Identification, cloning and characterization of a plasma membrane zinc efflux transporter, *TrZnT-1*, from fugu pufferfish (*Takifugu rubripes*)

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An orthologue of the mammalian ZnT-1 (zinc transporter-1) gene was cloned from the intestine of the torafugu pufferfish (*Takifugu rubripes*), demonstrating that this gene predates the evolution of land-living vertebrates. *TrZnT-1* (*T. rubripes* ZnT-1) shares overall topology with other members of the ZnT-1 family of zinc transporters, with six TMs (transmembrane domains) including a large histidine-rich intracellular loop between TM IV and V and intracellular C- and N-termini. Expression of *TrZnT-1* in a metallothionein acquiescent cell line suggested that this protein reduces intracellular Zn²⁺ levels. Manipulation of the transporting media showed that several externally applied hydrominerals had no effect on *TrZnT-1* activity. However, addition of *N*-ethylmaleimide increased *TrZnT-1*-mediated transport, possibly by increasing intracellular free Zn²⁺ levels by Zn²⁺ release from car-

rier proteins. Generation of a specific antibody and subsequent immunocytochemistry on fixed cells overexpressing *TrZnT-1* indicated that the protein is localized to the plasma membrane in these cells. The genomic organization of *TrZnT-1* is the same as that in mammals with two exons. The upstream regulatory region of the *TrZnT-1* gene contains several putative *cis*-acting elements, including metal-response elements and an Sp1 site. Analysis of the DNA contigs surrounding the *TrZnT-1* gene reveal limited synteny between corresponding regions in the rat, mouse and human; however, this was very low, with only two syntenic genes, ZnT-1 and NEK2 (never in mitosis gene A-related kinase).

Key words: fish, fugu pufferfish (*Takifugu rubripes*), slc30A1, zinc, zinc transporter-1 (ZnT-1).

INTRODUCTION

Zinc is an essential micronutrient involved in a wide range of biological processes, including enzyme catalysis, protein structure, protein–protein interactions and protein–oligonucleotide reactions [1–3]. The lack of redox activity facilitates the incorporation of zinc into biological systems without the risk of oxidant damage; therefore zinc is able to perform complex roles in intracellular signalling, reproduction, immunity, neuromodulation, cellular growth and differentiation. The precise mechanisms behind these roles have not yet been fully elucidated. However, the ubiquity and versatility of zinc in cellular metabolism suggest that zinc deficiency may well result in a generalized impairment of many metabolic functions [4,5]. Zinc deficiency is increasingly acknowledged as a widespread problem [5]; on the other hand, zinc toxicity in mammals is rare. Zinc toxicity, however, can be a major problem for aquatic organisms, such as fish, causing mortality, growth retardation, tissue alterations, respiratory and cardiac changes, inhibition of spawning and a multitude of additional detrimental effects [6]. Intestinal uptake of dietary zinc may be implicated in chronic toxicity [7,8]. However, mortality generally occurs due to ionoregulatory disturbances across the gill, resulting in the inhibition of calcium uptake [9,10]. This is caused by the impairment of basolateral transfer of calcium in the gill, owing to blockage of the high-affinity Ca²⁺-ATPase, resulting in hypocalcaemia [10]. Hypocalcaemia seems to be one of the most important physiological manifestations of acute zinc toxicity in fish.

In both mammals and aquatic organisms, the physiological mechanisms of zinc uptake have not been fully elucidated. The

mechanisms for zinc uptake at the molecular level may aid the understanding of how zinc is incorporated and absorbed, leading to an enhanced understanding of zinc homeostasis at the whole-body level. The cloning and characterization of zinc transporters responsible for zinc uptake and extrusion has facilitated these studies in mammals. Members of the ZIP (Zrt-, Irt-related protein) family of zinc transporters are characterized by similar topology, with six to eight TMs (transmembrane domains), extracellular N- and C-termini and an extracellular histidine-rich motif [11,12]. Studies of human ZIP transporters have shown that uptake of zinc is time-, temperature- and concentration-dependent [13,14], with localizations of the protein product that differ, depending on the cell type. Several members of the ZIP family have been identified in fish [15].

Another family of zinc transporters identified is the CDF (cation diffusion facilitator) family, and these are characterized by six TMs, intracellular N- and C-termini and a large cytoplasmic histidine-rich loop between TM IV and V [16]. CDF members include those identified in mammals, yeast and bacteria, and some of them transport metals other than zinc. The first mammalian member of the CDF family to be identified was ZnT-1 (zinc transporter-1), which was cloned by virtue of its ability to confer zinc resistance onto a zinc-sensitive cell line [17]. ZnT-1 is highly homologous with two genes from the CDF family, ZRC1 and COT1 of *Saccharomyces cerevisiae*, which confer resistance against zinc and cobalt respectively [18,19]. ZnT-1 resides on the basolateral membrane of enterocytes, which is consistent with its proposed function as an exporter [17,20].

ZnT-1 mRNA levels have been shown to be directly regulated by zinc. This may be due to the presence of MREs (metal-response

Abbreviations used: CDF, cation diffusion facilitator; CHSE-214, Chinook salmon embryonic 214; HGMP-RC, Human Genome Mapping Project Resource Centre; MEM, minimal essential medium; MRE, metal-response element; MT, metallothionein; MTF-1, metal-responsive element transcription factor-1; NEK2, never in mitosis gene A-related kinase; NEM, *N*-ethylmaleimide; ORF, open reading frame; RT, reverse transcriptase; TM, transmembrane domain; (Tr)ZnT-1, (*Takifugu rubripes*) zinc transporter-1; UTR, untranslated region; ZIP, Zrt-, Irt-related protein.

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elements) in the promoter region and subsequent regulation by the MTF-1 (metal-responsive element transcription factor-1) [17,21]. However, MTF-1 is not essential for ZnT-1 regulation, since homozygous knockout of MTF-1 only attenuates ZnT-1 expression, and does not abrogate it completely [21–23]. Therefore the basal level of the ZnT-1 gene is dependent on transcription factors other than MTF-1.

To date, there has been no reported characterization of members of the CDF family of metal ion transporters in fish. We report here the cloning and characterization of a piscine orthologue of mammalian ZnT-1.

EXPERIMENTAL

Cloning of *TrZnT-1* (*Takifugu rubripes* ZnT-1) cDNA

Mouse ZnT-1 cDNA [kindly given by Professor Richard Palmiter, HHMI (Howard Hughes Medical Institute), University of Washington School of Medicine, Seattle, WA, U.S.A.] was used as a probe to screen a *T. rubripes* intestinal cDNA spotted library [HGMP-RC (Human Genome Mapping Project Resource Centre), Cambridge, U.K.]. The library was screened with [³²P]_i-labelled probe (Prime-a-Gene[®] Labelling System, Promega). Low-stringency hybridization was performed for 16 h at 30 °C in DIG Easy Hyb buffer (Roche). Washes were performed at 55 °C in 2 × SSC [30 mM sodium citrate (pH 7.0)/0.3 M NaCl containing 0.1 % (w/v) SDS] and again in 0.1 × SSC/0.1 % (w/v) SDS. Positive clones were ordered from the HGMP-RC, and plasmid DNA was isolated using the Wizard[®] Plus SV Miniprep DNA purification system (Promega). Sequences were determined on a capillary DNA sequencer (CEQ2000; Beckman).

Homology searches were performed using BLASTX ([24]; see <http://www.ncbi.nlm.nih.gov/blast>). The presence and location of the ORF (open reading frame) was analysed using GeneMark [25]. Alignments and phylogenetic analyses were performed using ClustalW ([26]; see <http://www.ebi.ac.uk/clustalw>), and hydrophobicity was analysed with the topology prediction program TMHMM [27].

Intron–exon structure of the *TrZnT-1* gene was deduced by comparing the cloned cDNA with the genomic sequence obtained from the Ensembl *Fugu* Genome Browser, v27.2d.1 (<http://www.ensembl.org/Fugu>). The actual splice sites were annotated by the NNSSP (Splice Site Prediction by Neural Network; http://www.fruitfly.org/seq_tools/splice.html). Promoter analysis of the *TrZnT-1* gene was performed with NNPP (Neural Network Promoter Prediction; http://www.fruitfly.org/seq_tools/promoter.html) and Web Signal Scan Service ([28]; see <http://bimas.dcrn.nih.gov/molbio/signal>) to identify potential *cis*-regulatory elements in the genomic sequence. The region examined extended 2000 bp upstream of the ATG initiation codon.

Expression of *TrZnT-1* in pufferfish tissues

To examine the tissue distribution of *TrZnT-1*, 10 µg of each total RNA from pufferfish gill, gut, kidney and ovary (purchased from the HGMP-RC, Cambridge) was first treated with 1 unit of DNase I (Promega) and subsequently reverse-transcribed with Powerscript reverse transcriptase (Clontech) and primed with 1 µg of oligo(dT)₁₅ primer (Promega). PCR was then performed using forward 5'-CTGGAAGCCATCGAACGCTTC-3' and reverse 5'-GTCAAGGTAGAGGACCCAGCA-3' PCR primers. Cycle parameters for *TrZnT-1* were 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 52 °C for 40 s and 72 °C for 60 s, followed by a single cycle of 72 °C for 5 min. As a control, a 0.5 kb fragment of β -actin (Ensembl ID: SINFRUG00000081338)

was amplified using forward 5'-ACAGACCTCATGAAGATCCT-3' and reverse 5'-GAGGCCAGGATGGAGCCTCC-3' primers. These are specific for sequences in exons three and five respectively of pufferfish β -actin (β -actin primers were kindly given by Dr M. S. Clark, HGMP-RC, Cambridge). Cycling parameters for β -actin were 95 °C for 5 min, followed by 26 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, followed by a single cycle of 72 °C for 5 min. PCR products were run on a 1.5 % agarose/Tris/borate/EDTA gel, purified using the QIAquick Gel Extraction Kit (Qiagen) and sequenced (as described above).

Cell culture

A Chinook salmon (*Oncorhynchus tshawytscha*) cell line [CHSE-214 (Chinook salmon embryonic 214); purchased from the European Collection of Cell Cultures] was grown in MEM (minimal essential medium) with Earle's salts and L-glutamine (Sigma), supplemented with 10 % fetal bovine serum (Sigma), 1 % non-essential amino acids (Sigma) and 100 µg/ml penicillin/streptomycin (Invitrogen). Cells were cultured in 25 cm³ flasks at 20 °C and 5 % CO₂.

Transfection protocol

Before transfection, cells were seeded on to six-well plates (Corning Inc.) at a density of 5 × 10⁵ cells/ml in complete growth medium and left to grow for 24 h to reach 75 % confluence. *TrZnT-1* was subcloned into a mammalian expression vector, pCI-neo (Promega), and transfections were performed using Lipofectamine[™] reagent (Invitrogen), diluted with MEM according to the manufacturer's recommendation. Samples (1.5 µg) of DNA were used for each transfection with 16 µg of Lipofectamine[™] reagent. Transfection efficiency was controlled for by using the pGL2 control plasmid (1.5 µg) and protein content of cell samples was determined using Bradford reagent (Sigma).

Luciferase studies

Cells were co-transfected with the *TrZnT-1* construct and the pMT-793 construct ([29–31] kindly given by Dr Peter Kille, University of Cardiff, Wales, U.K.) in order to assess relative intracellular zinc levels. At 46 h post-transfection, supplemented medium was aspirated off, and cells were washed with MEM. Differing amounts of zinc (as ZnSO₄) were added to the cells with MEM and left for 6 h. After this period, the zinc-containing medium was aspirated off and the cells were washed twice with phosphate buffer. Cell preparation for luminescence analysis was performed using the Luciferase Assay System with Reporter Lysis buffer (Promega) according to the manufacturer's instructions. Luciferase activity was measured on a 1251 Luminometer (BioOrbit) set to perform a 10 s reading with a 2 s delay. Differences in luciferase activity between *TrZnT-1*-transfected and mock-transfected cells were statistically tested by pairwise comparison at each concentration using the Student's *t* test on log-transformed data.

Efflux studies with ⁶⁵Zn

TrZnT-1-transfected cells were allowed to recover for 24 h before complete medium was aspirated off, cells washed once with MEM and loaded with 100 000 c.p.m. ⁶⁵Zn for 24 h. At 48 h post-transfection, the ⁶⁵Zn-containing MEM was removed and cells were washed once with MEM. Differing amounts of zinc (as ZnSO₄) or NEM (*N*-ethylmaleimide; 1 mM) were added to the medium and after the required time, the medium was aspirated off and cells were prepared for gamma counting. Briefly, cells were washed twice with phosphate buffer, and 10 M NaOH was

added and left to incubate for 10 min at room temperature. Cells were lysed by pipetting, transferred to a microcentrifuge tube and counts were measured in a gamma counter (1282 compugamma; LKB Wallac). Differences in ^{65}Zn efflux between *TrZnT-1*-transfected and mock-transfected cells, or between treatments, were statistically tested by Student's *t* test on transformed data. The overall effect of *TrZn-1* on cellular ^{65}Zn efflux in the presence of different external concentrations of stable zinc isotope was statistically tested. First, raw data of efflux from individual replicates at each external zinc concentration were calculated as percentages of the mean efflux in mock-transfected cells at the corresponding zinc concentration. Student's *t* test was then used on square-root-transformed data of all *TrZnT-1*-transfected cells against all mock-transfected cells.

Generation of polyclonal anti-peptide antiserum

A peptide (ERFTEPHEIESC) encompassing part of the extracellular loop between transmembrane domains IV and V was used as the antigen. The peptide was conjugated to keyhole-limpet haemocyanin, and sera were produced in sheep (performed at the Babraham Institute, Cambridge, U.K.).

Purification of anti-peptide antibody

Purification of anti-*TrZnT-1* antibody was performed as described previously [33]. Briefly, the antigen-affinity gel was prepared by coupling the antigenic peptide to ω -aminohexyl-Sepharose 4B using *N*- γ -maleimidobutyryloxysuccinimide ester (Sigma, U.K.). The immunoglobulin fraction of the antiserum (10 ml) was first precipitated with 50 % ammonium sulphate, re-dissolved in 100 mM sodium phosphate buffer, pH 7.5 (10 ml), dialysed against 40 mM phosphate buffer, pH 8, and mixed with the antigen-affinity gel (5 ml) for 2 h at room temperature. The gel was transferred to a column, washed with cold TBS, pH 7.5, and 1 ml fractions were collected on ice until the absorbance at 280 nm fell to 0.05. After washing with 20 ml of cold distilled water, bound antibodies were eluted with 100 mM glycine/HCl, pH 2.5. The pH of the eluted fractions was adjusted with 20 μl of 1 M Tris/HCl, pH 8. The column fractions were analysed for antibody activity by direct ELISA using gelatin linked to the *TrZnT-1* peptide as the solid-phase antigen. Fractions showing antibody activity were pooled and dialysed against PBS. Several purifications were performed as described, and the resulting purified antibody fractions pooled.

Preparation of cell lysates

Approx. 10^7 cells (both *TrZnT-1*- and mock-transfected) were lysed by the addition of 1 ml of ice-cold lysis buffer [50 mM Tris/HCl (pH 8.0)/150 mM NaCl/1 % (v/v) Triton X-100] containing protease-inhibitor cocktail (500 ng/ml PMSF, 10 ng/ml aprotinin, 10 ng/ml leupeptin and 10 ng/ml pepstatin). Cell lysates were kept on ice for 30 min with occasional mixing, before being centrifuged at 4 °C at 10 000 *g*. The supernatant was removed and kept at -80 °C for further use. Protein content of cell lysates was measured using Bradford Reagent (as above).

SDS/PAGE and Western blotting

The cell lysates were analysed by SDS/PAGE and Western blotting. SDS/PAGE with 12 % polyacrylamide gels, transfer to nitrocellulose membranes and silver staining were performed using standard procedures [34,35]. The membranes were blocked in TBS-T buffer [0.1 % (v/v) Tween 20] containing 5 % milk, incubated with sheep anti-*TrZnT-1* antibody (1:250 dilution) and donkey anti-sheep horseradish peroxidase conjugate (1:2000 dilu-

tion), followed by detection using enhanced chemiluminescence [GE Healthcare (formerly Amersham Biosciences), U.K.].

Immunocytochemistry

TrZnT-1-transfected CHSE-214 cells were grown on coverslips, medium was aspirated off and the cells were washed twice with PBS. Cells were fixed for 3 min with 3.7 % formaldehyde. This was removed and cells were washed twice with PBS. Blocking was performed with 1 % normal donkey serum for 15 min at room temperature. Sheep anti-*TrZnT-1* antibody was added (1:50 dilution) and left in a humidified chamber for 90 min. Washes were performed with TBS-T buffer (as described above) and cells were subjected to secondary antibody incubation with goat anti-sheep FITC conjugate (1:250 dilution) for 45 min in a humidified chamber. Washes were performed as above and cells mounted with 50 % glycerol. Fluorescence was viewed under an inverted microscope (Eclipse TE200, Nikon) utilizing a digital camera (DXM1200, Nikon) and associated software (ACT-1; Nikon) to capture images.

Statistics

Differences between *TrZnT-1*- and mock-transfected cells were assessed statistically using the Student's *t* test. For the experiment exploring ^{65}Zn elimination from cells, *t* test analysis was performed on square-root-transformed data expressed as percentages of ^{65}Zn counts in mock-transfected control cells.

RESULTS

Isolation of a *T. rubripes* ZnT-1 clone, *TrZnT-1*

A *T. rubripes* intestinal cDNA library was screened with mouse ZnT-1 and one clone was isolated and sequenced. An uninterrupted ORF was found to encode a protein of 485 amino acids with a calculated molecular mass of 53 kDa. *TrZnT-1* displays the typical conserved structural properties of the ZnT family. There are six putative TMs (Figure 1, shaded in grey) and a large histidine-rich intracellular loop in between TMs IV and V. Homology searches using BLASTX identified this clone as the *Takifugu* homologue of ZnT-1 with 57 % identity (69 % positives) to mouse ZnT-1. Indeed, multiple sequence alignment suggested that the deduced *TrZnT-1* amino acid sequence shares about 50 % overall identity with other known members of the CDF family, with the putative TMs having the highest similarity (Figure 1). When depicted as a phylogenetic tree, it appears that *TrZnT-1* clusters together with ZnT-1 orthologues found in mammals (Figure 2). This lends further credence that the *Takifugu* clone is indeed a ZnT-1 orthologue.

Genomic organization of *TrZnT-1*

The gene structure of *TrZnT-1* was analysed by comparing its DNA sequence with the corresponding genomic contig on scaffold 230 in the MRC (Medical Research Council) HGMP-RC *Takifugu* database, version 27.2d.1. From this comparison, the intron-exon structure was revealed. The *TrZnT-1* gene, in common with the mouse ZnT-1 gene, has two exons. As expected, the *TrZnT-1* intron is smaller than that of mouse.

Analysis of scaffold 230 revealed the presence of 34 genes, only 13 of which returned high-scoring matches against the NCBI database. Analysis of the genes flanking *TrZnT-1* in *Takifugu* showed that the best matches to the surrounding genes were NEK2 (never in mitosis gene A-related kinase), rhesus-blood-group-associated glycoprotein and three other genes with no known homologues. Analysis of human, mouse and rat genes showed that

Figure 1 ClustalW alignment of *TrZnT-1* and other members of the *ZnT-1* family in mammals and yeast

Putative TMs are shaded grey. Conserved histidine residues are shaded black. Asterisks indicate exact amino acid matches. Dots indicate similar amino acids. Accession numbers for human, rat and mouse *ZnT-1* are Q9Y6M5, Q62720 and Q60738 respectively; ZRC1, P20107; COT1, P32798.

only *NEK2* has an orthologue in proximity to *ZnT-1*. In humans, *ZnT-1* and *NEK2* are located on chromosome 1 on 1q32–41 and 1q32.2–41 respectively. In the mouse, both genes are also located on chromosome 1, whereas in rat they are located on 13q27. It appears that some degree of synteny is conserved between all four species.

Promoter analysis of the *TrZnT-1* gene

The 5' upstream region of the ORF of the *TrZnT-1* gene was identified and analysed for regulatory elements. There is a potentially strong promoter, containing a typical TATA cassette, upstream of the 5'-UTR (untranslated region). Further upstream of the putative promoter, several *cis*-regulatory elements were identified (Figure 3). Four consensus MREs were identified, along with one Sp1 recognition element upstream from the MREs. MREs are binding sites for MTF-1, which is present and functional in Japanese pufferfish [36]. The *TrZnT-1* gene contains a CpG island at the 5' end, which covers the putative promoter region.

Expression of *TrZnT-1* in pufferfish tissues

Northern blot analysis of *TrZnT-1* expression in the gill, gut, kidney and ovary did not yield clear results, possibly because *TrZnT-1* is expressed at low levels (results not shown). However, RT (reverse transcriptase)–PCR analysis showed that *TrZnT-1* is expressed in all tissues examined, with the highest expression in the gut and ovary after β -actin normalization (Figure 4).

Functional analysis

The ability of *TrZnT-1* to lower loosely bound intracellular zinc levels was assessed by expressing *TrZnT-1* driven by a strong CMV promoter in CHSE-214 cells. This cell type was chosen

as its MT (metallothionein) genes are quiescent [37,38] and they therefore have a lower capacity to buffer intracellular zinc concentrations.

The promoter region of the MT-A gene has been studied extensively, and functional characterization of this region has shown that there are six MREs approx. 800 bp from the transcription start site [32]. One construct, pMT-793, includes 793 bp of the 5'-flanking region fused to a luciferase reporter gene. It has only the six MREs present, and other *cis*-acting elements, such as AP-1 (activator protein-1) and NF-IL6 (nuclear factor κ B–interleukin 6) sites that are present further upstream of the MREs, have been removed [32].

Cells co-transfected with *TrZnT-1* and the pMT-793 construct showed low luciferase activity at all extracellular zinc levels tested (Figure 5). This was in contrast with control cells co-transfected with the expression plasmid without *TrZnT-1* and pMT-793, which showed statistically higher luciferase activity at all zinc concentrations. Luciferase activity in *TrZnT-1*- and pMT-793-co-transfected cells increased steadily as the zinc concentration increased, but did not reach a peak or attain a plateau. The control cells, however, showed a luciferase activity peak at 60 μ M zinc, but this decreased markedly at 120 μ M zinc.

The ability of *TrZnT-1* cells to efflux zinc was measured using ^{65}Zn . Efflux was measured as the amount of ^{65}Zn remaining in ^{65}Zn -loaded cells after treatment with extracellular zinc. *TrZnT-1*-transfected cells showed higher efflux than mock-transfected (expression plasmid without *TrZnT-1*) cells, although the pattern of efflux was almost identical between cells overexpressing *TrZnT-1* and those that had been mock-transfected (Figure 6). With an increased concentration of cold zinc in the external medium, there appeared to be an exponential decline in the ^{65}Zn remaining in the cells 6 h after the start of the efflux experiment (Figure 6).

Figure 2 Phylogenetic tree of the eukaryotic members of the cation diffusion facilitator family of metal ion transporters

The branch length as indicated (in arbitrary units) is proportional to phylogenetic distance. The phylogenetic tree was constructed using ClustalW. Accession numbers are as follows: rat ZnT-2, Q62941; mouse ZnT-3, P97441; human, rat and mouse ZnT-4, Q14863, Q55174 and Q35149 respectively; human and mouse ZnT-5, AAL96437 and NP075023 respectively; mouse ZnT-6, AAM27917; human and mouse ZnT-7, AA017324 and AA017323 respectively; human ZnT-8, NP776250. Accession numbers for human, mouse and rat ZnT-1, and ZRC1 and COT1 are as described in the legend for Figure 1.

The effects of addition and depletion of various ions to the medium were assessed. The addition and depletion of calcium, magnesium, chloride, sodium and potassium ions showed no significant effects on efflux in either *TrZnT-1*-overexpressing cells or those of the control (results not shown). However, the addition of NEM to the extracellular medium had a stimulatory effect on zinc efflux (Figure 7). NEM appeared to statistically increase zinc efflux compared with control levels in both *TrZnT-1*-transfected and mock-transfected CHSE-214 cells. This increase was more pronounced in *TrZnT-1*-transfected cells, with an apparent increase of 50%; the increase was marginally less in mock-transfected cells (approx. 33%).

Localization of *TrZnT-1* in CHSE-214 cells

In order to ascertain the localization of *TrZnT-1* in CHSE-214 cells, a polyclonal antibody was produced against a peptide portion of the extracellular loop between TMs IV and V. The antibody

was affinity-purified, and immunoreactivity and specificity of the anti-*TrZnT-1* antibody to the peptide antigen was verified by ELISA. Western blot analysis was performed on cell extracts from both mock-transfected and *TrZnT-1*-transfected cells (Figure 8A). There appears to be a band of the predicted size (53 kDa) seen in both extracts from mock-transfected and *TrZnT-1*-transfected cells. The density of this band, however, was higher in *TrZnT-1*-transfected cells than in controls.

The hypothesized role of *TrZnT-1* as a zinc exporter suggests that this protein is found in the plasma membrane. To examine the localization of *TrZnT-1* within the cell, immunocytochemistry was performed. *TrZnT-1*-transfected cells were incubated with the anti-*TrZnT-1* antibody and then with an FITC-labelled conjugate. Results show that *TrZnT-1* is localized to the plasma membrane in CHSE-214 cells (Figure 8B), as ascertained from the dense fluorescence seen around the periphery of the cell.

DISCUSSION

Takifugu ZnT-1

The ZnT-1 family of zinc transporters has been found to lower intracellular zinc concentrations by efflux or transport into intracellular vesicles. A *T. rubripes* intestinal cDNA library was screened in order to identify a ZnT-1 homologue in fish. One positive clone was found to have high homology with rat and mouse ZnT-1, as well as with yeast members of the CDF family of heavy-metal-ion transporters.

This clone was sequenced, and has a nucleotide length of 3135 bp that codes for a protein of 485 amino acids. The 3' region of *Takifugu* ZnT-1 appears to be very long. This long 3'-UTR sequence is a consistent feature of mammalian ZnT-1, with rat, mouse and gerbil ZnT-1 cDNAs having a long 3' UTR [18,39].

Further analysis of the cDNA sequence revealed that, in common with other members of the CDF family, *TrZnT-1* has six TMs, and there seems to be the greatest degree of homology amongst the putative TMs. Between TMs IV and V, there is a large histidine-rich loop that is thought to play a role in zinc binding, since histidine is an amino acid that binds zinc ions strongly. Additional examination of this region revealed that the *TrZnT-1* histidine-rich loop is shorter than that of the rat, mouse, human and trout, and may denote that there are specific histidine residues that are sufficient for zinc binding. Specifically, there are four histidine residues that are conserved in mammalian, fugu and yeast orthologues present just after TM IV. The most variable regions are those in between the TMs. The loop in between TMs IV and V seems to have the highest variability among all the members of the CDF family examined here, indicating that the length, and not the exact sequence, is important for function, since in each protein this loop is large.

Phylogenetic analysis also showed that *TrZnT-1* is closely related to the members of the mammalian ZnT-1 family, as well as members found in yeast. Structural similarity between *TrZnT-1* and other known homologues of ZnT-1 may indicate that the *Takifugu* homologue has a similar function to that of other characterized ZnT-1 proteins.

Genomic organization of the *TrZnT-1* gene

The *Takifugu* ZnT-1 gene covers 2525 bp from the first to the last amino acid. The gene contains two exons, which encode the six TMs. This is consistent with the structure of the mouse ZnT-1 gene, which also has two exons. The position of the intron is almost the same in mouse as it is in *Takifugu*, although as expected, the *Takifugu* intron is shorter. There is a TATAAA sequence

Figure 3 Promoter region of the *TrZnT-1* gene

Regulatory elements, Sp1 and MREs, are indicated, with the orientation denoted by (–) for the minus strand and (+) for the plus strand. The putative TATA box is underlined, and the nucleotide section shaded grey represents the CpG island.

Figure 4 Expression distribution of *TrZnT-1* using RT-PCR

Representative PCR of *TrZnT-1* expression in pufferfish gill, gut, kidney and ovary. RNA was obtained from the HGMP-RC and represents RNA from several individuals. Upper panel: *ZnT-1* RT-PCR (annealing temperature 55 °C; 26 cycles). Lower panel: β -actin was used as an indication of mRNA amount and to normalize data (annealing temperature 55 °C; 26 cycles).

Figure 5 Relative luciferase activity of CHSE-214 cells transfected with *TrZnT-1* (□) or control (△) after 6 h of treatment with extracellular zinc (5, 20, 60 and 120 μ M ZnSO_4)

Luciferase activity is given as relative luminescence standardized for transfection efficiency by measurements with the control plasmid, pGL-2, and the protein amount (μ g of protein/ml). Results are presented as means $\pm$ S.E.M. ($n = 3$). Asterisks indicate a significant difference from mock-transfected cells based on the Student's t test (* $P < 0.05$).

located approx. 270 bp upstream of the ORF that may serve as a ‘TATA’ box. Regulatory elements such as Sp1 recognition sites and MREs were also found.

Figure 6 ^{65}Zn efflux from CHSE-214 cells after treatment with extracellular zinc (5, 20, 60, 120 μ M ZnSO_4)

^{65}Zn remaining in cells after treatment with extracellular zinc after 6 h. *TrZnT-1*-transfected cells are shown by open squares; mock-transfected cells are shown by open triangles. Results are presented as means $\pm$ S.E.M. ($n = 3$). There was significantly ($P < 0.01$) less ^{65}Zn left in cells transfected with *TrZnT-1* compared with that in mock-transfected cells.

Figure 7 Effects of 1 mM NEM on zinc efflux in CHSE-214 cells

The control condition is given as the percentage of ^{65}Zn left in *TrZnT-1* and mock-transfected cells respectively per μ g of protein per ml after no treatment. Results are presented as means $\pm$ S.E.M. ($n = 3$). Asterisks indicate significant difference from control (no NEM treatment) of either *TrZnT-1*-transfected or mock-transfected cells, based on the Student's t test (* $P < 0.05$).

Figure 8 Western blot of lysates prepared from *TrZnT-1*-transfected (lane 1) and mock-transfected CHSE-214 cells (lane 2) (A), and *TrZnT-1* fluorescence in CHSE-214 cells overexpressing *TrZnT-1* following fixation and staining with anti-*TrZnT-1* polyclonal antibody (B)

(A) The blotted membrane (25 μ g of loaded cell lysate) was probed with anti-*TrZnT-1* antibody and immunoreactivity was visualized using enhanced chemiluminescence. Molecular-mass markers are indicated on the left of the gel. (B) The top panel shows *TrZnT-1* localization with FITC-labelled secondary antibody; the bottom panel presents cells as shown under a light microscope ($\times 40$ magnification).

Mouse ZnT-1 has two MREs located approx. 100 bp upstream of the promoter region. *TrZnT-1* contains four consensus MRE-binding sites approx. 1 kb upstream of the promoter. Multiple MREs function synergistically to confer resistance to zinc, cadmium and oxidative stress in transfected mammalian cells [40,41], and it is thought that two or more MREs are required for a promoter to exhibit significant metal responsiveness. Neither the spacing nor the orientation of MREs in these promoters appears to be critical for function. Metal responsiveness is dependent on the MRE core consensus sequence, TGRNC [40,41]. The *TrZnT-1* gene may contain more MREs because fish are required to respond more quickly to toxic zinc insult than mammals, where zinc is rarely toxic. The functionality of the MRE in the *TrZnT-1* gene has not been investigated, but mRNA for the rainbow trout ZnT-1 homologue increased in abundance in gill tissue following elevation of the zinc concentration in the water [42]. There is one Sp1 recognition element upstream of the MREs. The 5'-upstream region consists of a CpG island. CpG islands are found to represent promoter regions of housekeeping genes, as well as some other ones [43]. In these, Sp1 seems to contribute to maintaining CpG islands free of methylation [44,45]. The 5' promoter region of the mouse ZnT-1 gene also consists of a CpG island, with a GC content of 72 % [18].

Conservation of synteny means that orthologous gene loci are linked in two species, regardless of gene order or the presence of intervening genes. When evolutionary distance is large, scrambling of gene order and the presence of non-syntenic intervening genes become frequent. A conservation of gene synteny between *Takifugu*, mouse, rat and human would bring further advantages in terms of structural analysis of continuous gene clusters. In *Takifugu*, the gene upstream of and adjacent to *TrZnT-1* is *NEK2*. *NEK2* plays a vital role in cell cycle progression,

particularly with respect to the centrosome cycle (for a review, see [46]). Although in the human, mouse and rat, *NEK2* is not situated directly adjacent to ZnT-1, both genes are on the same segment of the same chromosome. It has been noted previously that the short-range gene order is conserved between the *Takifugu* and human genomes in many, but not all loci [47]. However, analysis of the 13 annotated genes present on the scaffold that contained *TrZnT-1* showed that only *NEK2* and ZnT-1 were present in close proximity in any of the other species examined.

Tissue specificity of *TrZnT-1*

The expression of *TrZnT-1* mRNA in *Takifugu* varied among different tissues. Highest amounts of *TrZnT-1* mRNA were seen in the ovary and gut, with the smallest amount in the gill and kidney. A high level of *TrZnT-1* mRNA in the gut is not surprising, since the gastrointestinal tract is the primary site for zinc uptake and reabsorption. This is probably why only low levels of *TrZnT-1* are expressed in the gills, which is an auxiliary pathway for zinc uptake. Low amounts in the kidney may be expected, since *T. rubripes* is a marine organism and excretes only very small amounts of urine. Many marine fish rely on renal secretion of excreta, rather than filtration. If this is true for pufferfish, there would be little need to reabsorb zinc. Expression of rat ZnT-1 in different tissues [48] showed that rat ZnT-1 levels were highest in the kidney, small intestine and placenta. This shows that relative distribution of mammalian ZnT-1 mRNAs in other tissues is similar to that of its piscine orthologue.

TrZnT-1 decreases weakly bound cytoplasmic zinc levels

TrZnT-1 appears to decrease the weakly bound cytoplasmic zinc level in CHSE-214 cells. This was demonstrated by the use of a zinc-sensitive promoter fused to a luciferase reporter gene [32]. Results from these experiments indicated that cells overexpressing *TrZnT-1* produced much lower luciferase activity than those that had been mock-transfected. Owing to the presence of six MREs in this reporter plasmid, and previous reports [32] showing that it is very sensitive to cellular zinc concentrations, this reporter gene is a very good indicator of intracellular labile zinc. It is assumed that the higher the luciferase activity, the higher the intracellular concentration of labile zinc in cells.

The pattern of luciferase activity in *TrZnT-1*-transfected cells shows a steady increase as the zinc concentration increases. This implies that, as the extracellular zinc concentration increases, the intracellular labile zinc concentration increases accordingly. However, this increase is only marginal and it appears that, at 120 μ M ZnSO₄, *TrZnT-1*-transfected cells are still capable of maintaining low intracellular zinc concentrations. In contrast, relative luciferase activity in mock-transfected cells was very high, reaching a peak at 60 μ M. This is surprising, since it would have been thought that, at 120 μ M, luciferase activity would be at its peak, owing to high levels of cytoplasmic zinc. It may be that this effect is one of toxicity. However, work investigating the effects of zinc exposure on CHSE-214 and the activation of the MT-A promoter [33] found that maximal luciferase activity was achieved at 125 μ M zinc, and that a 50 % decrease in luciferase activity occurred at a concentration of 2.9 mM zinc in the medium. These findings do not coincide with those in the present study. Perhaps the decline in luciferase activity observed with a medium concentration of 120 μ M zinc presented here may be due to the presence of other zinc-binding entities, such as GSH, being up-regulated, or endogenous zinc transporters being activated, thereby reducing the level of weakly bound cytoplasmic zinc. In mammalian studies of ZnT-1 [18], it was shown that zinc-sensitive

cells overexpressing rat ZnT-1 could withstand elevated medium zinc levels of up to 200 μM , whereas medium zinc levels of 50 μM were toxic to those cells not expressing rat ZnT-1.

The assessment of efflux was made via ^{65}Zn remaining in cells after the addition of extracellular cold zinc. Six hours after the addition of various concentrations of extracellular zinc (5, 20, 60 and 120 μM), cellular ^{65}Zn amounts were measured. In *TrZnT-1*-transfected cells, there was less ^{65}Zn than in the mock-transfected cells, indicating that the former were able to remove more ^{65}Zn than the latter. This was the case at all extracellular zinc concentrations added. The pattern of extrusion both for cells overexpressing *TrZnT-1* and for mock-transfected cells is identical, indicating an endogenous export mechanism similar to that of *TrZnT-1* in CHSE-214 cells. The ^{65}Zn retained in the cells after the 6 h efflux period decreased with increasing zinc concentrations up to 60 μM , above which there was no further decline. The concentration-dependent increase in ^{65}Zn elimination could be interpreted as an activation of zinc-efflux system(s) in the face of elevated external zinc levels or increased zinc influx. The fact that there was no further increase in elimination of ^{65}Zn when external zinc concentrations of 60–120 μM were added might be the result of dilution of the cellular ^{65}Zn with unlabelled zinc from the medium.

Extracellular ions do not affect zinc efflux, but NEM does

In agreement with a study on mouse ZnT-1 [18], addition of hydromineral to, or depletion of hydromineral from, the extracellular medium does not appear to affect *TrZnT-1*-mediated efflux. None of the ions tested in this study had effects on ^{65}Zn export that were statistically significant. ^{65}Zn export in mock-transfected cells was also not affected by the modifications of the concentrations of anions and cations in the extracellular medium.

The addition of NEM to the extracellular medium increased zinc efflux from both *TrZnT-1*- and mock-transfected CHSE-214 cells by 50 % and 33 % respectively. NEM is a highly reactive thiol reagent, binding to any thiol group when the pH is within the range of pH 6–10, although it is more reactive at pH 7.5–9.5. NEM is able to cross cell membranes, and it is likely that NEM binds to and disrupts the co-ordination of zinc with zinc-binding proteins, resulting in increased, intracellular weakly bound zinc levels. The effect on intracellular zinc release of NEM has been documented previously [49], where increased export of cellular zinc was shown both by decreased intracellular zinc-specific fluorescence and increased extracellular zinc-specific fluorescence.

TrZnT-1 protein is expressed in CHSE-214 cells

In a study by Cousins and McMahon [50], after screening a wide range of cells in culture, none were found to express detectable levels of ZnT-1 protein. We were unable to detect ZnT-1 mRNA in CHSE-214 cells by Northern blot analysis. However, Western blot analysis showed that the CHSE-214 cell line contained endogenous ZnT-1 protein. In support of this finding, transport experiments using ^{65}Zn showed that the pattern of extrusion of mock-transfected cells was the same as that of cells overexpressing *TrZnT-1*. The ability of CHSE-214 cells to withstand elevated zinc levels, even though they express no endogenous MT [37,38], may be due to the expression of endogenous ZnT-1 protein, which enables the extrusion of toxic zinc, thereby preventing zinc toxicity. High expression of *TrZnT-1* protein derived from transfected cells demonstrates that, once transfected, *TrZnT-1* can be translated successfully, and this improved the ability of these cells to extrude zinc, as shown in the functionality assays.

TrZnT-1 is localized to the plasma membrane

Immunocytochemistry experiments using the anti-*TrZnT-1* antibody on fixed transfected CHSE-214 cells revealed a dense fluorescence around the plasma membrane. This location for *TrZnT-1* is consistent with a role of *TrZnT-1* as a zinc exporter protein, extruding zinc from the cytoplasm. One caveat to this approach is that the protein is overexpressed, and may be directed incorrectly towards the plasma membrane, whereas the distribution of the protein when expressed at normal levels may be different. However, previous studies of the localization of ZnT-1 in rats have shown that, in enterocytes and renal tubular cells, it is localized to the basolateral membrane [21,50]. This localization of rat ZnT-1 in enterocytes would ensure zinc delivery to the circulation, and the localization of rat ZnT-1 in the kidney indicates a role in zinc reabsorption, not delivery into urine.

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