene expression

The CTAB method described above was adapted to produce mRNA from frond tips by using a poly(T) magnetic bead separation protocol (Dynal, Oslo, Norway) after selective LiCl precipitation of total RNA. cDNA was generated and expression profiles were determined for MT and actin (EMBL accession number X98885) by fluorescence-based quantitative PCR using a Lightcycler (Idaho Technology) as described previously [23]. 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 quantification.

Cloning of *F. vesiculosus* MT for expression studies

F. vesiculosus MT was engineered with restriction enzyme sites directly proximal to the coding region of the gene, to facilitate cloning into the expression vector. This was performed by PCR amplification with a primer encoding the first six amino acids of the coding region with a 5' extension resulting in the formation of an *Nco*I restriction site encompassing the start codon of the gene (5'-CATGCCATGGCGGGCACTGGCTGC-3') and a primer complementary to the sequence encoding the last six amino acids of the protein with a 5' extension containing an *Eco*RI restriction site (5'-CGGAATTCGGCATTCTGAGGC-GGTGGG-3'). The amplification reaction was performed in a volume of 50 μl ; the final concentrations of the reaction components were 0.4 mM dNTPs, 1 pmol of MT primers, 7 units of

Expand High Fidelity *Taq* (Boehringer Mannheim) and 2.5 μ l (250 ng) of plasmid DNA. The optimal $MgCl_2$ concentration was found to be 2.5 mM. A thermocycler (Perkin-Elmer Cetus) was used with conditions of 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. The 300 bp PCR product was extracted with phenol/chloroform and precipitated prior to digestion with *EcoRI* and *NcoI* (New England Biolabs). A sample (1 μ g) of the purified expression vector (pET29a; Novagen) was also digested with *EcoRI* and *NcoI* according to the manufacturer's instructions. The engineered gene fragment and vector were purified from 2% and 1% agarose gels respectively, using the Jetsoorb gel purification protocol (Genomed, Bad Oeynhausen, Germany) according to manufacturer's instructions. Purified vector and fragment were subsequently ligated and transformed into competent *Escherichia coli* BL21 DE3 [24]. Prior to expression, the sequence of the cloned product was determined in order to authenticate the orientation.

Recombinant expression and purification of *F. vesiculosus* MT

An overnight culture of *E. coli* (BL21 DE3) containing the pET29a vector was diluted 1:100 (v/v) in fresh LB medium supplemented with 50 μ g/ml kanamycin. Cells were grown at 37 °C until an A_{600} of 0.6 was reached, and expression of the recombinant protein was induced by addition of isopropyl β -D-thiogalactoside (IPTG) to a final concentration of 1 mM. The resulting recombinant MT was stabilized by addition of $CdSO_4$ to a final concentration of 200 μ M after a further 30 min of growth.

For the purposes of purification of the recombinant protein, MT-expressing *E. coli* were grown for 16 h at 37 °C with shaking (125 rev./min) after the addition of $CdSO_4$ (200 μ M final concentration). The cells were then harvested by centrifugation at 6000 g for 15 min and resuspended in 10% of the original volume of 10 mM Tris/HCl, pH 8.6. Cells were lysed by freeze-thawing and sonication, and complete lysis was confirmed by light microscopy. Cell debris was removed by ultracentrifugation at 132 000 rev./min for 45 min at 4 °C (Beckman L-80 Ultrafuge). Heat-labile proteins were denatured by heating the supernatant at 80 °C for 15 min and removed subsequently by ultracentrifugation, as before. The fusion protein was purified from the supernatant by applying the latter to a 5 ml Hi-Trap Q-Sepharose column (Pharmacia). The protein was initially eluted with a steep linear gradient of 0–150 mM NaCl in 20 mM Tris/HCl, pH 8.6, over 70 ml. The metal concentration of samples was determined by atomic absorption spectrophotometry, and fractions containing high levels of cadmium were pooled. The ionic strength of the sample was then reduced 10-fold by dilution into 20 mM Tris/HCl, pH 8.6, and the ion exchange was repeated with a 1 ml Hi-Trap Q-Sepharose column and a more gentle salt gradient (0–120 mM NaCl, spanning 22 ml). Fractions containing high cadmium levels were pooled, freeze-dried under vacuum, resuspended in 1–2 ml of MilliQ water, pH 7.6, and applied to a Sephadex G-50 column (50 cm $\times$ 1 cm) equilibrated with MilliQ water, pH 7.6. Fractions containing purified recombinant MT were identified by metal and UV spectral analyses.

Protein analysis, amino acid composition and N-terminal sequencing.

Proteins were resolved by SDS/20%-PAGE by the method of Laemmli [25], and relative molecular masses were determined by comparison with prestained low-molecular-mass protein markers in the range 2850–43 000 Da (Gibco BRL). Proteins were visualized by Coomassie Blue staining. Proteins containing the S-

peptide sequence were detected by Western blot analysis using the S-Tag system (Novagen) according to the manufacturer's instructions. Transfer of the proteins on to Hybond-C Super (Amersham International) was carried out using a semi-dry blotter (Sartoblot II-S; Sartorius).

Duplicate samples of protein for amino acid analysis were hydrolysed in 6 M HCl *in vacuo* as described previously [26]. For amino acid sequence analysis, protein samples were size-fractionated by SDS/20%-PAGE, as before [25], but substituting tricine for glycine in the running buffer. Proteins were then transferred to an Immobilon membrane (Millipore) by semi-dry blotting. Transfer was confirmed by staining the membrane with Coomassie Blue [0.1% (w/v) Coomassie Blue in 50% methanol for 5 min, followed by destaining in 50% methanol/10% acetic acid]. Appropriate bands were excised and subjected to 15 cycles of Edman degradation in an Applied Biosystems Model 470A Protein Sequencer. The phenylthiohydantoin derivatives obtained were identified by HPLC using a methanol/water gradient system as described previously [27].

pH titration

An aliquot of *Fucus* MT recombinant protein (1 μ g) in a final volume of 350 μ l of MilliQ water, pH 7.6, was added to 2.15 ml of a buffer of appropriate pH (pH ranging between 1.1 and 7.6) and incubated for 60 min. The pH of the sample was measured before application to a Sephadex G-25 column (PD-10; Pharmacia) previously equilibrated with 25 ml of the specific buffer. The MT protein was eluted with 3.5 ml of elution buffer. The cadmium concentration was measured by atomic absorption spectrophotometry, as described above.

RESULTS

Metal bioaccumulation by marine macroalgae

The ability of macroalgae to accumulate metals from the surrounding waters has been described previously [7–9]. The present study confirms the bioaccumulation of Zn, Cu and Cd by *Fucus serratus* and *Fucus vesiculosus* from the waters they inhabit (Table 1). The levels detected were correlated with the metal content of the water from which the samples were taken. Cadmium levels in all samples were at the limit of detection, and a significant increase could only be detected in *F. serratus* inhabiting the most polluted site compared with samples from the control (non-polluted) site. However, Zn and Cu levels were shown to increase significantly in both *Fucus* species from the

Table 1 Metal levels in water and algal samples collected from different sites

Abbreviation: n.d., none detected.

		Concn. in macroalgae ($\mu\text{g/g}$ dry weight)	
Metal ion	Concn. in water ($\mu\text{g/ml}$)	<i>F. vesiculosus</i>	<i>F. serratus</i>
(a) Oxwich Bay			
Cd	n.d.	3	1
Cu	< 0.1	18	29
Zn	< 0.1	160	103
(b) Devoran			
Cd	n.d.	2	11
Cu	0.3	300	173
Zn	4.0	647	1670

Devoran site (polluted by run-off from old copper mines), although the pattern of metal accumulation was species-specific. *F. vesiculosus* showed only a 4-fold increase in Zn in the Devoran site compared with the control, whereas the sequestered Zn levels in *F. serratus* were increased by 16-fold. Interestingly, this pattern was reversed for copper accumulation, with *F. vesiculosus* exhibiting a much greater (17-fold) increase than *F. serratus* (6-fold) when comparing the two sites. These data suggest that the two species have differential efficiencies in their metal handling pathways that are not simply related to their position on the shore.

Both the yeast *Saccharomyces cerevisiae* and the fungus *Neurospora crassa* use gene-encoded MTs to sequester copper. Therefore we examined the algae to see if they possess a similar physiological system. Accordingly, it could be predicted that *F. vesiculosus* exposed to copper would exhibit a high level of MT expression.

Isolation of an MT sequence from the *F. vesiculosus* cDNA library

A cDNA library was constructed from *F. vesiculosus* harvested from the Devoran site. Degenerate probes were designed to regions encoding the conserved cysteine-rich regions of known plant MTs (Figure 1). These probes were combined and used to screen the cDNA library (80000 recombinants) using a non-isotopic method. Initial screening identified 10 hybridizing plaques, and subsequent rounds of screening allowed these clones to be purified to homogeneity. The isolated phagemids were auto-excised to facilitate the purification of plasmid DNA for sequencing. Of the 10 clones, one had a putative open reading frame of 204 bp which encoded a putative 67-amino-acid sequence that contained 16 cysteine residues (24%) (Figure 2). A high cysteine content is a well documented feature of MT proteins [2,5]. Only one aromatic residue (tryptophan) was present in the predicted protein; such an absence is also characteristic of other MT proteins [2,5]. The gene comprised a total length of 1229 bp, of which 64 bp was upstream of the initiator codon and a further 961 bp followed the termination codon, including a poly(A)⁺ tail of 19 bp.

The putative protein sequence of 67 amino acid residues was shown to have high identity with certain plant and invertebrate MT proteins (Figure 3). Comparisons using the FASTA algorithm (as implemented by the Genetics Computer Group [28]) between the *Fucus* MT sequence and those of MTs from *Arabidopsis thaliana* (EMBL accession no. P43392) and *Brassica napus* (accession no. P43402) showed sequence identities of 42 and 38% respectively at the protein level. A high degree of similarity was also detected with a group of invertebrate MTs already identified as a subgroup, which have a high percentage of glycine residues and exhibit identity with the class I MTs of vertebrates [29]. The *Fucus* MT peptide had 43% identity with an oyster MT sequence (*Crassostrea virginica*; accession no. P23038 [30]), and 36% identity with snail (*Helix pomatia*; accession no. P33187 [31]) and mussel (*Mytilus edulis*; accession no. P80248 [29]) MT proteins.

Presence of putative metal-binding domains

The *Fucus* MT protein has two domains which contain the cysteine residues, and therefore are presumably involved in the metal-binding function. The N-terminal domain contains three Cys-Xaa-Cys motifs, and the C-terminal domain contains three Cys-Xaa-Cys motifs and one Cys-Cys-Xaa-Cys motif. An unusual feature of *Fucus* MT is the presence of a region between

Figure 1 Design of degenerate probes using plant MTs

The nucleotide sequences corresponding to the two plant MT cysteine-rich regions are aligned. Panels (A) and (B) depict the primers designed to the most conserved areas of the N-terminal and C-terminal cysteine-rich regions respectively. Schematic diagrams, indicating the approximate positions of the primers in the plant sequences, are shown above the sequence alignments. The probe sequence is given above the alignment, with degenerate positions indicated by base codes R (A/G), S (C/G), Y (C/T), W (A/T), I (inosine) and N (A/C/G/T). Where the aligned plant sequences are consistent with the probe, the base is not given in the alignment. Conserved amino positions are shown under the appropriate alignment.

the two putative metal-binding domains which contains no cysteine residues (Figure 4). This is a feature of plant MTs, but not of vertebrate MTs, which characteristically have a three-amino-acid linker region. In the *Fucus* MT this linker region consists of 14 amino acids, which is much smaller than the 40 residues commonly seen in plant MTs.

Expression and characterization of the recombinant MT protein

To express the protein, primers flanking the coding region of the *Fucus* MT sequence were designed with additional restriction enzyme sites. The 300 bp MT PCR product was amplified from plasmid DNA and digested with the restriction enzymes *NcoI* and *EcoRI*. The MT coding region was cloned in-frame as a C-terminal fusion to a sequence encoding the S-peptide (S-Tag) in the expression vector pET29a (Figure 5). Induction of T7-RNA-polymerase-based transcription of gene fusion was performed in the BL21 (DE3) *E. coli* expression strain by the addition of IPTG when bacterial cultures were at mid-exponential phase. Previous work had demonstrated that it is necessary to add cadmium to

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-64 AATTCCGGCAGTCCAACTTCAAGTCTCTCTACAAGCAATACAGCACCGATCGAAGAAA -4
-3 AAAATGGCGGGCACTGGCTGCAAGATCTGGGAAGACTGCAAGTGCAGGAGCGGCGTGCAGC 56
    M A G T G C K I W E D C K C G A A C S 19
57 TGC GGCGACTCGTGCACTGCGGAAGTGTCAAGAAGGGCACCACCTCTCGCGCGGCGCG 116
20 C G D S C T C G T V K K G T T S R A G A 39
117 GGCTGCCCTGCGGCCCAAGTGCAAATGCACGGCCAAGGCAGCTGCAACTGCGTCAAG 176
40 G C P C G P K C K C T G Q G S C N C V K 59
177 GACGACTGCTGCGGCTGCGGCAAGTAAGATAAGAGGCATTCCACCGCCTCAGAATGCC 236
60 D D C C G C G K *
237 CCTGCTCGGGAGTGAAGGGATGAAATGGGCAAGCGACATCCAGGAGGCTGGGATAGTCT 296
297 CAACGGTGTAGACAAGGAAACAACCTCGTATGTTCTTCGGCGACTCAAACGATATGGTTCC 356
357 GGCACGAGGGTGGGCTCCTAGGAATAGAAGTTGTCTGAACGTTTCCACGAAGCAGAA 416
417 GGACATACTCTGTGGCGTACCCATGTATGTATGGTTACTCGTATACCTGATTATATCGGG 476
477 ACAGCGGTGCTGATTTGGGAAGAAATCTGGCTTTTGGTCTGGCTTCACTCT 536
537 CTACTTTAGCGGACACTTTTTTCCCCCTCCTGCTGGGAGTGTGGCCTAGTGCTTTGG 596
597 CCGCTTCAGGCGAGTTGAAACAGTCGAATGGGCAATCAACGGTTTCTTGGGCGATGAA 656
657 CGATGAAAGATTGGCTGAAACGCCCGAGAATTGCTGTGACGTTTGCACCTTGACATGGGC 716
717 TTGCTGCAGTTTACGTCGACATCACTAGGCTGTGGAGTGGACGAAACGTATCCGTGCGCT 776
777 TCAGAAATAGTTATGTTGCCGTTTACTAGGCTTCCATAATATAATATGAAAAGGGTTTT 836
837 GAAAAAATAGCCGTCGAAGTTGTTCTGATAGTGGATTGACCTCAGGGGTGCTTTTAAAC 896
897 AAATCTCGCGAGCATGGCACTCGGTTCTGTCGGCCCTGCTCTCTCGTTCAAGTGTGCATG 956
957 GGTACTGGATTTTTTTTCTGTCTGATACCGCTCAGGAGTACGGCCCAAGTCTTGTTGT 1016
1017 AGAGTACGTTGGAGACGACAGCTCGAAATCGCTCGGCGGGTTATCATCTGCTACCTCC 1076
1077 ACCTTTGAAACGAGCACTGTTTGTACTGTACTCTGGAGAAGAGAAAGTAAATCTATACAC 1136
1137 TGTCTCTCAAAAAAAAAAAAAAAAAAAAA 1164

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Figure 2 Full-length cDNA and predicted amino acid sequence of *F. vesiculosus* MT

cDNA numbering starts from the predicted initiation methionine residue. Cysteine motifs are underlined, and the putative polyadenylation signal sequence is double-underlined. The symbol * indicates the predicted TAA stop codon.

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Fucus      MAGTGCKIWERDCHGAAACGDSGTCGTVKKGTTSPAGA-CGCG---GPNKCT---GGGSCGKVKDDDCGCGK
Arabidopsis MADSKCCSSSCGDSGSCCKNYNKEC-----DNCGC---GSNGCG-----SNKC
Rape       MAGSKCCSSSCGDSGSCCKNYNTEC-----DSGCG---GSNGCG-----DSGCG
Snail      SSGKKGKCKTCSAC-RSEKCCGSKKCCGEGCTGAA-----CCT-----CCT-SDGKCGKCTGPDSCGSS--GGC-K
Mussel     PCPCMCITETVCTGCTGC-SGKCCGSDGSCGSCGKVKRC--SGTGC-----GCTCT---GPTNCGGSG--GSC-K
Oyster     SDPCMCITETGCASSSCG-PATGCTGKPCGSDGAGGAG-CKVKCCTSGEGGCG---GKCTG-----PATCGSGG--GSCKK
Horse N-term MDPCSCPTGGSCGAGSGKRE-DRGTSCKK
Horse N-term MDPCSCPTGGSCGCA-----GSCGCKE---GCTGTSCKK

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Figure 3 Alignment of MT protein sequences

Sequence alignment of the predicted protein sequence of *F. vesiculosus* MT with sequences from representative plant [*Arabidopsis thaliana* (accession no. P43392) and *Brassica napus* (rape) (P43402)] and invertebrate [snail (*Helix pomatia*) (P33187), mussel (*Mytilus edulis*) (P23038) and oyster (*Crassostrea virginia*) (P23038)] MTs, and the N-terminal region of a mammalian [horse (*Equinus caballus*) (P02800)] MT. Dashes indicate where spaces have been introduced to maximize alignment.

Figure 4 Schematic diagram of MT domain structure

The known two-domain structure of vertebrate MTs is compared with a putative single metal-binding cluster proposed for plant and *Fucus* MTs, which contain an extended linker region. Cysteine-rich metal-binding domains are shaded. aa, amino acid.

the medium after induction in order to stabilize MT-like clusters and prevent their degradation [32]. Time-course samples were taken from the point of induction in analytical cultures, and

Figure 5 Temporal expression of recombinant *F. vesiculosus* MT

E. coli cells were harvested and prepared for electrophoresis, as described in the Materials and methods section, at the point of induction (T_0) and subsequently 2 h (T_2), 5 h (T_5) and 16 h (T_{16}) after induction with cadmium (200 μ M final concentration). After size separation by SDS/PAGE (20% gel), proteins were visualized by staining with Coomassie Blue (A). An identical separation was performed in parallel and proteins were transferred to a nitrocellulose membrane. The S-peptide-MT fusion was then detected by interaction with an S-protein-alkaline-phosphatase conjugate (B). Arrows indicate sizes of molecular mass standards (kDa).

recombinant protein accumulation was investigated by SDS/PAGE analysis of total cell extracts. A substantial protein component with a molecular mass of ~ 14 kDa was clearly evident 2 h after inducing expression with IPTG, and this was still the predominant protein 16 h after induction (Figure 5A).

Figure 6 Purification of recombinant *F. vesiculosus* MT

Anion-exchange chromatography was carried out on a heat-treated soluble cell extract from *E. coli* expressing the plasmid harbouring the *F. vesiculosus* MT gene. Pooled Cd-containing fractions from an initial separation on a 5 ml Hi-Trap Q-Sepharose column were diluted in 20 mM Tris/HCl, pH 8.6, and loaded on a 1 ml Hi-Trap Q-Sepharose column pre-equilibrated in the same buffer. After extensive washing with 20 mM Tris/HCl, pH 8.6, a linear gradient of 0–120 mM NaCl was used for desorption. Fractions of 1 ml were collected. Protein was monitored by measuring A_{254} (●; broken line), and cadmium concentrations were determined (♦; solid line).

The identity of the induced protein was confirmed by Western blot analysis using an S-protein–alkaline-phosphatase conjugate that binds specifically to the S-peptide fusion component (Figure 5B).

A soluble cell extract was prepared from a large-scale culture of bacteria harvested 16 h after induction with cadmium. Further purification was achieved by heat treatment of the sample followed by ultracentrifugation, as previous studies have shown that both S-peptide and MT proteins are extremely heat-stable [2]. The recombinant protein was purified from the resultant mixture by two rounds of anion-exchange chromatography (Q-Sepharose). The final gradient showed clearly a peak of protein (as monitored by absorbance at 254 nm) coincident with high levels of cadmium (Figure 6). SDS/PAGE analysis of the pooled cadmium-containing fractions showed the continuing presence of higher-molecular-mass proteins (~23 and ~57 kDa) co-purifying with the recombinant fusion protein. Size separation and desalting was facilitated by gel filtration on a G-50 Sephadex matrix. This gave rise to two cadmium-containing peaks (results not shown); the low-molecular-mass peak contained large amounts of cadmium, but no protein bands could be detected on an SDS/PAGE gel. The higher-molecular-mass peak contained predominantly the 14 kDa protein when analysed by SDS/PAGE. N-terminal sequencing of this 14 kDa band confirmed the presence of the S-peptide sequence only, showing that this fraction contained the MT fusion protein. Duplicate amino acid analysis of the peak gave a good correlation with the predicted ratios (results not shown). This allowed the calculation of an accurate concentration of the fusion protein which, together with cadmium analysis performed in duplicate, was used to calculate the amount of Cd bound to the S-peptide–MT fusion as 7 Cd ions per protein molecule. As no metal binding has previously been attributed to the S-peptide, this metal co-ordination can be

Figure 7 Characterization of purified recombinant *F. vesiculosus* MT

(A) Absorbance spectrum of cadmium-containing recombinant *F. vesiculosus* MT, showing a shoulder at 254 nm characteristic of Cd–S energy transfer. The insert shows SDS/PAGE analysis (20% gel) of the purified protein (Coomassie Blue staining). (B) Copper-bound MT, showing the shift of the energy-transfer band to 240 nm. (C) Spectrum of the apo-protein produced by acidification of the recombinant MT–Cd protein to pH 2.0.

Figure 8 pH-dependent metal dissociation from recombinant *F. vesiculosus* MT

A detailed analysis of the pH-dependent loss of cadmium from thiol–metal clusters compares recombinant *F. vesiculosus* MT (■) with recombinant human MT 1A (○) expressed in a similar manner. Gel filtration was used to separate proteins from free cadmium after equilibration at a range of H^+ concentrations. The percentage of metal recovered in the protein fraction is related to the H^+ concentration, as measured by pH.

attributed to the putative *Fucus* MT. The sequestered Cd could not be displaced by incubation of the protein with a weak chelating agent (Chelex resin) followed by gel filtration (results not shown). However, incubation of the protein under argon with an excess of Cu(I) led to the displacement of > 95% of the bound Cd and association of the fusion protein with 13 mol equivalents of Cu. The involvement of thiol co-ordination in the binding of the metal ions was confirmed by measuring the UV spectrum, which showed the characteristic energy transfer of S–Cd bonds at 254 nm [33] (Figure 7A). This spectrum is shifted towards 240 nm in the Cu(I)-containing protein (Figure 7B). Production of the apo-protein by acidification removed these features, leaving only minor absorbance at 280 nm due to the aromatic amino acids within the S-peptide (Figure 7C). The stability of the cadmium cluster was investigated further by performing a pH titration (H^+ ions compete out metal ions bound to cysteine residues) to determine the H^+ concentration at which half of the cadmium was lost from the metal ion cluster (Figure 8). The S-peptide–MT fusion exhibited 50% dissociation at approx. pH 4.1, similar to the value estimated for pea MT

Figure 9 Quantification of *F. vesiculosus* MT induction in response to copper

Quantitative amplification of the actin (A) and MT (B) genes from *F. vesiculosus*, using fluorescence-based PCR. Dashed lines show a representation of exponential amplifications for a dilution series of plasmid DNA containing either the MT or the actin sequence (5000, 500, 50, 5 and 0.5 pg of plasmid DNA). Solid lines show the amplification of cDNA samples from control and exposed *Fucus* (2.5 mg/l copper chloride; 72 h), as indicated by arrows. All amplifications were performed in duplicate; only a representative example of the standard quantification is shown for clarity. The insets show computer-generated regressions, allowing extrapolation of the unknown sample concentration over a diluted standard range.

(PsMT_A–glutathione-S-transferase fusion), which showed 50 % dissociation of cadmium at pH 3.95 [34].

Quantification of MT gene expression *in vivo*

A fluorescence-based PCR technique [23] was used to investigate the expression of *Fucus* MT mRNA during copper exposure. By allowing constant monitoring of the formation of PCR products, this technique enables amplification to be related directly to the quantity of starting material present (Figure 9). *F. vesiculosus* was incubated in sterilized seawater over a 72 h period with or without 2.5 mg/l copper chloride. The absolute expression profiles in cDNA samples from the exposed and control specimens were determined in duplicate for MT and also for actin, which was used as an invariable endogenous standard (Figure 9). cDNA was diluted so that the quantity of actin present was constant between the two samples (Figure 9A) prior to measuring MT expression (Figure 9B). An approx. 2-fold increase in MT expression was observed in *F. vesiculosus* acutely exposed to copper over this short time period, demonstrating that the *F. vesiculosus* MT gene is responsive to copper in the environment.

DISCUSSION

This study describes the isolation and characterization of the first MT sequence from marine macroalgae. Features of both invertebrate and plant MTs were identified in the *Fucus* MT, such as a high proportion (19 %) of glycine residues (a characteristic of mussel MTs) [29], and the presence of two cysteine-rich regions containing at least three Cys-Xaa-Cys motifs. These regions show significant identity with the N-terminal domain of vertebrate MTs, suggesting a relationship with class I MTs (see Figure 3).

Fucus MT, like plant and invertebrate MTs, contains an extended linker region (14 amino acids), the implications of

which for the cluster structure have not yet been determined. Protein engineering experiments with vertebrate MTs that placed different-sized linker regions between the two metal–thiolate clusters showed that linkers of over 16 amino acids decreased the ability of the protein to confer metal stability in yeast. However, linkers of 12 amino acids or less did not affect the stability of the molecule [35]. Plant linker regions vary in size up to 40 residues in length and do not prevent metal binding, but have been shown to be hypersensitive to cleavage by proteinases [34,36]. It is intriguing that the *Fucus* MT is not cleaved by endogenous proteinases when expressed in *E. coli* and that the length of the linkers is within the stability limits required for stable expression of recombinant mammalian MTs in yeast [35].

Other similarities of the *Fucus* MT sequence with those of plant MTs include the presence of a Cys-Cys-Xaa-Cys motif, which would classify it as a 'type II' plant MT. The stability of the metal-bound cluster (50 % dissociation at pH 4.1) also shows more similarity with the pea MT (PsMTA–glutathione-S-transferase fusion) (50 % dissociation at pH 3.95 [34]) compared with the human MT (50 % dissociation at pH 2.5).

The *Fucus* protein therefore belongs to a group of proteins for which the precise metallo-structure has not yet been elucidated. Each cysteine-rich region contains fewer cysteines than the N-terminal domain of the mammalian counterpart, which might prevent formation of independent clusters. However, the numbers of bivalent ions (seven) and monovalent ions (13) bound per protein molecule are comparable with those in vertebrate MT proteins. The cleaved portions of the plant MTs are thought not to dissociate, but are held together in an extended single-cluster formation co-ordinated to the bound metal, thus incorporating all of the cysteine residues (Figure 4). However, there is currently no direct structural evidence for the formation of the one- or two-domain cluster in plant MTs.

Laboratory exposure of *F. vesiculosus* to elevated copper levels resulted in induction of the MT gene. The displacement of cadmium by copper under anaerobic conditions also suggests

that the *Fucus* MT is preferentially a copper-binding protein. These results implicate the MT protein in the biological response to copper pollution. The role of MT may be as a 'trafficking' metal chaperone, targeting the metal-bound protein to an intracellular compartment, and/or it may be involved in the long-term storage of metal ions in macroalgae. Macroalgae have been shown to sequester metal in cellular compartments called physodes, which have been shown to contain high concentrations of copper and sulphur [9]. The identity of the chelator responsible for binding the metal is not known, but it may be an MT-like protein. Whether or not this protein is gene-encoded, such as the *Fucus* MT reported in the present paper, or synthesized enzymically, like the phytochelatin isolated previously from macroalgae [22], remains to be established. The biological significance of the linker region is not known, but it has been speculated that cleavage of the linker could allow targeting of the protein to a specific intracellular location [36].

The cellular functions of gene-encoded and enzymically synthesized MTs have not been resolved, except in a limited number of cases. Gene-encoded MTs are the predominant metal-binding proteins induced in response to metal exposure in vertebrate systems. In *Candida glabrata*, both systems are observed working in a metal-ion-specific manner; phytochelatin are synthesized in response to cadmium salts and two MT isoforms are synthesized in response to copper [19]. This contrasts with the situation in another yeast species, *Schizosaccharomyces pombe*, where phytochelatin are synthesized in response to both copper and cadmium [37,38], but there is no evidence that this species synthesizes MTs. It is interesting to note also that *S. pombe* is more sensitive to cadmium than to copper ions, which it tolerates well. However, an MT protein from copper-resistant *S. cerevisiae* has been purified [39]. Our results show that *Fucus vesiculosus* has a functional MT gene, which is induced in response to copper. However, the metal specificity of this response needs to be investigated, along with the importance of phytochelatin in the biological adaptation to metal pollution.

The reason why some species use both systems and others only one is not clear. The functional distinction of gene-encoded and enzymically synthesized MTs may lie in their temporal relationship to metal exposure. Phytochelatin are synthesized within 5–15 min of exposure [20,40,41], suggesting that the enzyme responsible must be constitutively expressed. Such a mechanism allows an immediate response to excess metal, whereas gene induction leading to gene-encoded MT production could be elicited as a long-term response. Therefore it is likely that the macroalgae use both MTs and phytochelatin in the biological response to metal exposure, but in different capacities.

In conclusion, this paper describes for the first time an MT gene identified from macroalgae, which is induced by copper. The identification of gene-encoded MTs in macroalgae raises a myriad of questions concerning both the structures of these unique metalloproteins and their role in conserving cellular metal homeostasis. The isolation of the *Fucus* MT gene and the ability to produce recombinant metalloprotein will enable these important questions to be addressed.

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