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Increased abundance of labile intracellular zinc during cell proliferation was due to increased retention of extracellular zinc in 3T3 cells☆

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Abstract

Platelet-derived growth factor (PDGF)-, epidermal growth factor (EGF)- and insulin-like growth factor I (IGF-I)-stimulated cell proliferation in 3T3 cells was accompanied by increased abundance of labile intracellular pool of zinc (LIPZ). However, the origin and regulation of this cell proliferation-associated increase in the abundance of LIPZ are unknown. Cellular zinc homeostasis involves zinc transporters and metallothionein. The objectives of this study were to determine whether cell proliferation-associated increase in the abundance of LIPZ was a result of an increased zinc uptake and to assess the involvement of zinc transporters and metallothionein in this cell proliferation-associated increase in the abundance of LIPZ in 3T3 fibroblasts. Zinc transporters assessed included both zinc importer (Zip1) and zinc exporters (ZnT1, ZnT2 and ZnT4). Growth factors increased the abundance of LIPZ while total cellular zinc concentration remained unaffected, demonstrating that LIPZ was responsive to the increased needs for zinc during growth factor-stimulated cell proliferation. Growth factors also increased net zinc retention as indicated by higher ^{65}zinc radioactivity and elevated mRNA levels of Zip1, ZnT1 and ZnT4. Although zinc is essential to cell proliferation, excessive cellular zinc accumulation causes cytotoxicity. Collectively, these observations suggest that increase in the abundance of LIPZ during growth factor-stimulated cell proliferation was due to increased net retention of extracellular zinc, which was apparently achieved through a coordinated up-regulation of the expression of transporters involved in both zinc influx and efflux to ensure adequate supply of zinc to sustain cell proliferation, yet to prevent potential zinc cytotoxicity in 3T3 cells.

Keywords: Cell proliferation; Labile intracellular pool of zinc; ^{65}Zn; Zip1; ZnT1; ZnT2; ZnT4

1. Introduction

We have previously shown an increase in the abundance of labile intracellular pool of zinc (LIPZ) when 3T3 fibroblasts are stimulated to proliferate by treating the cells with a combination of platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) [1,2]. This suggests that the abundance of LIPZ is important for cell proliferation. Indeed, depleting LIPZ in 3T3 fibroblasts by culturing the cells in a low-zinc medium suppresses DNA synthesis and cell proliferation. In contrast, when the low-zinc medium is supplemented with zinc, the abundance of LIPZ is increased, and DNA synthesis and cell proliferation are promoted in a concentration-dependent manner [1]. Since zinc is required for DNA synthesis [3–5] and cell proliferation [6], it is logical that there is an increased need for zinc when cell proliferation is stimulated. However, most of the intracellular zinc is protein-bound and not readily available to meet this increased need for zinc. In contrast, LIPZ consists of both free zinc ions and zinc loosely bound to macromolecules. It is then possible that increases in the abundance of the LIPZ during growth factor-stimulated cell proliferation may be an important cellular event for providing sufficient zinc to sustain increased DNA synthesis and cell proliferation. However, the origin and regulation of this cell proliferation-associated increase in the abundance of LIPZ are unknown.

Cellular zinc homeostasis involves numerous proteins, including Zip proteins, ZnT proteins and metallothionein. Zip proteins are a family of transporters involved in zinc uptake. Increased expression of hZip1 or hZip2 results in increased ^{65}Zn accumulation in K562 erythroleukemia cells

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[7,8]. Upon uptake, excess zinc is bound to metallothionein, the major intracellular zinc binding protein. Metallothionein-bound zinc can be donated to other proteins such as enzymes [9,10] and zinc-finger proteins [11,12]. More recently, we have provided evidence showing that metallothionein-bound zinc is apparently part of the LIPZ [13]. Cellular zinc efflux involves ZnT proteins. This family of proteins is involved in transporting zinc from the cytoplasm either into the extracellular space or into intracellular vesicles [14–16]. For example, overexpression of ZnT1 in zinc-sensitive baby hamster kidney cells results in zinc resistance due to increased ^{65}Zn efflux [17]. It is possible that cell proliferation-associated increase in the abundance of LIPZ results from an altered cellular zinc homeostasis, involving zinc importers and exporters and metallothionein, to ensure sufficient supply of zinc to sustain cell proliferation and yet prevent zinc cytotoxicity.

The objectives of this study were to determine whether cell proliferation-associated increase in the abundance of LIPZ was a result of an increased zinc intake and to assess the involvement of zinc transporters and metallothionein in the cell proliferation-associated increase in the abundance of LIPZ in 3T3 fibroblasts. In this study, zinc transporters assessed included both zinc importer (Zip1) and zinc exporters (ZnT1, ZnT2 and ZnT4).

2. Materials and methods

2.1. Cell culture system and treatments

3T3 Swiss fibroblasts (3T3; passage number 122–124; CCL-92; ATCC, Manassas, VA) were cultured using the same culture system reported earlier [1,2] with modifications. Briefly, the cells (1×10^4 cells/T75 flask) were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS), D-glucose (4.5 g/L), L-glutamine (292 mg/L), sodium pyruvate (110 mg/L), sodium bicarbonate (1.5 g/L), penicillin G sodium (50,000 U/L) and streptomycin sulfate (50 mg/L) at 37°C in an atmosphere containing 5% CO_2 for 72 h. The cells were then cultured in a low-serum DMEM (1% FBS) for 48 h to induce quiescence. To stimulate cell proliferation, the cells were cultured for 48 h in the DMEM+10% FBS supplemented with a combination of growth factors (PDGF 100 ng/ml; EGF 50 ng/ml; IGF-I 20 ng/ml; Sigma, St. Louis, MO). The control cells received vehicles for the growth factors, which were phosphate buffered saline (PBS), bovine serum albumin (BSA, 0.1%; Sigma) in PBS, and BSA (0.1%) in 4 mM HCl. Prior to changing to the low-serum medium and growth factor treatment, cells were thoroughly rinsed with PBS (pH 7.4).

2.2. Labile intracellular pool of zinc assay

Zinquin ethyl ester (Calbiochem, San Diego, CA) was reconstituted in dimethyl sulfoxide (DMSO) as stock solution (1 mM), which was further diluted with DMSO to prepare the working solution (50 μM). Both the stock and working solutions were stored at −20°C until use. The working solution was used within 1 month after preparation. The abundance of LIPZ was assessed using both microscopic assay and spectrophotometric assay.

For the microscopic assay, the cells were stained with Zinquin ethyl ester working solution using the same procedure described previously [1,2]. The fluorescent images of the cells were obtained by passing the fluorescent emissions through a long-pass emission filter (cutoff: 395 nm) using a fluorescent microscope (Axiovert 200M, Carl Zeiss, Oberkochen, Germany). The images were captured with a digital camera (AxioCam MRm, Carl Zeiss) and evaluated using Axiovision Imaging Software (release 4.1, Carl Zeiss). The camera's aperture and shutter speed were kept consistent for all samples. Overall brightness of zinc-dependent Zinquin fluorescence was used to assess LIPZ abundance.

For the spectrophotometric assay, cells were harvested and resuspended in Hank's Balanced Salt Solution (HBSS). The cells were then transferred to a 96-well dark microplate (1×10^6 cells/well; 200 μl/well) and labeled with Zinquin at a final concentration of 8 μmol/L. The cells were then incubated in the dark followed by incubation at room temperature on a thermal shaker (Eppendorf Thermomixer R, Brinkman Instruments, Westbury, NY) at 100 rpm for 30 min. The fluorescence intensity was measured using a fluorescence microplate reader (SpectraMAX GEMINI XS, Molecular Devices, Sunnyvale, CA) at excitation and emission wavelengths of 365 and 475 nm, respectively. Reagent blank was subtracted from the fluorescence intensity of the samples.

2.3. RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR) assay

Total RNA was isolated using Trizol (5 ml/T75 flask; Invitrogen, Burlington, ON) according to the manufacturer's instructions. The RNA pellets were allowed to dry at room temperature and dissolved in diethyl-pyrocarbonate-treated water and stored at −80°C until analyses. Integrity of RNA was ensured by running the samples on an agarose gel (1%) stained with ethidium bromide and viewed under UV light.

RT-PCR was performed using the ThermoScript RT-PCR kit plus Platinum Taq polymerase (Invitrogen) according to the manufacturer's instructions. Total RNA (2 μg) was used for reverse transcription. The resulting cDNA was stored at −20°C until analyses. To assess the mRNA level of Zip1, ZnT1, ZnT2, ZnT4 and β-actin, cDNA (1 μl, which was equivalent to 0.1 μg of total RNA) was amplified with a thermocycler (Eppendorf Gradient Mastercycler, Brinkman Instruments). The PCR conditions used were adopted from the conditions described earlier [18] with modifications to optimize the amplification. Briefly, after a hot start (94°C, 2 min), the samples were denatured at 95°C for 1 min, annealed at 55°C for β-actin, 57°C for ZnT2 and 59°C for

Zip1, ZnT1 and ZnT4 for 1 min, and elongated at 72°C for 1 min followed by a final extension at 72°C for 10 min. For each target, preliminary studies were carried out to determine the relationship between amplification efficiency and the number of cycles. Based on the results of these preliminary studies, the number of cycles used was chosen on the bases that the amplification was within the liner phase of the amplification curve. The R^2 for the number of cycles tested was 0.97, 0.89, 0.99, 0.97 and 0.99 for Zip1, ZnT1, ZnT2, ZnT4 and β-actin, respectively. The amplification did not yield any nonspecific PCR product. The number of PCR cycles for β-actin and ZnT4 was 27, ZnT1 was 30, Zip1 was 34 and ZnT2 was 35.

The sequences for the sense and antisense primers used were as follows: Zip1: 5′-CTGCGTGCTTGTGTCCTGGTC-3′ and 5′-CTCTGCCAGAGCTGCACC-3′ (256 bp) [19]; ZnT-1: 5′-TGAAGGCGGACCAGGCAGAG-3′and 5′-AGACATGTAGCTCATGGACTTC-3′ (519 bp; Genbank accession code BC052166); ZnT-2: 5′-AGCCATTGCCCAGAATGCTG-3′ and 5′-CATGGATCTTGTTCAATTTTTGG-3′ (473 bp; Genbank accession code AK031425); ZnT-4: 5′-TTGCAGTTAATGTAATAATGGGGTT-3′ and 5′-GACAATTTGCACAAGTTCTGATC-3′ (600 bp; Genbank accession code AF003747); β-actin: 5′-TATGGAGAAGATTTGGCACC-3′ and 5′-CCACCAATCCACACAGAGTA-3′ (786 bp) [18]. Two negative controls, primer blank and cDNA blank, were also included for each PCR amplification. β-Actin was used as the control to verify the integrity of the RNA and as an indicator for equal loading. The PCR product was separated on agarose gel (2%) stained with ethidium bromide and visualized with Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290, Scientific Imaging Systems Eastman Kodak, New Haven, CT). The optical density of the bands was quantified with Kodak 1D Image Analysis Software (release 3.6). The mRNA level of the targets was normalized on the optical density of the corresponding β-actin band.

2.4. Net zinc retention assay

For the net zinc retention assay, cells were cultured using the same culture system described above at an initial seeding density of 3.3×10^3 cells/T25 flask. To prepare for the ^{65}Zn-containing media, ^{65}Zn in HCl (0.11 mCi/μg $ZnCl_2$; Brookhaven National Laboratory, US Department of Energy, Upton, NY) was added to the culture media with a final activity of 0.39 μCi/ml culture medium. The media was stored overnight at 4°C to facilitate equilibrium prior to use.

Upon the induction of quiescence, the cells were cultured in the ^{65}Zn-containing media (5 ml with a total activity of 1.95 μCi/flask) under the same conditions described above. After 48 h of culture, cells were rinsed twice with cold PBS containing 2.84 μM $ZnSO_4$. This zinc concentration was equivalent to the media zinc concentration. The purpose of adding zinc to the PBS was to displace ^{65}Zn bound to the outside of the cell, therefore giving a more accurate picture of intracellular ^{65}Zn. Cells were then lysed with 2 ml cell lysis solution (0.2% sodium dodecyl sulfate) in 0.2 M NaOH. The cell lysate was quantitatively transferred to a scintillation vial, after which an aliquot (150 μl) was removed for protein concentration determination. Scintillation fluid (3.5 ml) was added to each sample, and each sample was counted for 5 min with a liquid scintillation counter (Beckman LS6500, Beckman Instruments, Mississagua, ON). The radioactivity of ^{65}Zn was expressed as counts per minute and normalized to protein concentration.

2.5. Other assays

For counting cell numbers, cells were harvested with 3 ml trypsin-EDTA (0.25% trypsin, 1 mM EDTA•4 Na; Gibco, Grand Island, NY) followed by neutralization with an equal volume of trypsin inhibitor (0.25 mg/ml, Gibco) dissolved in HBSS. An aliquot of the cell suspension (0.5 ml) was diluted with PBS (9.5 ml) and counted for cell numbers using a particle counter (Z1 Coulter Particle Counter, Beckman Coulter, Fullerton, CA) with a cutoff point of 5.0 μm. The remaining cell suspension was pelleted by centrifugation (500 rpm, 5 min) at room temperature for the determination of total cellular zinc concentration as reported previously [20]. Briefly, the cell pellet was digested with concentrated nitric acid (500 μl). The digest was quantitatively transferred to a 1-ml volumetric flask. The zinc concentration was determined using flame atomic absorption spectrophotometer.

Metallothionein levels were determined using the cadmium (^{109}Cd)-hemoglobin affinity assay [21] with modifications described earlier [13]. The metallothionein level was normalized to total cellular protein concentration, which was quantified using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). For the net zinc retention assay, total cellular protein concentration was quantified using the Bio-Rad DC Protein Assay (Bio-Rad), which is compatible with detergent in the cell lysis solution.

2.6. Statistical analyses

The results are expressed as mean±S.E.M. ($n=6$). The significant difference between the means was determined by

Table 1
Effects of PDGF, EGF and IGF-I on cell number, total cellular zinc concentration, ^{65}zinc radioactivity and metallothionein protein level in 3T3 cells

	Treatment	
	Control	+GFs
Cell number ($\times10^5$/ml)	0.88±0.02	1.31±0.02*
Total cellular zinc concentration (ng/10^6 cells)	117±7	114±8
^{65}Zinc radioactivity (cpm/μg protein)	40,352±2,207	48,353±1,412*
Metallothionein protein (μg/mg protein)	0.17±0.02	0.15±0.01

Values are means±S.E.M. ($n=6$). +GFs: PDGF-, EGF- and IGF-I-treated cells.

* Significantly different from the control ($P<.05$).

Student's *t*-test (SPSS for Windows, version 11.5). Differences were considered significant at $P<.05$.

3. Results

3.1. Cell proliferation

Treating 3T3 cells with a combination of PDGF, EGF and IGF-I for 48 h significantly increased the cell numbers by 49% ($P<.05$; Table 1). This increase in cell numbers indicated an increased cell proliferation in response to growth factor treatment.

3.2. LIPZ abundance, total cellular zinc concentration and net zinc retention

The relative intensity of zinc-dependent Zinquin fluorescence in the growth factor-treated 3T3 cells was generally noticeably greater than in the control cells (Fig. 1A,B). The size of the growth factor-treated 3T3 cells was less uniform compared to the control cells. The difference in the size of the cells had no apparent effect on the relative intensity of zinc-dependent Zinquin fluorescence as, at similar sizes, the relative intensity of zinc-dependent Zinquin fluorescence was still greater in the growth factor-treated 3T3 cells than in the control cells (Fig. 1A,B). Spectrophotometric analysis also showed an increased intensity of zinc-dependent Zinquin fluorescence in the growth factor-treated 3T3 cells (31%) compared to that in the control cells (Fig. 2). Zinc-dependent Zinquin fluorescence was primarily found in the cytoplasm (Fig. 1C,D). In the cytoplasm, zinc-dependent Zinquin fluorescence was clearly much more intense in the area surrounding the nucleus. This was especially evident in the growth factor-treated 3T3 cells.

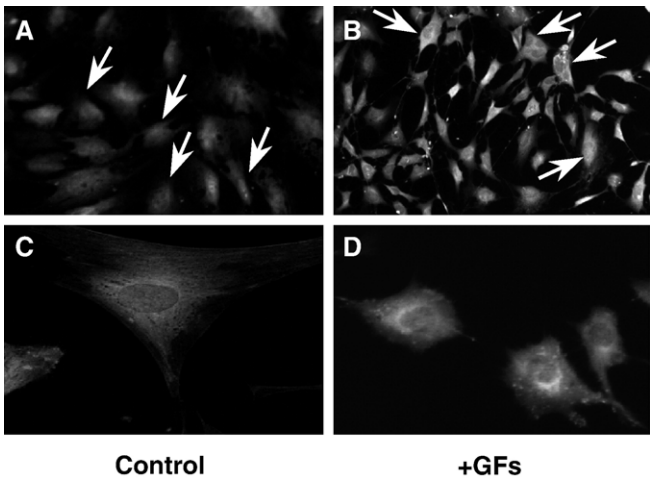

Fig. 1. Microscopic analysis of the relative intensity and distribution of Zinquin-dependent fluorescence in 3T3 cells. (A) The control cells (100×). (B) Cells treated with a combination of PDGF, EGF and IGF-I for 48 h (100×). Control and PDGF-, EGF- and IGF-I-treated cells with similar sizes are indicated with arrows. Cellular distribution of Zinquin-dependent fluorescence is shown in (C) and (D) (200×). +GFs: PDGF, EGF and IGF-I-treated cells.

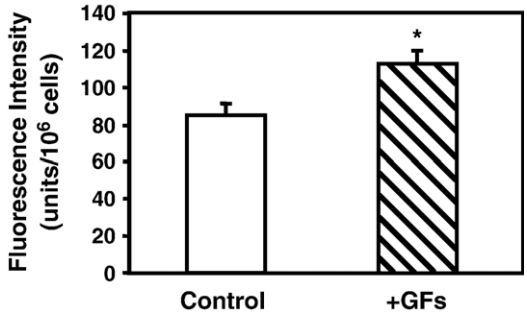

Fig. 2. Intensity of Zinquin-dependent fluorescence in 3T3 cells. Values represent mean±S.E.M. ($n=5$). An asterisk indicates significantly different from the control cells ($P<.05$). +GFs: PDGF-, EGF- and IGF-I-treated cells.

Total cellular zinc concentration was similar between the control and growth factor-treated cells (Table 1). The radioactivity of ^{65}zinc was increased by 20% in growth factor-treated cells compared to the control cells ($P<.05$; Table 1).

3.3. Metallothionein protein level and Zip1, ZnT1, ZnT2 and ZnT4 mRNA levels

Metallothionein protein level was unaffected by growth factor treatment (Table 1). The expression of *Zip1*, *ZnT1*, *ZnT2* and *ZnT4* was assessed by their corresponding steady-state mRNA level. Treating 3T3 cells with a combination of growth factors resulted in 33% increase in Zip1 mRNA

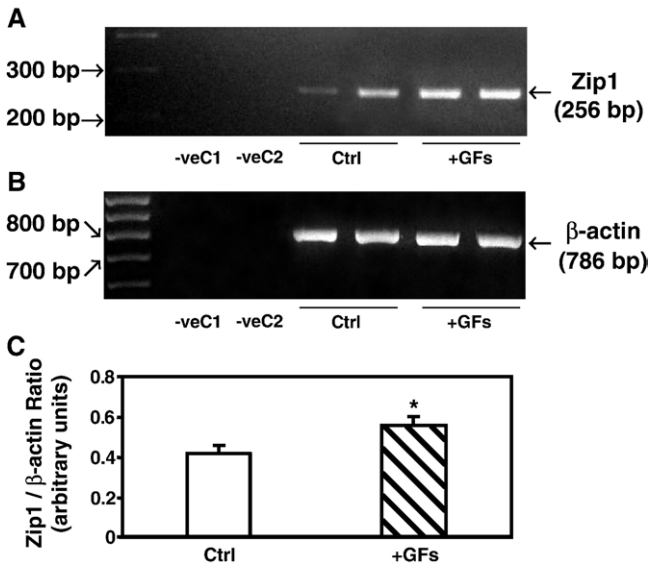

Fig. 3. RT-PCR analysis of the mRNA level of Zip1 in 3T3 cells. Representative ethidium bromide-stained agarose gel showing the representative mRNA level of Zip1 (A) and β-actin (B). (C) Relative abundance of Zip1 mRNA levels. The optical density of bands representing Zip1 mRNA was normalized to the optical density of corresponding β-actin mRNA band. Values represent mean±S.E.M. ($n=6$). An asterisk indicates significantly different from the control cells ($P<.05$). −veC1: negative control 1 (no primers); −veC2: negative control 2 (no cDNA); Ctrl: control; +GFs: PDGF-, EGF- and IGF-I-treated cells.

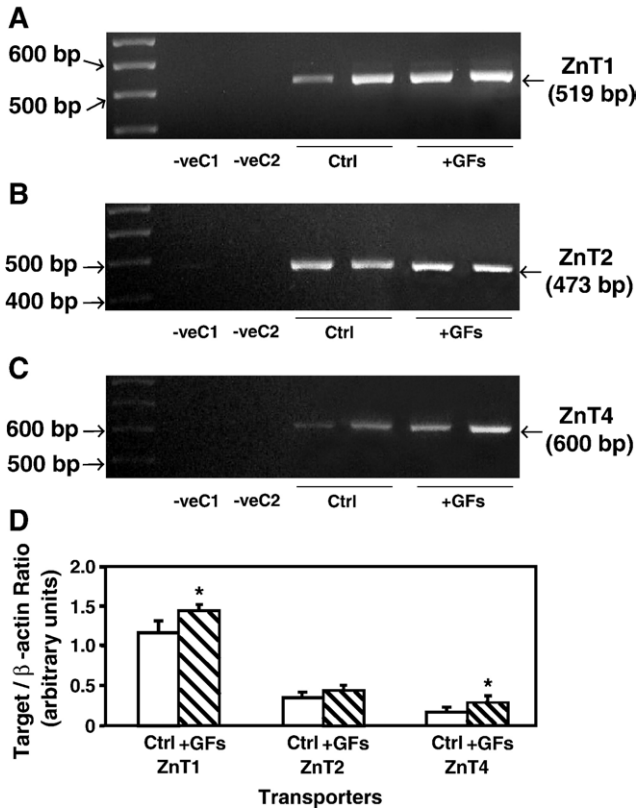

Fig. 4. RT-PCR analysis of the mRNA level of ZnT1, ZnT2 and ZnT4 in 3T3 cells. Representative ethidium bromide-stained agarose gel showing the representative ZnT1 (A), ZnT2 (B) and ZnT4 (C) mRNA level. The optical density of bands representing ZnT1, ZnT2 and ZnT4 mRNA was normalized to the optical density of corresponding β-actin mRNA band and expressed as relative abundance of ZnT1, ZnT2 and ZnT4 mRNA levels (D). Values represent mean±S.E.M. ($n=6$). An asterisk indicates significantly different from the control cells ($P<.05$). +GFs: PDGF-, EGF- and IGF-I-treated cells.

levels compared to the control cells ($P<.05$; Fig. 3). The mRNA level of ZnT1 and ZnT4 in the growth factor-treated cells was also increased by 24% and 67%, respectively, compared to the control cells ($P<.05$; Fig. 4). The ZnT2 mRNA level remained unaffected by growth factor treatment (Fig. 4).

4. Discussion

The results reported herein showed that the intensity of zinc-dependent Zinquin fluorescence was elevated in growth factor-treated cells compared to the control cells. This observation is consistent with our earlier report showing an increased abundance of LIPZ in PDGF-, EGF- and IGF-I-treated 3T3 cells using *N*-(6-methoxy-8-quinolyl)-*para*-toluenesulfonamide, another membrane-permeable fluorescence probe for zinc [1,2]. Zinquin is a fluorescent probe that is specific to zinc in biological systems, and the intensity of zinc-dependent Zinquin fluorescence is indicative of the abundance of labile intracellular zinc [22–24]. A higher intensity of zinc-dependent Zinquin fluorescence in the growth factor-treated cells indicates a greater abundance of LIPZ in response to the growth factor treatment. Coincided with the increased abundance of LIPZ, ^{65}Zn retention was also increased in growth factor-treated cells compared to the control cells. An adequate size of LIPZ is important for both DNA synthesis and cell proliferation [1,2], and growth factor promotes cell proliferation [2]. Therefore, the concomitant increase in the abundance of LIPZ and ^{65}Zn retention suggests that, in response to increased growth signal, there was an increased net zinc uptake to maintain adequate size of LIPZ to ensure sufficient zinc to meet the needs of new cells and to sustain increased cell proliferation. It is interesting to note that this increased abundance of LIPZ occurred in the absence of an increase in the total cellular zinc concentration. This lack of concomitant change in the total cellular zinc concentration and the abundance of LIPZ are possibly due to the fact that most intracellular zinc is bound to macromolecules such as proteins, and LIPZ is only a small fraction of the total cellular zinc. In addition, LIPZ is highly dynamic and transitional in response to cell needs. Therefore, the increase in the abundance of LIPZ is quantitatively too small to have an effect on the total cellular zinc concentration under the conditions employed in the present study. Hence, the occurrence of a concomitant increase in cell proliferation and an abundance of LIPZ in the absence of an increase in total cellular zinc concentration suggest that LIPZ is more responsive to the increased need for zinc during cell proliferation and affirms the concept that the abundance of LIPZ is important for cell proliferation.

In response to the treatment of a combination of PFDG, EGF and IGF-I, ^{65}zinc radioactivity was increased. Coinciding with an increased ^{65}zinc radioactivity, Zip1 mRNA levels were also elevated in response to the growth factor treatment. Zip1 is a member of the ZIP family of transporters [7,25–27]. Overexpression of hZip1 results in increased ^{65}Zn accumulation due to increased zinc uptakes in human K562 erythroleukemia cells [8] and prostate adenocarcinoma cells [27], while inhibition of hZip1 expression markedly reduced ^{65}Zn uptake [8]. Similarly, yeast mutant (ZHY3) transformed to express *Arabidopsis thaliana* Zip1 also results in accumulation of ^{65}Zn [28]. Interestingly, growth factor treatment also resulted in elevated ZnT1 and ZnT4 mRNA levels. ZnT1 and ZnT4 belong to the ZnT transporter family involved in zinc export [16,25]. For example, the overexpression of ZnT1 results in increased zinc efflux in zinc-sensitive strain of baby hamster kidney [17]. Hence, increased ^{65}zinc radioactivity in the presence of this concomitantly up-regulated expression of the transporters involved in both zinc influx and efflux indicates that the higher ^{65}zinc radioactivity in the growth factor-treated cell was a result of increased net retention of extracellular zinc in response to growth factor treatment. Moreover, zinc is essential for DNA synthesis and cell proliferation, but excessive cellular zinc accumulation causes cytotoxicity [29]. MacDiarmid et al. [30] have reported that induction of

ZRC1, which encodes a transporter involved in zinc efflux, is required for resistance to zinc shock in yeast. Therefore, concomitantly up-regulated expression of these zinc transporters perhaps reported herein suggested that these are coordinated cellular events aimed to ensure adequate zinc supply to sustain growth factor-stimulated cell proliferation while preventing potential zinc cytotoxicity.

In this study, growth factor treatment had no effect on metallothionein level. Consistent with this lack of increase in metallothionein level, total cellular zinc concentration also remained unaffected by growth factor treatment. Metallothionein is a main intracellular zinc storage protein, and its synthesis is known to be up-regulated under conditions of excess zinc to lower the concentration of free zinc ions to prevent zinc cytotoxicity. Moreover, our previous observations [1,2] and the data reported herein suggest that the increased zinc retention in response to growth factor stimulation is likely a means of ensuring that there is sufficient LIPZ to meet the increased needs for zinc required for DNA synthesis and cell proliferation. This dynamic nature of LIPZ would prevent intracellular accumulation of free zinc ions. Thus, the absence of an increase in metallothionein levels is likely because zinc retention and the concentration of free zinc ions are not sufficiently high to trigger up-regulation of metallothionein synthesis.

Labile intracellular zinc was clearly more abundant in the area surrounding the nucleus than in the rest of the cytoplasm. This perinuclear distribution pattern of LIPZ is especially noticeable in the growth factor-treated cells. The specific cause and biological significance of a higher abundance of labile zinc in the perinucleus region are presently not understood. It is possible that this perinuclear distribution pattern is related to cell proliferation. Existing evidence has shown that treating 3T3 cells with a combination of PDGF, EGF and IGF-I stimulates DNA synthesis and cell proliferation [2]. DNA synthesis and cell proliferation require an adequate supply of zinc [4–6]. Moreover, metallothionein is translocated from the cytoplasm into the nucleus in proliferating tissues [31–33] and in EGF- and insulin-stimulated rat hepatocytes [34]. The nuclear translocation of metallothionein probably serves as a vehicle for achieving a high nuclear zinc level in the S-phase of the cell cycle [35]. Furthermore, metallothionein-bound zinc contributes to the LIPZ [13,23]. Therefore, it is possible that the higher intensity of Zinquin-dependent fluorescence in the perinuclear region might be indicative of this intracellular translocation of zinc. Besides, it is also possible that this perinuclear distribution pattern indicates an accumulation of zinc in the Golgi apparatus. Kirschke and Huang [36] have shown that, in ZnT7-Myc-expressing CHO cells cultured in a zinc-supplemented medium, ZnT7 facilitates zinc accumulation in the Golgi apparatus, which has a perinuclear distribution pattern of Zinquin-dependent fluorescence. Interestingly, zinc deficiency, not zinc supplement, in HeLa cells induces a ZnT5- and/or ZnT7-facilitated zinc accumulation in the Golgi apparatus, which also has a perinuclear distribution pattern of Zinquin-dependent fluorescence [37]. In contrast, zinc supplement in HeLa cells resulted in a strong punctuate Zinquin-dependent fluorescence, most of which seems to be vesicular [37]. Golgi apparatus is involved in the packaging of newly synthesized proteins. Some of the zinc metalloenzymes are important to DNA synthesis and cell proliferation, and zinc-finger proteins are mostly transcription factors. Thus, it is possible that during growth factor-stimulated growth, zinc is accumulated in the Golgi apparatus to provide zinc to neo-synthesized zinc metalloenzymes and zinc-finger proteins. Further studies are warranted to reveal the nature of this perinuclear distribution pattern of LIPZ.

In summary, treating 3T3 cells with a combination of PDGF, EDGF and IGF-I resulted in a concomitant increase in the abundance of LIPZ and cell proliferation. However, total cellular concentration remained unchanged. These observations indicate that the abundance of LIPZ is responsive to the increased need for zinc during cell proliferation. Moreover, growth factor treatment also increased net zinc retention and mRNA level of Zip1, ZnT1 and ZnT4. Zinc is essential to DNA synthesis and cell proliferation, but excessive zinc accumulation in cells causes cytotoxicity. These observations collectively show that increase in the abundance of LIPZ during growth factor-stimulated cell proliferation was due to increased net retention of extracellular zinc, which was apparently achieved through a coordinated up-regulation of the expression of transporters involved in both zinc influx and efflux to ensure adequate supply of zinc to sustain cell proliferation, yet to prevent potential zinc cytotoxicity in 3T3 cells.

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