Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 68 (2004) 699–709
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Differential regulation of zinc efflux transporters ZnT-1, ZnT-5 and ZnT-7 gene expression by zinc levels: a real-time RT–PCR study

Séverine Devergnas[a], Fabrice Chimienti[a], Nadège Naud[b], André Pennequin[b], Yoann Coquerel[c], Jocelyne Chantegrel[a], Alain Favier[a], Michel Seve[a,*]

[a]*Laboratoire des lésions des acides nucléiques, DRFMC/SCIB/LAN, CEA/Grenoble, 17, rue des Martyrs, 38000 Grenoble, France*
[b]*Département de radiobiologie et de radiopathologie, CRSSA, 38700 la Tronche, France*
[c]*Laboratoire RéSO, UMR CNRS SYMBIO, Centre Universitaire de St Jérôme, 13000 Marseille, France*

Received 20 January 2004; accepted 4 May 2004

Abstract

Intracellular zinc levels are strictly regulated by zinc channels and zinc-binding proteins to maintain cellular zinc-dependent functions. We demonstrated a correlation between extracellular zinc concentration and intracellular exchangeable zinc levels using the fluorescent zinc-specific probes zinquin and zinpyr-1. The effect of extracellular zinc status on the regulation of the two *trans*-Golgi network directed zinc transporters ZnT-5 and ZnT-7 was next studied by real-time RT–PCR in zinc supplemented or depleted HeLa cells. While sub-toxic extracellular zinc addition strongly induced the efflux transporter ZnT-1 gene expression, consistent with its activation by the transcription factor MTF-1, treated HeLa cells did not display any change in ZnT-5 and ZnT-7 mRNA levels compared to control cells. In contrast, zinc depletion induced by non-toxic doses of the zinc chelator TPEN (*N*,*N*,*N*′,*N*′ tetrakis-(2 pyridylmethyl) ethylene diamine) resulted in a up to eight-fold induction of transporters ZnT-5 and ZnT-7 mRNA levels, providing the first evidence of a transcriptional control of these two zinc efflux transporters by zinc deficiency in cultured cells.

Keywords: ZnT; Zinc transporter; Zinc chelation; Gene expression; Real-time PCR

1. Introduction

Zinc, an essential trace element, is involved in many cellular processes as a cofactor of enzymes, nuclear factors and hormones and, hence, is a very important component of cell viability [1–4]. Intracellular zinc concentration is correlated to cell fate, i.e. proliferation, differentiation or apoptosis [5,6], and modifications of zinc homeostasis are linked to several pathologies affecting humans at any stage of life [7–9]. This homeostasis results from a coordinated regulation by different proteins involved in uptake, excretion and intracellular storage/trafficking of zinc [10]. These proteins are transmembrane transporters, belonging to the ZIP and ZnT families, and metallothioneins. Zip proteins are involved in cellular uptake of zinc. They were first discovered in plants and yeast and several members were later described in mouse and human [11]. ZnT proteins are responsible for the extrusion of zinc outside the cytoplasm, i.e. in the extracellular space or intracellular organelles. They belong to the CDF family (cation diffusion facilitator) and most of them share six membrane-spanning domains and a histidine-rich intracellular loop. In human cells, the seven homologous zinc export proteins which have been identified (ZnT-1 to ZnT-7) belong to the *SLC30* gene family. ZnT-1, a ubiquitous zinc transporter located in the plasma membrane, ensures zinc efflux from the cell [12]. ZnT-2 equally confers zinc resistance, although it is located in acidic endosomal/lysosomal vesicles and allows vesicular zinc accumulation inside the cell [13]. ZnT-3 and ZnT-4 have quite the same functions as ZnT-2. ZnT-3 is tissue specific and mainly located in brain, in the membranes of zinc-rich synaptic vesicles within mossy fiber boutons of hippocampus [14], and in testis [15]. ZnT-4 is largely expressed [16], but higher levels of ZnT-4 are found

Abbreviations: CDF, cation diffusion facilitator; MRE, metal response elements; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide; PBS, phosphate buffered saline; TPEN, *N*,*N*,*N*′,*N*′ tetrakis-(2 pyridylmethyl) ethylene diamine

* Corresponding author. Tel.: +33 4 38 78 21 59; fax: +33 4 38 78 50 90.
E-mail address: seve@drfmc.ceng.cea.fr (M. Seve).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.05.024

in brain and epithelial cells. This transporter has been shown to be essential in mammary epithelia for regulating milk zinc content [17]. ZnT-5 is also widely expressed, but more abundantly in pancreatic beta cells, and localized in intracellular non-acidotropic vesicles [18]. Mice deficient for this gene show osteopenia and male-specific sudden cardiac death [19], suggesting a role in maturation of osteoblasts and in maintenance of the cells involved in the cardiac conduction system for this transporter. Recently, ZnT-6 has been described as a protein responsible for the relocation of cytoplasmic zinc into the *trans*-Golgi network and the vesicular compartment [20], while ZnT-7 is involved in zinc transport from the cytoplasm into the Golgi apparatus [21].

The controlled expression at the transcriptional level of these ZnT proteins regulates, at least in part, cellular zinc homeostasis. Experiments in rats demonstrated the influence of zinc deficiency or supplementation on ZnTs zinc transporters mRNA expression levels [16,22]. In rats fed a high zinc level, intestinal ZnT-1 and ZnT-2 mRNA expression levels were increased [23]. A same ZnT-1 induction was obtained in the LNCaP and PC-3 human prostate cell lines treated by zinc [24]. The induction of ZnT-1 gene transcription was explained by the binding of the metal-specific transcription factor MTF-1 on two metal response elements (MRE) in ZnT-1 promoter [25]. Conversely, ZnT-4 expression in intestine was not influenced by dietary zinc intake [16], but in rats fed a low zinc diet, a higher ZnT-4 mRNA and protein expression was detected in mammary gland [26].

However, the control of ZnT-5 and ZnT-7 gene expression by zinc ions, which appears to be of significant importance for zinc delivery to the Golgi apparatus, has not been investigated yet. Herein are presented the results of our study on the influence of extracellular zinc concentration (from depletion to supplementation) on cell viability and intracellular exchangeable zinc levels, together with a real-time RT–PCR study of the transcriptional response of ZnT-1, ZnT-5 and ZnT-7. In contrast with the results obtained with the MTF-1 driven zinc transporter ZnT-1, which was confirmed to be induced by zinc ions, we showed that ZnT-5 and ZnT-7 gene expression did not respond to zinc supplementation, while they are strongly up-regulated by zinc depletion in HeLa cells. Up-regulation of zinc transporters by zinc deficiency may be induced by a zinc-responsive transcription factor yet to be identified.

2. Material and methods

2.1. Chemicals

All chemicals were reagent grade from Sigma or Merck. Zinquin ethyl ester was purchased from TRC.

2.2. Zinpyr-1 synthesis

Zinpyr-1 was prepared in high yield (84%) by slight modification of the reported procedure [27]. The crude product obtained according to the described procedure (scaled up by four times) was thoroughly washed five times with hot ethanol, then twice with cold water and dried under vacuum to afford homogeneous zinpyr-1, as proven by analytical data (not shown). Due to significant decomposition of the material upon silica gel chromatography, this modified procedure should be preferred for multi-gram scale synthesis.

2.3. Determination of zinc concentration

Total zinc concentrations in both the control and supplemented media were determined by flame atomic absorption spectroscopy (Perkin-Elmer). Cell culture medium zinc concentration was 6.0 ± 0.5 μM. For $[Zn(II)]_{free}$ determination, the fluorescence of solutions of 1 μM Zynpir-1, 25 μM TPEN (*N*,*N*,*N*′,*N*′ tetrakis-(2 pyridylmethyl) ethylene diamine) and different concentrations of zinc in cell culture medium or buffer (50 mM Tris–HCl, pH 7.4, 0.1 M NaCl) was measured with a Perkin-Elmer LS50B spectrofluorimeter. The parameters were: excitation wavelength = 507 nm with 5 nm slit width, emission wavelength = 530 nm with 5 nm slit width and a 515 nm cut-off filter. $[Zn(II)]_{free}$, $[TPEN]_{free}$ and [Zn-TPEN] were calculated with the program MINEQL+ (Environmental Research Software).

2.4. Cell culture methods

HeLa epithelial cells (ATCC number CCL-2) were grown in Opti-MEM medium (Modified Eagle's Medium, Invitrogen) supplemented with 5% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37 °C in a 5% humidified CO_2-enriched atmosphere and routinely splitted at a 1:5 ratio.

2.5. Cell viability assay

Cellular viability in the presence or absence of experimental agents was determined using the Mosmans's MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide; Sigma) assay. This assay is based on the ability of living cells to convert dissolved MTT to insoluble formazan [28]. Briefly, following experimental treatment (zinc sulphate or TPEN), 10 μL of a 5 mg/mL MTT solution were added in each well, and the plate was incubated in the dark for 3 h at 37 °C. The medium was then removed, and the coloured reaction product was solubilized in 100 μL DMSO. Absorbance was measured at 570 nm using a V_{max} plate reader (Labsystems Multiskan RC). The percentage viability was calculated as follows:

percentage specific viability $= [(A - B)/(C - B)] \times 100$ where $A = OD_{570}$ of the treated sample, $B = OD_{570}$ of the medium, and $C = OD_{570}$ of the control (phosphate buffer saline (PBS)-treated cells). The values were expressed as percentage viability relative to vehicle-treated control cultures.

2.6. Intracellular zinc staining by zinquin and zinpyr-1

Cells were grown in Lab-tek II chambered cover glass systems (Nunc) and incubated with either 50 μM zinc, or 5.67 μM TPEN for 2 h. After washing with PBS, cells were stained with the zinc-specific fluorescent probes zinquin ethyl ester or zinpyr-1 as previously described [6,27]. Cells in PBS with calcium and magnesium were then observed under an inverted fluorescence microscope (for zinquin stained cells: excitation wavelength 365 nm, emission wavelength 485 nm; for zinpyr-1 stained cells: excitation wavelength 507 nm and emission wavelength 535 nm). Cells were photographed with a cooled digital camera ORCA-100 (Hamamatsu).

2.7. Zinc or TPEN treatment and RNA extraction

HeLa cells were treated either by zinc sulfate or TPEN at different concentrations or fixed concentrations for different time before total RNA extraction. Zinc dilutions were prepared from a 20 mM stock $ZnSO_4$ solution, in which zinc was measured by atomic absorption spectroscopy for concentration accuracy. TPEN was solubilized in ethanol/water (50/50, v/v). Dilutions were prepared with medium from a 5 mM stock solution (ethanol final concentration <0.1%). Treated cells (10^6) in 60 mm Petri dishes were washed twice with cold PBS, scraped and centrifuged 3 min at 2000 × *g*. Total RNA was extracted from the cell pellet by the High Pure RNA Isolation kit (Roche) as described by the manufacturer. The RNA eluted with 50 μL of elution buffer, was quantified and stored in aliquots at −80 °C.

2.8. Quantitative RT–PCR analysis

Primer design and optimization regarding to dimerization, self-priming and melting temperature was carried out using MacVector software (Accelrys). PCR products were controlled using Agilent 2100 Bioanalyzer with DNA 500 Assay (Agilent Technology). In addition, for each separate sample, specificity of PCR product was checked with Lightcycler melting curve (Lightcycler Software v.3.5, Roche Applied Science). All primers used in this study were synthesized at Eurogentec (Saraing). The PCR was carried out with the LC Fast Start DNA Master SYBR Green kit (Roche Applied Science) using 0.1 μL of cDNA (equivalent to 3.75 ng total RNA) in a 20 μL final volume, 4 mM $MgCl_2$ and 0.4 μM of each primer (final concentration). Quantitative PCR was performed using a Lightcycler (Roche Applied Science) for 45 cycles at 95 °C for 20 s, 58 °C (Cyclophilin A and ZnT-1) or 60 °C (ZnT-5 and ZnT-7) for 5 s, and a final step of 10 s at 72 °C. Quantification was achieved with the comparative threshold cycle method [29] using RealQuant Software (Roche Applied Science).

2.9. Statistics

Results were presented as mean ± S.D. of at least five experiments. Significance was assessed by the Student's *t*-test.

3. Results

3.1. Determination of free zinc concentration in extracellular medium

The $[Zn(II)]_{free}$ was calculated according to C.E. Outten [30], from Eq. (1) with $[TPEN]_{total}$, $[Zn(II)]_{Total}$, and $K'_{Zn\text{-}TPEN}$, the apparent binding constant at a given pH and ionic strength.

$$Zn(II)_{free} + TPEN \rightarrow Zn\text{-}TPEN \quad (1)$$

The value of $K'_{Zn\text{-}TPEN}$, $2.5 \times 10^{15}\ M^{-1}$, was calculated from the absolute binding constants for Zn-TPEN [31,32] and at pH 7.4, 0.1 M ionic strength (50 mM Tris–HCl, pH 7.4, 0.1 M NaCl). The values for $[Zn(II)]_{free}$, $[TPEN]_{free}$ and [Zn-TPEN] were calculated with 25 μM TPEN in the buffer (Fig. 1A). To determine $[Zn(II)]_{free}$ in medium, in vitro assays were conducted with buffer or medium, at 1 μM zynpir-1, 25 μM TPEN and different concentrations of zinc, and fluorescence of the solution was measured (Fig. 1B). As the K_d of zinpyr-1 for zinc is 0.7 nM [27], the increase of zinpyr-1 fluorescence (50% of F_{max}) in this system is directly proportional to the increase of $[Zn(II)]_{free}$. So, a shift of the curve to the right indicates a higher buffering capability for zinc of the medium and a lower $[Zn(II)]_{free}$ for the same value of $[Zn(II)]_{total}$. Atomic absorption spectrophotometry allowed measurement of $[Zn(II)]_{total}$ in buffer and medium for each condition. The free zinc concentration was then calculated at given concentration of zinc added in the medium (Fig. 1C), accordingly to the range of the values used in the following experiments.

3.2. Effect of zinc and TPEN treatment on HeLa cells viability

Since zinc and TPEN can be toxic for cells, we determined the optimal concentrations of these substances for subsequent experiments, i.e. experimental conditions for a non-toxic zinc supplementation or depletion. HeLa cells were treated with different concentrations of zinc or TPEN for 24 h. Cell viability was assessed using the MTT assay.

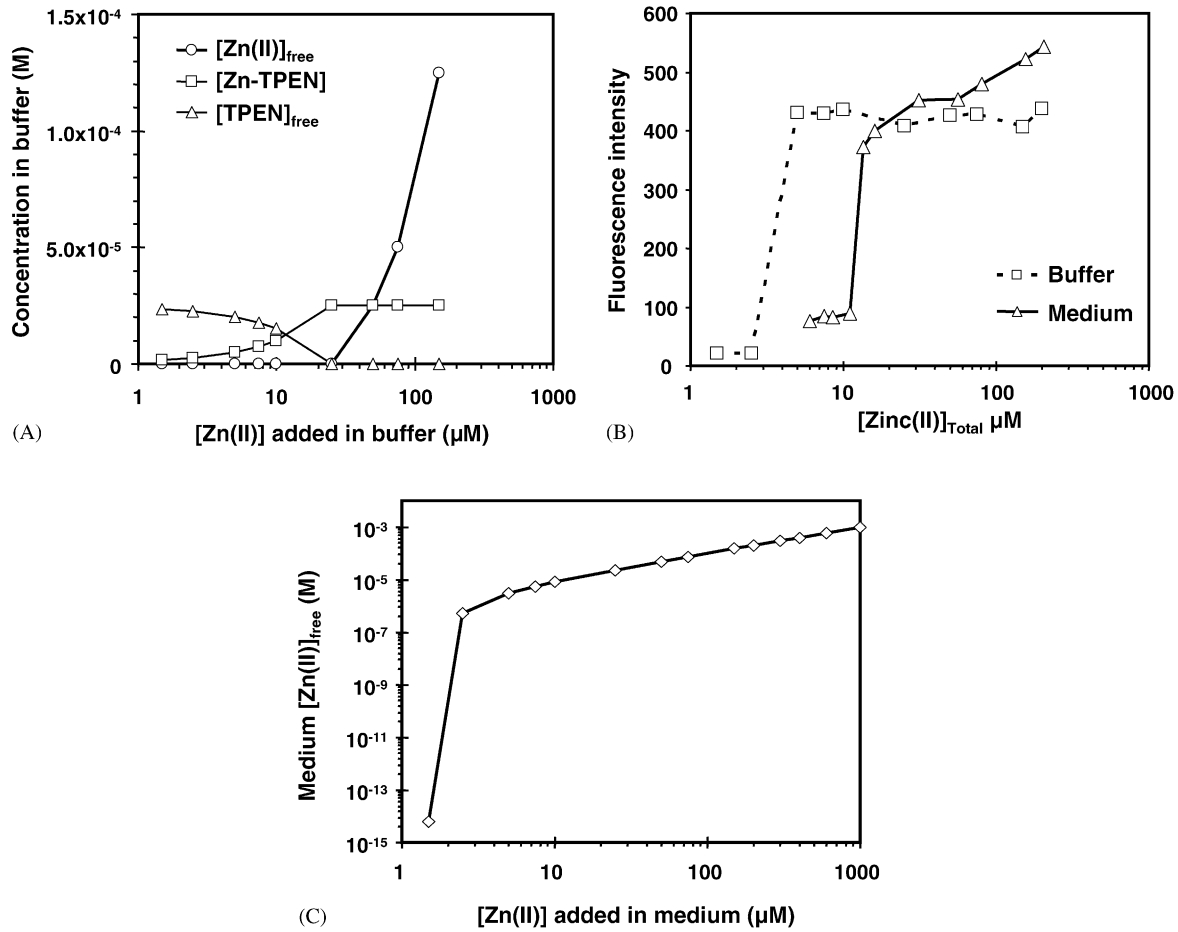

Fig. 1. Determination of free zinc concentration in buffer and medium. (A) The values for $[Zn(II)]_{free}$, $[TPEN]_{free}$ and [Zn-TPEN] in 50 mM Tris–HCl, pH 7.4, 0.1 M NaCl buffer were calculated according to O'Halloran et al. (see Section 2) using $K'_{Zn\text{-}TPEN} = 2.5 \times 10^{15}\ M^{-1}$ and $[TPEN]_{total} = 25\ \mu M$. (B) The fluorescence of 1 μM zynpir-1 in medium or buffer was measured with a spectrofluorimeter using 25 μM TPEN and different concentrations of zinc. (C) $[Zn(II)]_{free}$ was calculated for a given concentration of zinc added to the medium.

Increasing zinc concentration in the medium increased cell viability up to 125 μM zinc added (123 μM free zinc), but induced cell death for higher concentrations, with a calculated LD_{50} (concentration with 50% viability) of 154 μM zinc added (152 μM free zinc) (Fig. 2A). Similarly, 24 h treatment with increasing concentration of TPEN, a specific zinc chelator decreased cell viability. Cell viability remained stable up to 5 μM TPEN. The LD_{50} after 24 h was 6.25 μM TPEN (Fig. 2B). These results indicated that zinc and TPEN induced cell death in a dose-dependent manner. We then determined the overall shape of cell viability compared to zinc available in the medium, by treating cells simultaneously with 10 μM TPEN and different concentrations of zinc (Fig. 2C). Cell viability increased with zinc addition and reached a plateau for concentrations of zinc added ranging from 12 to 150 μM (2–140 μM free zinc). For concentrations higher than 200 μM (190 μM free zinc), the cell viability decreased with zinc added in the medium. This curve illustrated the correlation between extracellular zinc or TPEN and cell viability and confirms a biphasic effect of zinc ions.

3.3. Exchangeable intracellular zinc is correlated to extracellular zinc levels

We used zinquin ethyl ester, a membrane-permeant derivative of TSQ, to visualize labile Zn^{2+} in living cultured HeLa cells (Fig. 3). Examination of control cells revealed background fluorescence with some cells showing localized regions with higher Zn^{2+} levels (Fig. 3c). Higher magnification of control cells showed a diffuse cytoplasmic localization of zinquin fluorescence, with a strong staining in the perinuclear region (Fig. 3d). Cells loaded with Zn^{2+} displayed higher zinquin fluorescence, thus indicating zinc added in the extracellular medium increased the intracellular labile Zn^{2+} (Fig. 3a). Moreover, at higher magnification these Zn^{2+}-loaded cells display a strong punctuate fluorescence, most of which seems to be vesicular, contrasting with the diffuse staining of control cells (Fig. 3b). Conversely, in zinc-depleted cells by addition of TPEN for 2 h in the medium, the zinquin fluorescence remained only perinuclear, and very low when compared to control cells (Fig. 3e and f). Thus, zinquin fluorescence in cells was significantly quenched by

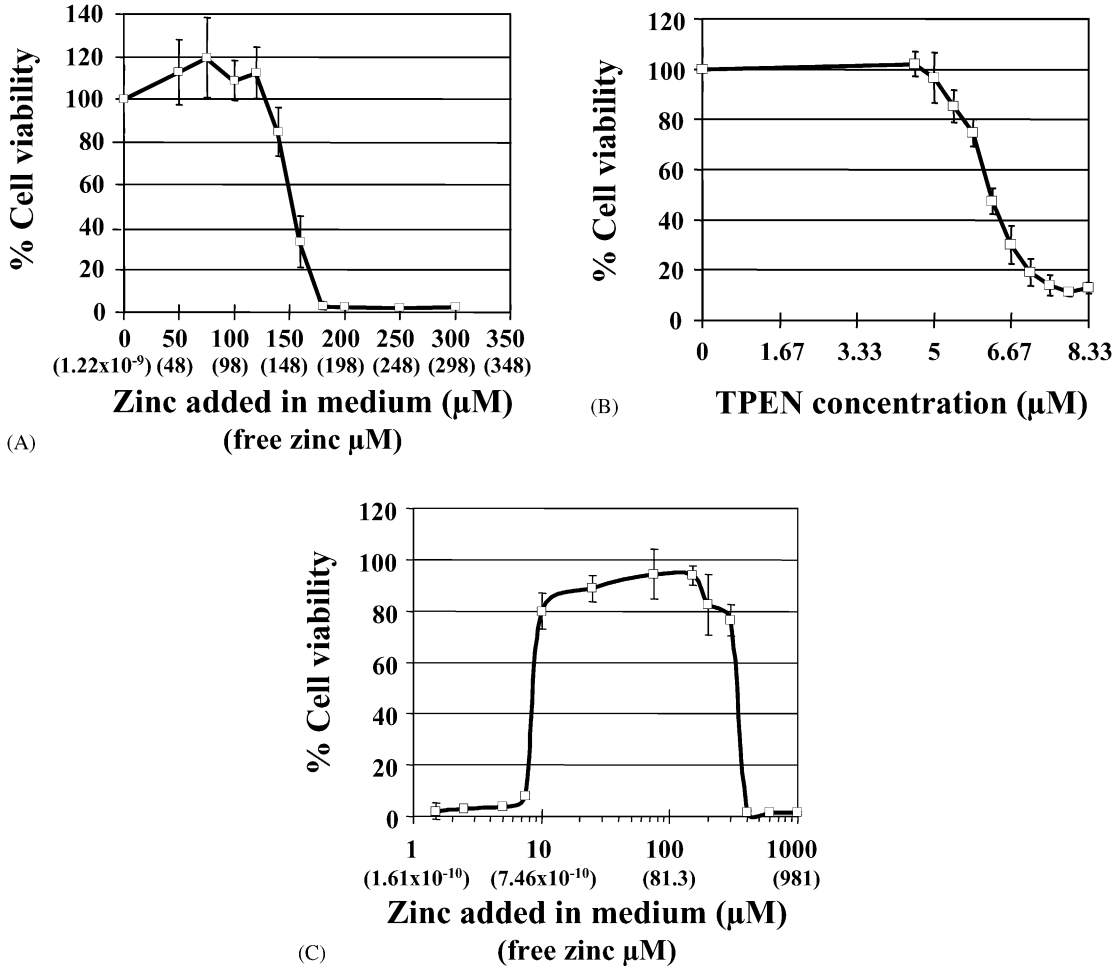

Fig. 2. Relationship of HeLa cell viability to intracellular zinc concentration. Cell Viability was determined by MTT assay as described in Section 2. Results are expressed as percentage viability, with 100% viability for non treated cells. Results are the mean ± S.D. of six experiments. (A and B) HeLa cells were incubated with the indicated concentration of zinc or TPEN added in the medium for 24 h. (C) HeLa cells were treated simultaneously with 10 µM TPEN and increasing zinc concentrations (0–2000 µM) for 24 h.

additional TPEN, which has a much higher affinity for zinc than zinquin ethyl ester [6,10]. These experiments confirmed that observed zinquin fluorescence in HeLa cells is due to the presence of intracellular labile Zn^{2+}, which was modified by high or low zinc extracellular concentration. The entire set of experiments was reproduced with zinpyr-1, a new membrane-permeant fluorescent sensor for Zn^{2+} (Fig. 4). Control zinpyr-1-loaded cells looked different from zinquin ethyl ester-stained control cells and revealed more localized regions with higher fluorescence. A higher magnification allowed localization of this fluorescence area solely in the Golgi network (Fig. 4c and d). Contrary to zinquin ethyl ester, zinpyr-1 reveals very slight, if any, cytoplasmic fluorescence. Again, we observed an increase in fluorescence when cells were cultured with 50 µM zinc added in the medium for 2 h (Fig. 4a and b). The fluorescence was not located exclusively around the nucleus, but scattered in the cytoplasm. In zinc depleted cells, zinpyr-1 fluorescence was almost completely quenched (Fig. 4e). Though, observation of cells at a higher magnification and longer time of integration (5 s compared to 500 ms for control cells) indicated that remaining zinpyr-1 fluorescence was located at the same area as control cells (Fig. 4f).

3.4. Effect of zinc ions on ZnT-1, ZnT-5 and ZnT-7 gene expression

To test the effect of zinc ions on the transcription level of different ZnTs, we treated HeLa cells with non-toxic concentrations of zinc sulfate, ranging from 0 to 100 µM (1.22×10^{-3} µM to 98 µM free zinc in the medium), for 10 h and prepared total RNAs. For kinetics analysis, HeLa cells were treated with 100 µM zinc for different times. cDNAs were then prepared on total poly-A+ RNAs by reverse transcription with poly dT primer. Then, the levels of ZnT transcripts were measured with real-time PCR using specific oligonucleotides for ZnT-1, ZnT-5 and ZnT-7 sequence and specific primer for cyclophylin A (Fig. 5). PCR products were analyzed using

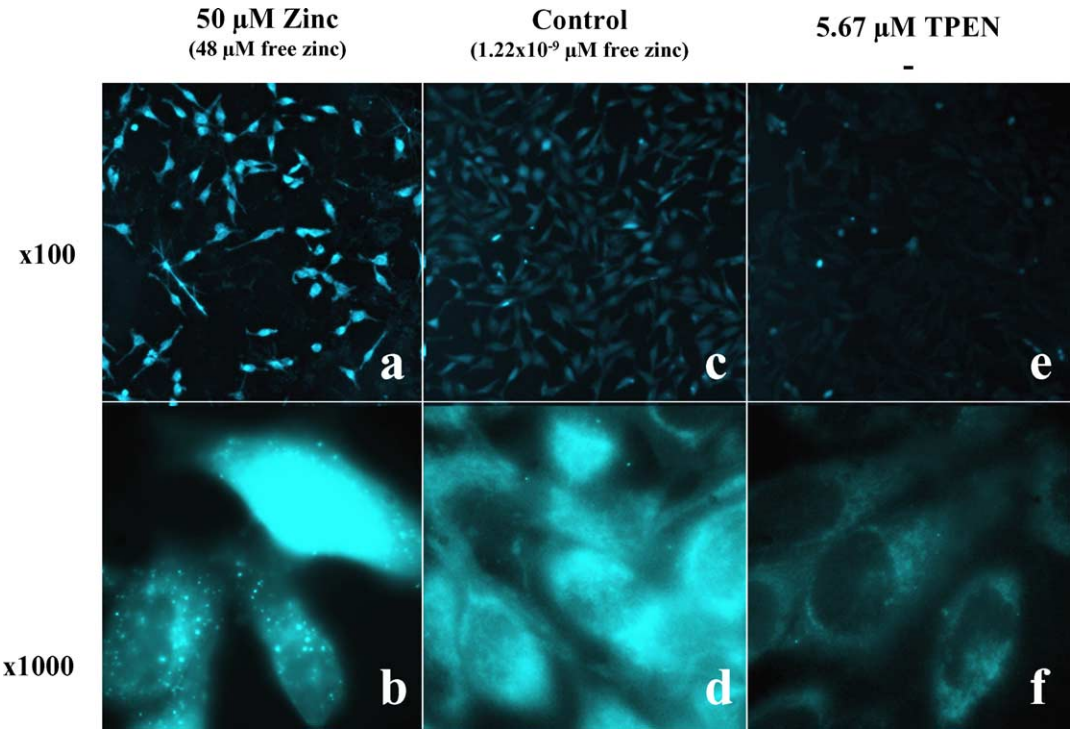

Fig. 3. Effect of extracellular zinc status on intracellular exchangeable zinc concentration analyzed by zinquin in HeLa cells. (a and b) HeLa cells were incubated for 2 h with 50 µM $ZnSO_4$, washed, and zinquin was added to visualize intracellular labile zinc. (c and d) HeLa cells were washed and zinquin was added to visualize intracellular labile zinc in untreated control cells. (e and f) HeLa cells were incubated for 2 h with 5.67 µM TPEN, washed, and zinquin was added to visualize intracellular labile zinc.

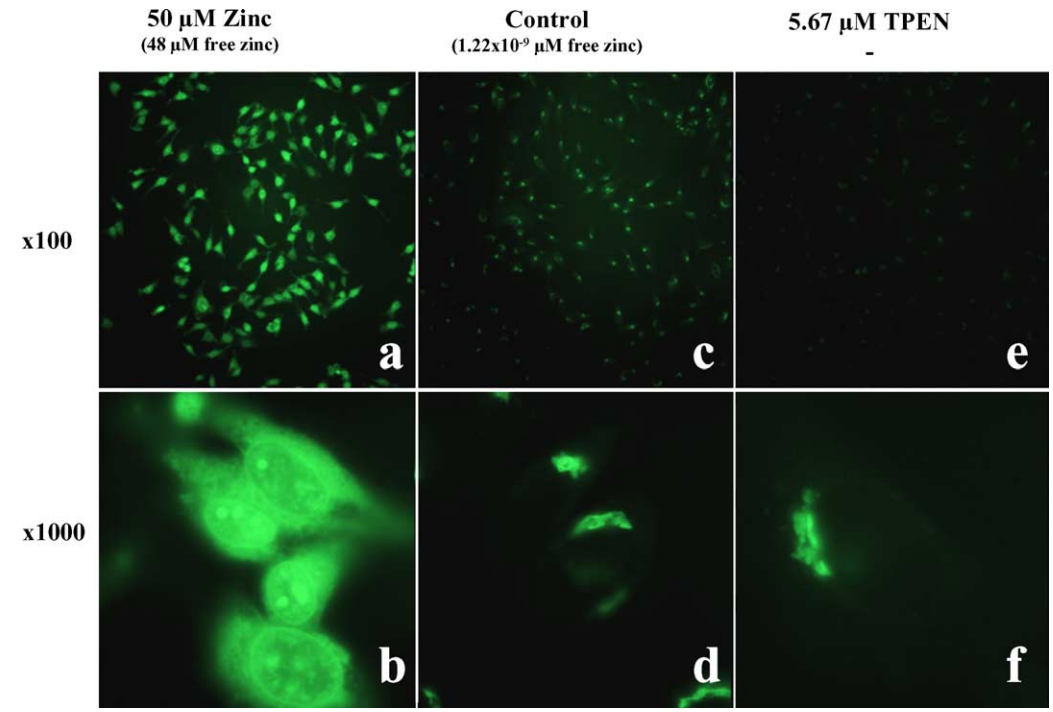

Fig. 4. Effect of extracellular zinc status on intracellular exchangeable zinc concentration analyzed by zinpyr-1 in HeLa cells. (a and b) HeLa cells were incubated for 2 h with 50 µM $ZnSO_4$, washed, and zinpyr-1 was added to visualize intracellular labile zinc. (c and d) HeLa cells were washed and zinpyr-1 was added to visualize intracellular labile zinc in untreated control cells. (e and f) HeLa cells were incubated for 2 h with 5.67 µM TPEN, washed, and zinpyr-1 was added to visualize intracellular labile zinc.

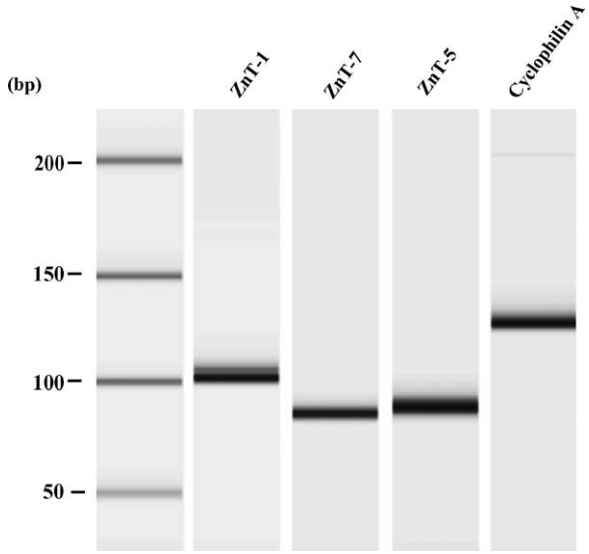

Fig. 5. Evaluation of PCR products by bioanalysis gel. cDNAs prepared from total poly-A+ RNAs were amplified by PCR using specific primers for ZnT-1, ZnT-5, ZnT-7 and cyclophylin A and analyzed by Agilent 2100 Bioanalyzer with DNA 500 Assay. Expected DNA sizes for ZnT-1, ZnT-5, ZnT-7 and cyclophylin A were 108, 83, 87 and 127 bp, respectively.

Agilent 2100 Bioanalyzer with DNA 500 Assay. PCR products were at the expected size for each gene (Table 1). Additionally, derived melting curve analyses were also performed to assess the specificity of amplification reaction. As control experiments for zinc treatment specificity of transcription levels modifications, we used both no zinc added and a change of medium, to ensure that fetal bovine serum had not any effect on ZnT transcription level (data not shown). Addition of zinc in the medium had not any effect on ZnT-5 and ZnT-7 transcription levels (Fig. 6), either at different time and doses. On the other hand the transcription level of ZnT-1, used here as a positive control, increased after addition of 100 μM zinc to the culture medium (Fig. 7A). After 1 and 3 h incubation, ZnT-1 transcription level was three-fold higher compared to controls ($P < 0.01$). After 6 h the transcription level of ZnT-1 decreased and returned to the basal level after 12 h. These results confirmed an inductive effect of zinc on ZnT-1 transcription.

3.5. Effect of zinc deficiency on ZnT-5 and ZnT-7 expression

To test the effect of zinc depletion on the transcription level of ZnT-5 and ZnT-7, we treated HeLa cells with non-toxic concentrations of TPEN ranging from 0 to 5.83 μM for 10 h or with 5.67 μM TPEN for different times. Real-time RT–PCR was conducted as described above. In cells treated with different concentrations of TPEN for 10 h (Fig. 8A and C), ZnT-5 and ZnT-7 transcriptional levels were increased comparatively to untreated cells. For both transporters, the higher effect was observed for 5.67 μM TPEN, with a 2.5-fold increase for ZnT-5 and an eight-fold increase for ZnT-7, compared to the controls. These data provide the first evidence that cells attempt to adapt to intracellular zinc deficiency by increasing the expression of those two transporters. We did not observe any effect of zinc depletion on ZnT-1 transcription level (Fig. 7B). When TPEN concentration was fixed at 5.67 μM for different times (Fig. 8B and D), the higher level of expression for ZnT-5 was observed after 12 h treatment, while Znt-7 gene expression was significantly increased after only 3 h. Thus, both ZnT-5 and ZnT-7 expressions were found to be up-regulated by zinc deficiency, in a time- and dose-dependent manner. However, the induction of ZnT-7 was faster and stronger than the induction measured for ZnT-5.

4. Discussion

The effect of extracellular sub-toxic concentrations of zinc and specific-zinc chelator TPEN on intracellular labile zinc localization was observed using two different fluorescent probe specific for Zn(II) ions, zinquin and zinpyr-1, [6,27]. These probes do not localize in the same compartments. Zinquin is able to diffuse across the membranes [33]. However, it is present in cells in its acid form and mainly accumulated into acidic vesicles [13]. HeLa cells cultured in normal zinc containing medium showed a diffuse cytoplasmic localization of zinquin fluorescence, with a stronger staining in the perinuclear region. We

Table 1
Sequences of the primers used in real-time PCR assays

Gene name	GenBank accession number	Sequence		Product size (bp)
ZnT-1 (SLC30A1)	AF364518	Forward Reverse	GAG ATG CCT TGG GTT CAG TGA TTG GGT CAG GGA AAC ATG GAT TCA CAC	108
ZnT-5 (SLC30A5)	AY089991	Forward Reverse	GGA GGC ATG AAT GCT AAC ATG AGG GTG GAT ACG ATC ACA CCA ATG CTG	83
ZnT-7 (SLC30A7)	AY094606	Forward Reverse	TTT CTT CCT GTG CCT GAA CCT CTC GAG TCG GAA ATC AAG CCT AAG CAG	87
Cyclophylin A	NM_021130	Forward Reverse	CAT CTG CAC TGC CAA GAC TGA GTG CTT CTT GCT GGT CTT GCC ATT CC	127

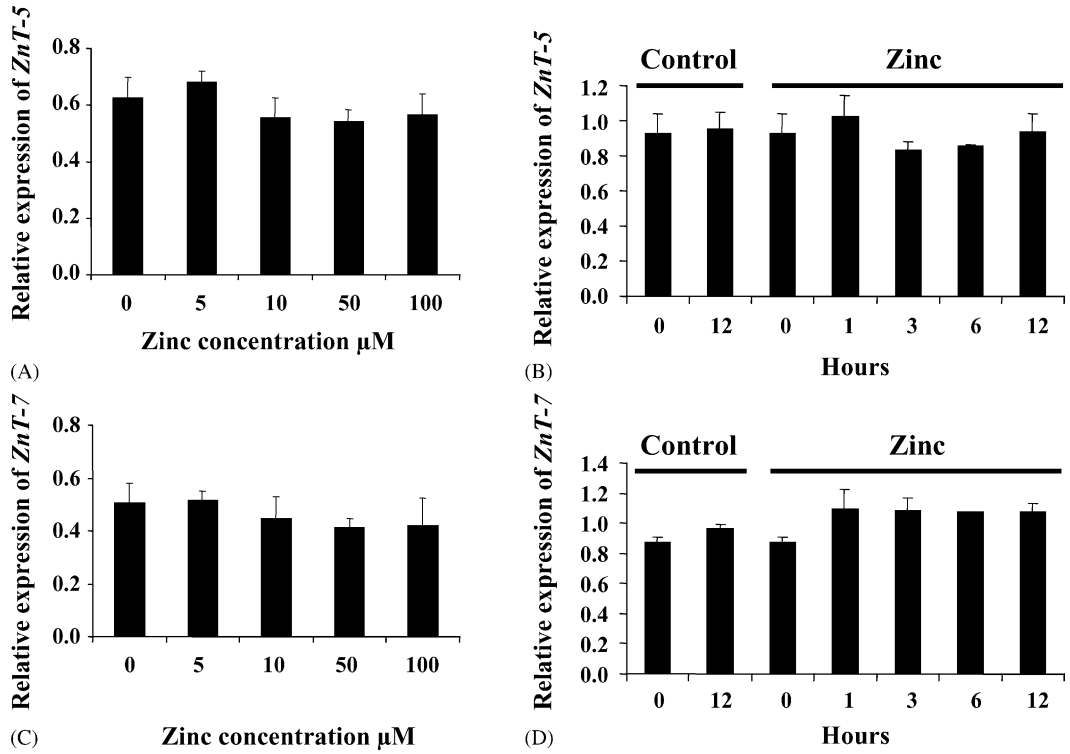

Fig. 6. Influence of zinc supplementation on ZnT-5 and ZnT-7 mRNA expression in HeLa cells. HeLa cells were cultured with various concentration of zinc for 10 h (A and C) or with 100 μM zinc for different times as indicated (B and D). Total poly-A+ RNA was reverse transcribed and the cDNA amplified by real-time PCR using specific primers for ZnT-5 (A and B) or ZnT-7 (C and D). The PCR products were detected with SYBR green fluorescence chemistry. PCR values were normalized to those produced with primers for cyclophylin. (A) Values are means ± S.D.; $n = 5$.

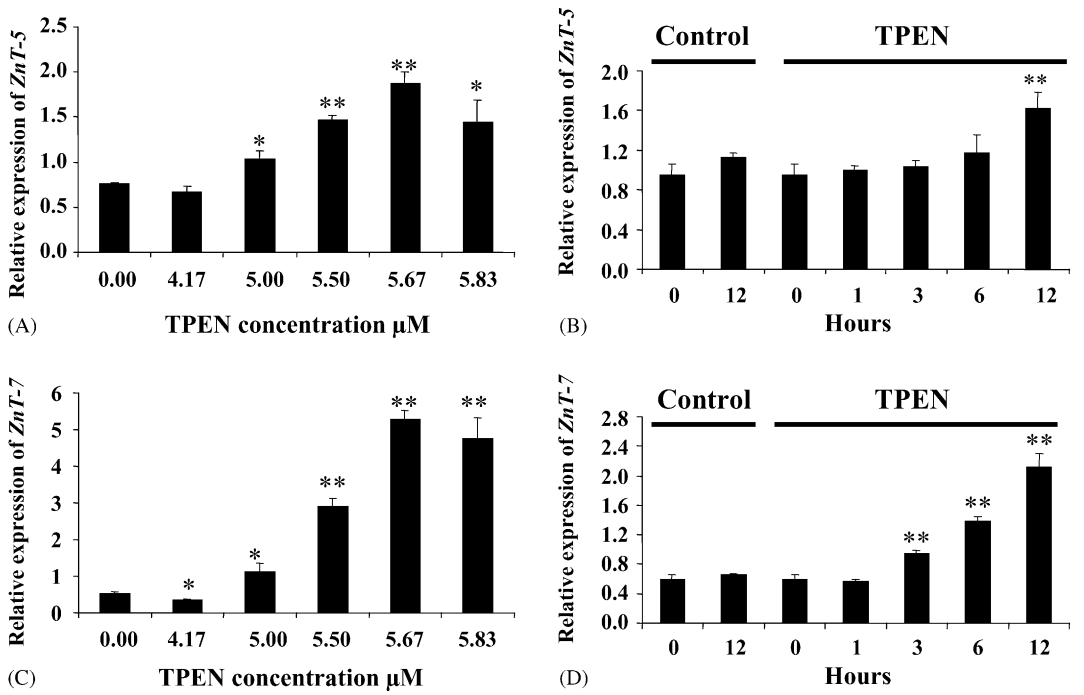

Fig. 7. Influence of zinc depletion on ZnT-5 and ZnT-7 mRNA expression in HeLa cells. HeLa cells were cultured with various concentration of TPEN for 10 h (A and C) or with 5.67 μM TPEN for different times as indicated (B and D). Total poly-A+ RNA was reverse transcribed and the cDNA amplified by real-time PCR using specific primers for ZnT-5 (A and B) or ZnT-7 (C and D). The PCR products were detected with SYBR green fluorescence chemistry. PCR values were normalized to those produced with primers for cyclophylin A. Values are means ± S.D.; $n = 5$. Asterisks indicate values significantly different from those for control culture at $^{*}P < 0.05$ or $^{**}P < 0.01$.

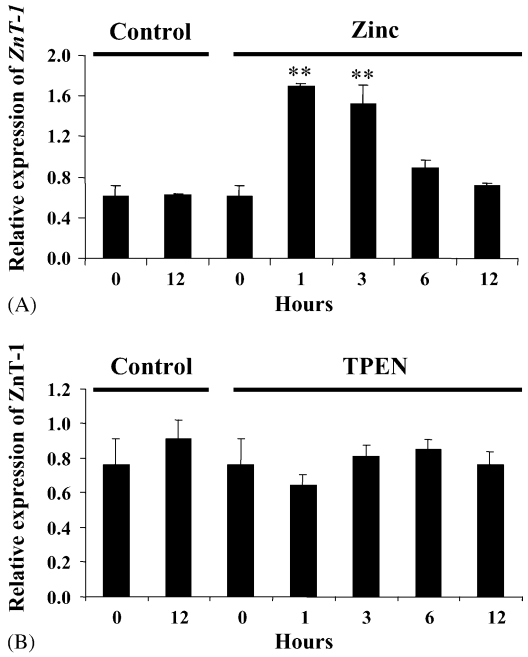

Fig. 8. Influence of zinc supplementation and depletion on ZnT-1 mRNA expression in HeLa cells. HeLa cells were cultured with 100 μM zinc (A) or 5.67 μM TPEN (B) for different times as indicated. Total poly-A+ RNA was reverse transcribed and the cDNA amplified by real-time PCR using specific primers for ZnT-1. The PCR products were detected with SYBR green fluorescence chemistry. PCR values were normalized to those produced with primers for cyclophylin A. Values are means ± S.D.; $n = 5$. Asterisks indicate values significantly different from those for control culture at $^{*}P < 0.05$ or $^{**}P < 0.01$.

observed a punctuate zinquin staining only in cells cultured in high zinc containing medium. Such an intense zinquin staining concentrated in intracellular vesicles has been observed with normal medium in other cell types, including airway epithelial cells [34], kidney and MDCK cells [35] and in an epithelial model resistant to zinc toxicity [36]. Thus, zinquin fluorescence level and localization are cell-type dependent. Zinpyr-1 is a new fluorescent probe, combining a fluorescein moiety as a reporting group and four pyridyl groups for zinc chelation [27]. Zinpyr binds Zn^{2+} in a 1:2 stoechoiometric ratio with a three- to five-fold fluorescent enhancement. It is membrane permeant and its fluorescence is mainly localized in Golgi network in several cell lines, including HeLa cells or COS-7 [27]. When combined, the fluorescent properties of zinquin and zinpyr-1 are a good indication of the global intracellular zinc status. Moreover, they have different excitation/emission wavelengths, which may explain in part the slower fading effect for zinpyr-1. The choice of a zinc fluorescent marker is dependent on which cells and organelles are studied and may be of significant importance depending on the experimental conditions.

We next examined the effect of zinc supplementation or deficiency on the transcriptional response of ZnT-1, ZnT-5 and ZnT-7 by real-time PCR. It was found that an increased intracellular zinc level, resulting from extracellular zinc supplementation, do not induce any up- or down-regulation of ZnT-5 and ZnT-7 gene expression. Yet, the inducing effect of zinc supplementation on ZnT-1 gene over-expression appears to be general (see above) as we also observed a significant one with a short kinetics. Induction of ZnT-1 expression has been described as a protection mechanism against high intracellular zinc levels provoked by extracellular zinc treatment [12] or after neuronal ischemia [37], and was explained by the binding of the metal-specific transcription factor MTF-1 on two metal response elements (MRE) in ZnT-1 promoter [25]. From our experiments, we conclude that ZnT-5 and ZnT-7 are not involved in a cellular protection mechanism against high extracellular zinc level in cells previously cultured in medium with normal zinc concentration.

Conversely, we observed a huge increase in ZnT-7 and a moderate increase in ZnT-5 genes expression after extracellular sub-toxic zinc chelator treatment (Fig. 8). A previous study indicated that ZnT-5 and ZnT-7 gene expressions were not changed or slightly decreased in THP-1 cells upon TPEN treatment [38]. But this study was performed with 10 μM TPEN, a concentration known to induce apoptosis in several cell lines [5,6,39] and to remove zinc from all zinc-containing proteins, and hence from transcription factors [40]. Another study reports a very slight increase in ZnT-5 gene expression by zinc ions, as measured by semi-quantitative RT–PCR. However, in this report this effect seems to be cell-type dependent, since ZnT-5 was up-regulated by zinc ions in intestinal Caco-2 cells, but not in placental JAR cells [41].

The Golgi network only, zinc deficiency induced, zinpyr-1 fluorescence combined with the ZnT-5 and ZnT-7 gene over-expression, confirm the hypothesis for a function of ZnT-5 and/or ZnT-7 in regulating intra-golgi zinc level [18,21]. This adaptative mechanism might help providing zinc to neo-synthesised proteins and keep some important biochemical reaction during zinc deficiency. An other hypothesis, similar to what has been described for ZRC1 in *Saccharomyces cerevisiae* [42], could be a mechanism for protecting the cell from zinc efflux (or zinc shock) following a zinc deficiency period, preventing a deleterious increase of cytosolic zinc due to a massive influx of zinc. In zinc-limited cells, ZRC1 transcription, required for resistance to this zinc shock, was found to be induced by the zinc-responsive transcription factor Zap1 [42,43].

In conclusion, while the MTF-1 driven zinc transporter ZnT-1 was confirmed to be induced by zinc ions, we showed that ZnT-5 and ZnT-7 gene expression did not respond to zinc supplementation, but they are strongly up-regulated by zinc depletion in HeLa cells (Fig. 9). This work represents the first evidence of a regulating mechanism in response to zinc deficiency at the transcriptional level for ZnT-5 and ZnT-7 in cultured cells. We also point out the possible occurrence of a low zinc level transcription factor similar to zap1 but yet to be identified.

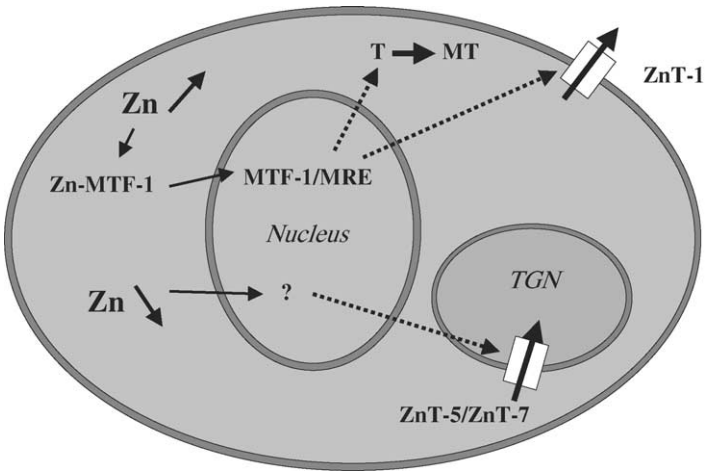

Fig. 9. Adaptative mechanism of zinc regulating proteins ZnT-1, ZnT-5 and ZnT-7 gene expression following extracellular zinc supplementation or depletion.

Acknowledgments

We thank Dr. J. Arnaud for determination of total zinc concentration by atomic absorption spectroscopy. This work has been supported by a grant from the ''Centre Evian Pour l'Eau'' to M.S.

References

[1] Maret W. Cellular zinc and redox states converge in the metallothionein/thionein pair. J Nutr 2003;133:1460S–2S.
[2] Truong-Tran AQ, Carter J, Ruffin RE, Zalewski PD. The role of zinc in caspase activation and apoptotic cell death. Biometals 2001;14: 315–30.
[3] Truong-Tran AQ, Ho LH, Chai F, Zalewski PD. Cellular zinc fluxes and the regulation of apoptosis/gene-directed cell death. J Nutr 2000;130:1459S–66S.
[4] Beyersmann D, Haase H. Functions of zinc in signaling, proliferation and differentiation of mammalian cells. Biometals 2001;14:331–41.
[5] Chimienti F, Seve M, Richard S, Mathieu J, Favier A. Role of cellular zinc in programmed cell death: temporal relationship between zinc depletion, activation of caspases, and cleavage of Sp family transcription factors. Biochem Pharmacol 2001;62:51–62.
[6] Zalewski PD, Forbes IJ, Betts WH. Correlation of apoptosis with change in intracellular labile Zn(II) using zinquin [(2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy)acetic acid], a new specific fluorescent probe for Zn(II). Biochem J 1993;296:403–8.
[7] Vallee BL. Zinc: biochemistry, physiology, toxicology and clinical pathology. Biofactors 1988;1:31–6.
[8] Prasad AS. Zinc deficiency. Br Med J 2003;326:409–10.
[9] Hambidge M. Human zinc deficiency. J Nutr 2000;130:1344S–9S.
[10] Chimienti F, Aouffen M, Favier A, Seve M. Zinc homeostasis-regulating proteins: new drug targets for triggering cell fate. Curr Drug Targets 2003;4:323–38.
[11] Eide DJ. The SLC39 family of metal ion transporters. Pflugers Arch 2003.
[12] Palmiter RD, Findley SD. Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. EMBO J 1995;14:639–49.
[13] Palmiter RD, Cole TB, Findley SD. ZnT-2, a mammalian protein that confers resistance to zinc by facilitating vesicular sequestration. EMBO J 1996;15:1784–91.
[14] Wenzel HJ, Cole TB, Born DE, Schwartzkroin PA, Palmiter RD. Ultrastructural localization of zinc transporter-3 (ZnT-3) to synaptic vesicle membranes within mossy fiber boutons in the hippocampus of mouse and monkey. Proc Natl Acad Sci USA 1997;94:12676–81.
[15] Palmiter RD, Cole TB, Quaife CJ, Findley SD. ZnT-3, a putative transporter of zinc into synaptic vesicles. Proc Natl Acad Sci USA 1996;93:14934–9.
[16] Liuzzi JP, Blanchard RK, Cousins RJ. Differential regulation of zinc transporter 1, 2, and 4 mRNA expression by dietary zinc in rats. J Nutr 2001;131:46–52.
[17] Huang L, Gitschier J. A novel gene involved in zinc transport is deficient in the lethal milk mouse. Nat Genet 1997;17:292–7.
[18] Kambe T, Narita H, Yamaguchi-Iwai Y, Hirose J, Amano T, Sugiura N, et al. Cloning and characterization of a novel mammalian zinc transporter, ZnT-5, abundantly expressed in pancreatic beta cells. J Biol Chem 2002;19:19.
[19] Inoue K, Matsuda K, Itoh M, Kawaguchi H, Tomoike H, Aoyagi T, et al. Osteopenia and male-specific sudden cardiac death in mice lacking a zinc transporter gene, Znt5. Hum Mol Genet 2002;11: 1775–84.
[20] Huang L, Kirschke CP, Gitschier J. Functional characterization of a novel mammalian zinc transporter, ZnT6. J Biol Chem 2002;277:26389–95.
[21] Kirschke CP, Huang L. ZnT7, a novel mammalian zinc transporter, accumulates zinc in the Golgi apparatus. J Biol Chem 2003;278:4096–102.
[22] Pfaffl MW, Windisch W. Influence of zinc deficiency on the mRNA expression of zinc transporters in adult rats. J Trace Elem Med Biol 2003;17:97–106.
[23] McMahon RJ, Cousins RJ. Regulation of the zinc transporter ZnT-1 by dietary zinc. Proc Natl Acad Sci USA 1998;95:4841–6.
[24] Hasumi M, Suzuki K, Matsui H, Koike H, Ito K, Yamanaka H. Regulation of metallothionein and zinc transporter expression in human prostate cancer cells and tissues. Cancer Lett 2003;200: 187–95.
[25] Langmade SJ, Ravindra R, Daniels PJ, Andrews GK. The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene [In Process Citation]. J Biol Chem 2000;275:34803–9.
[26] Kelleher SL, Lonnerdal B. Zinc transporters in the rat mammary gland respond to marginal zinc and Vitamin A intakes during lactation. J Nutr 2002;132:3280–5.
[27] Burdette SC, Walkup GK, Spingler B, Tsien RY, Lippard SJ. Fluorescent sensors for Zn(2+) based on a fluorescein platform: synthesis, properties and intracellular distribution. J Am Chem Soc 2001;123: 7831–41.

[28] Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 1987;47:936–42.
[29] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402–8.
[30] Outten CE, O'Halloran TV. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 2001;292: 2488–92.
[31] Martell AE, Smith RM. NIST critical stability constants of metal complexes. In: NIST Standard Reference Database 46, v5.0. New York: Plenum; 1998.
[32] Fahrni CJ, O'Halloran TV. Aqueous coordination chemistry of quinoline-based fluorescence probes for the biological chemistry of zinc. J Am Chem Soc 1999;121:11448–4458.
[33] Snitsarev V, Budde T, Stricker TP, Cox JM, Krupa DJ, Geng L, et al. Fluorescent detection of Zn(2+)-rich vesicles with zinquin: mechanism of action in lipid environments. Biophys J 2001;80:1538–46.
[34] Truong-Tran AQ, Ruffin RE, Zalewski PD. Visualization of labile zinc and its role in apoptosis of primary airway epithelial cells and cell lines. Am J Physiol Lung Cell Mol Physiol 2000;279:L1172–83.
[35] Ranaldi G, Perozzi G, Truong-Tran A, Zalewski P, Murgia C. Intracellular distribution of labile Zn(II) and zinc transporter expression in kidney and MDCK cells. Am J Physiol Renal Physiol 2002;283: F1365–75.
[36] Chimienti F, Jourdan E, Favier A, Seve M. Zinc resistance impairs sensitivity to oxidative stress in HeLa cells: protection through metallothioneins expression. Free Radic Biol Med 2001;31:1179–90.
[37] Tsuda M, Imaizumi K, Katayama T, Kitagawa K, Wanaka A, Tohyama M, et al. Expression of zinc transporter gene, ZnT-1, is induced after transient forebrain ischemia in the gerbil. J Neurosci 1997;17: 6678–84.
[38] Cousins RJ, Blanchard RK, Popp MP, Liu L, Cao J, Moore JB, et al. A global view of the selectivity of zinc deprivation and excess on genes expressed in human THP-1 mononuclear cells. Proc Natl Acad Sci USA 2003;100:6952–7.
[39] Parat MO, Richard MJ, Pollet S, Hadjur C, Favier A, Beani JC. Zinc and DNA fragmentation in keratinocyte apoptosis: its inhibitory effect in UVB irradiated cells. J Photochem Photobiol B 1997;37: 101–6.
[40] Seve M, Favier A, Osman M, Hernandez D, Vaitaitis G, Flores NC, et al. The human immunodeficiency virus-1 Tat protein increases cell proliferation, alters sensitivity to zinc chelator-induced apoptosis, and changes Sp1 DNA binding in HeLa cells. Arch Biochem Biophys 1999;361:165–72.
[41] Cragg RA, Christie GR, Phillips SR, Russi RM, Kury S, Mathers JC, et al. A novel zinc-regulated human zinc transporter, hZTL1, is localized to the enterocyte apical membrane. J Biol Chem 2002; 277:22789–97.
[42] MacDiarmid CW, Milanick MA, Eide DJ. Induction of the ZRC1 metal tolerance gene in zinc-limited yeast confers resistance to zinc shock. J Biol Chem 2003;278:15065–72.
[43] Miyabe S, Izawa S, Inoue Y. Expression of ZRC1 coding for suppressor of zinc toxicity is induced by zinc-starvation stress in Zap1-dependent fashion in *Saccharomyces cerevisiae*. Biochem Biophys Res Commun 2000;276:879–84.