PTEN interacts with metal-responsive transcription factor 1 and stimulates its transcriptional activity

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MTF-1 (metal-responsive transcription factor 1) is an essential mammalian protein for embryonic development and modulates the expression of genes involving in zinc homeostasis and responding to oxidative stress. We report in the present paper that PTEN (phosphatase and tensin homologue deleted on chromosome 10) associates with MTF-1 in the cells. These two proteins interact via the acidic domain of MTF-1 and the phosphatase/C2 domain of PTEN. Depletion of PTEN reduced MT (metallothionein) gene expression and increased cellular sensitivity to cadmium toxicity. PTEN did not alter the nuclear translocation, protein stability or DNA-binding activity of MTF-1. Zinc increased MTF-1–PTEN interaction in a dose-dependent manner. The interaction elevated within 2 h of zinc addition and declined afterwards in the cells. The enhanced binding activity occurred mainly in the cytoplasm and reduced after translocating the MTF-1 into the nucleus. Blocking signalling through the PI3K (phosphoinositide 3-kinase) pathway did not alter the zinc-induced MT expression. Analysis of enzymatically inactive PTEN mutants demonstrated that protein but not lipid phosphatase activity of PTEN was involved in the regulation of MTF-1 activity. The same regulatory role of PTEN was also noted in the regulation of ZnT1 (zinc transporter 1), another target gene of MTF-1.

Key words: gene transcription, metallothionein, metal-responsive transcription factor 1 (MTF-1), phosphatase and tensin homologue deleted on chromosome 10 (PTEN), zinc transporter 1 (ZnT1).

INTRODUCTION

MTF-1 (metal-responsive transcription factor 1) is an essential gene required for the development of mouse embryos [1]. The N-terminal region of MTF-1 has six zinc (Zn)-finger motifs that serve as the DNA-binding domain for the recognition of MREs (metal-responsive elements) at the promoter region of target genes. The activation domain consists sequentially of acidic, a proline-rich and a serine/threonine-rich regions and locates penultimately to the DNA-binding motif [2]. MTF-1 resides mainly in the cytoplasm and translocates into the nucleus upon challenging with certain metals and stressors. Zn and cadmium (Cd) are the most potent metal activators of MTF-1 even though they might employ different activation mechanism. Upon metal stimulation, MT (metallothionein) genes are rapidly transcribed and translated into cysteine-rich proteins that firmly bind metal ions and sequester them from attacking cellular molecules [3]. In addition to MT genes, MTF-1 regulates the expressions of genes involving in metal homeostasis and oxidative stress, such as those encoding ZnT1 (Zn transporter 1) and the glutamate-cysteine ligase heavy chain [4,5].

MTF-1 is constitutively expressed in cells and its expression is not regulated by metals and stressors [6]. There is no indication that translational control modulates the activity of MTF-1. Post-translational modification, such as phosphorylation, can possibly alter the activity of the factor. MTF-1 can be phosphorylated at multiple sites and certain kinases may be involved in the modification [7]. However, none of the studies elucidated the exact phosphorylation sites and assigned a function to the modification. Interacting proteins, such as HIF-1α (hypoxia-inducible transcription factor 1α), C/EBPα (CCAAT/enhancer binding protein α) and NF-κB (nuclear factor κB), reportedly bind with MTF-1 in the nucleus and co-ordinately activate MT expression [8–10].

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a tumour suppressor that has a dual lipid and protein phosphatase activities. PI3K (phosphoinositide 3-kinase) phosphorylates PIP2 (phosphatidylinositol 4,5-bisphosphate) to produce PIP3 (phosphatidylinositol 3,4,5-trisphosphate) and subsequent phosphorylation of Akt [11]. The phospho-Akt activates signalling pathways involving cell growth, differentiation, motility and survival [12]. PTEN dephosphorylates and converts PIP3 into PIP2. The reaction down-regulates the signalling pathway transduced through PI3K/Akt. Depletion or dysfunction of PTEN allows constitutive activation of the PI3K/Akt signalling pathway, resulting in a loss of control in cellular proliferation with consequential tumour formation [13]. In addition to the lipid phosphatase activity, PTEN also regulates focal adhesion, cell migration, cell proliferation, apoptosis, angiogenesis, the DNA damage response and chromosome...
stability through its potential protein phosphatase activity and/or protein–protein interaction [14].

PTEN has N-terminal catalytic (phosphatase) and C-terminal regulatory (C2 and C-tail) domains. The catalytic domain contains a conserved signature motif (HCXXGXXR) found in dual-specific protein phosphatases [15]. Mutations within this catalytic domain, such as a C124S mutation, abrogate PTEN catalytic activity [11]. A PDZ-binding domain located at the C-terminus of PTEN and interacts with PDZ-containing proteins such as the MAGI (membrane-associated guanylate kinase with inverted structure) protein family and NHERF (Na+/H+ exchanger regulatory factor) [14]. In addition to the PDZ-binding domain, several key serine and threonine phosphorylation sites (Ser380, regulatory factor) [14]. In addition to the PDZ-binding domain, supplementation as above and 0.15 DMEM (Dulbecco’s modified Eagle’s medium) with the same HEK (human embryonic kidney)-293 cells were cultured in Other chemicals were obtained from Sigma unless specified. Reagents for cell culture were purchased from Invitrogen/Gibco.

MTF-1 is required for the integral function of MTF-1 that investigated in the present study. We demonstrate that PTEN interacts with and modulates MTF-1 activity. This interaction with PTEN is required for the integral function of MTF-1 that positively stimulates MT and ZnT1 expression. Our results reveal a novel mechanism for PTEN in regulating MTF-1 activity.

**MATERIALS AND METHODS**

**Cell culture and chemicals**

CHO (Chinese-hamster ovary) K1 cells were cultured as monolayers at 37°C in McCoy’s 5A medium supplemented with 10% heat-inactivated FBS (fetal bovine serum), 0.22% sodium bicarbonate, 100 units/ml ampicillin and 100 mg/ml streptomycin in 5% CO₂/95% air and 100% humidity. HEK (human embryonic kidney)-293 cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) with the same supplementation as above and 0.15% sodium bicarbonate. Reagents for cell culture were purchased from Invitrogen/Gibco. Other chemicals were obtained from Sigma unless specified. ZnSO₄ and CdCl₂ were purchased from SERVA and Merck respectively. Anti-His and anti-HA (haemagglutinin) antibodies were purchased from Santa Cruz Biotechnology. Antibody B1 (ab16048) was purchased from Abcam. Anti-tubulin-α antibody (Clone DM1A) was from Thermo Scientific. Anti-PTEN, p-Akt (S473) and Akt (Akt1/2) antibodies were from Cell Signaling Technology. Anti-FLAG (M2) antibody was from Sigma–Aldrich. Inhibitors of PI3K and Akt (LY294002 and Akt inhibitor IV) were from Sigma–Aldrich and Merck respectively. Transfection was performed using Lipofectamine™ 2000 (Invitrogen) and Lipofectamine™ RNAiMAX (Invitrogen) following the manufacturer’s instructions. siRNAs (small interfering RNAs) targeting PTEN, MTF-1, Akt1, Akt2, Akt3 and Stealth RNAi™ siRNA Negative Control were purchased from Invitrogen.

**Plasmid construction**

Mouse MTF-1 was amplified by PCR and inserted into the pcDNA4/V5-HisB plasmids (Invitrogen) that had been digested with HindIII and AgeI, to produce the MTF-1–HisB expression plasmids. MTF-1 of different lengths (1–317, 1–405, 1–498 and 1–620) and deletions (Δ317–405 and Δ317–329) were amplified with gene-specific primers and cloned into pcDNA4/V5-HisB plasmids for the expression of various truncated forms of MTF-1. The acidic domain (329–405) and the proline-rich domain (406–498) of MTF-1 were amplified with gene-specific primers and cloned into pcDNA4-Egfp-HisB plasmids to produce GFP (green fluorescent protein)–AcD (acidic domain)–HisB, and the GFP–ProD (proline-rich domain)–HisB, expression plasmids. Human PTEN, SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase 2) and PTP1B (protein tyrosine phosphatase 1B) cDNAs were amplified by PCR with primers containing EcoRI or XhoI sites, and inserted into pIRE5–FLAG, or pcDNA3-HA vectors to produce PTEN–FLAG, SHP-2–FLAG and HA–PTP1B expression plasmids. Expression plasmids encoding various PTEN fragments (1–351, 1–185, 186–351, 186–403 and 351–403) were amplified with gene-specific primers and cloned into the pIRE5–FLAG vectors. The PTEN mutants C124S, G129R, G129E and Y138L were created with the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions.

**Transfection and real-time PCR**

For plasmid and siRNA transfection, 5 × 10² and 4 × 10² HEK-293 cells were seeded in six-well plates for 48 and 24 h respectively. Transfections were conducted using 4 μg of plasmid DNA and Lipofectamine™ 2000, or 10 nM siRNA and Lipofectamine™ RNAiMAX following the manufacturer’s instructions. To express proteins in CHO K1 cells, 6 × 10⁵ cells were seeded in 6 mm dishes for 48 h. Transfection was then performed using Lipofectamine™ 2000.

Total RNA was extracted with TRIzol® reagent (Invitrogen) following the manufacturer’s instructions. The extracted RNA (1.5 μg) was reverse-transcribed with a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). The resulting complementary DNA was used for quantitative real-time PCR. SYBR® Green PCR Master Mix (Applied Biosystems) was used for the reactions. Real-time PCR experiments were performed on an Applied Biosystems 7500 Real-Time PCR System with the primers listed in Supplementary Table S1 (available at http://www.BiochemJ.org/bj/441/bj4410367add.htm). The expression of human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was determined for each sample and used as internal reference. Expression of MT-IIA or ZnT1 mRNA was compared on the basis of equivalent GAPDH transcripts.

**Cell viability assay**

HEK-293 cells (7 × 10⁴) were seeded in 96-well plates for 24 h and then transfected with PTEN siRNA. At 2 days after transfection, cells were treated with various concentrations of CdCl₂ and cultured for an additional 24 h. Premixed WST-1 (water-soluble tetrazolium salt) 1 Cell Proliferation Reagent (Clontech) was added at 1/10 of the medium volume 4 h before the end of the culture period. The plates were then shaken thoroughly for 1 min before the attendance (D) at 450 nm and the reference wavelength at 600 nm were recorded using a ThermoMax microplate reader (Molecular Devices).

**Preparation of whole-cell extracts**

Harvested cells were pelleted and resuspended in five cell volumes of extraction buffer [20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM
PMSF, 50 mM NaF, 0.5 mM Na<sub>2</sub>VO<sub>4</sub>, mixed protease inhibitors (2 μg/ml aprotinin, 5 μg/ml leupeptin and 1 μg/ml pepstatin) and 0.5 % Nonidet P40. The tubes were rocked vigorously at 4°C for 40 min. Cell debris was removed by centrifugation at 16000 g for 10 min at 4°C. The supernatant (whole cell extracts) was collected and stored at −70°C. The extracts were used for immunoprecipitation and EMSA (electrophoretic mobility-shift assay). Protein concentration was determined using a dye-binding assay with chemicals purchased from Bio-Rad Laboratories. The procedures for EMSA have been described previously [21].

Immunoprecipitation

Whole-cell extracts were mixed with PBS containing 0.4 % Nonidet P40 and mixed protease inhibitors to the volume of 1 ml. The mixture was incubated at 4°C with 10 μl of ImmunoPure<sup>®</sup> immobilized Protein A/G–agarose beads (Pierce) for 30 min then centrifuged. The supernatant was collected and incubated with specific antibodies overnight at 4°C on a rocking platform. Fresh Protein A/G–agarose beads (20 μl) were then added to the reaction mixture and incubated at 4°C for 2 h. Thereafter, Protein A/G–agarose beads were collected and rinsed three times with PBS containing 0.4 % Nonidet P40. The beads were resuspended in 2× SDS loading buffer [0.125 M Tris/HCl (pH 6.8), 4 % SDS, 20 % glycerol, 0.02 % Bromophenol Blue and 0.2 M DTT (dithiothreitol)], and boiled for 5 min. The supernatants were analysed by Western blotting as previously described [21].

Metal-affinity purification

Harvested cells were pelleted and resuspended in 200 μl of equilibration buffer [50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.0), 300 mM NaCl, 10 mM imidazole, 0.4 % Nonidet P40, 10 % glycerol and mixed protease inhibitors]. Cells were lysed by rocking vigorously at 4°C for 40 min and debris was removed by centrifugation at 16000 g for 10 min at 4°C. The supernatant was collected and the volume was brought to 1 ml with equilibration buffer. TALON<sup>™</sup> metal-affinity resins (50 μl, Clontech) was added to the sample and incubated at 4°C for 1 h. Thereafter, the TALON<sup>™</sup> resins were collected and sequentially washed twice with buffers containing 20, 30 or 40 mM imidazole. The proteins bound on to the resins were eluted with 300 mM imidazole and analysed by Western blotting.

Separation of cytosolic and nuclear fractions

Harvested cells were resuspended in a hypotonic buffer [10 mM Hepes (pH 7.9), 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 50 mM NaF, 0.5 mM Na<sub>2</sub>VO<sub>4</sub>, mixed protease inhibitors and incubated on ice for 15 min before Nonidet P40 was added to a final concentration of 0.5 %. After shaking vigorously for 10 s, the homogenate was centrifuged at 16000 g for 5 min at 4°C and the supernatant was removed and designated as the cytosolic fraction. The pellet was resuspended in extraction buffer [20 mM Hepes (pH 7.9), 0.4 M NaCl, 0.5 mM DTT, 1 mM PMSF, 50 mM NaF, 0.5 mM Na<sub>2</sub>VO<sub>4</sub> and mixed protease inhibitors] and rocked vigorously at 4°C for 40 min. The mixture was centrifuged for 10 min and the supernatant was collected as the nuclear fraction.

ChIP (chromatin immunoprecipitation) assay

An EZ ChIP<sup>TM</sup> Chromatin Immunoprecipitation Kit (Millipore) was used for the assay according to the manufacturer’s instructions. Briefly, HEK-293 cells (2 × 10<sup>5</sup>) transfected with plasmids encoding MTF-1-His<sub>6</sub> and PTEN-FLAG were treated with or without 100 μM ZnSO<sub>4</sub> for 3 h before fixing with 1 % formaldehyde. After quenching with 0.125 M glycine, cells were washed and resuspended in lysis buffer, and genomic DNAs were sheared to 200–1000 bp in length with an ultrasonic processor (model UP50H, Dr. Hielsgers). Cell debris was removed by centrifugation and 100 μl of the supernatant was diluted with 900 μl of dilution buffer. Then a 10 μl aliquot from each sample was removed for target DNA quantification (designated as input) and the rest was incubated with 4 μg of anti-His<sub>6</sub> or anti-FLAG antibodies at 4°C overnight. The protein–DNA complexes were pulled down with Magna ChIP<sup>™</sup> Protein A magnetic beads (Millipore) at 4°C for 2 h. After washing with different buffers, the protein–DNA complexes were eluted. The input and the immunoprecipitated samples (with the NaCl concentration brought to 0.2 M) were incubated at 65°C overnight to disrupt the DNA–protein linkages, and then treated sequentially with RNase A and proteinase K. DNA in each sample was then extracted by Wizard<sup>®</sup> SV Gel and PCR Clean-Up System (Promega) before being used as templates in PCR. Primers were used to yield fragments covering the −203 to +28 region of the human MT-IIA gene. After 30 cycles of amplification, the products were analysed on 1.5 % agarose gels.

RESULTS

MTF-1 interacts with PTEN

It has been reported that MTF-1 can be phosphorylated at multiple sites [7]. We hypothesize that phosphatases are present to reverse the modifications. In initial experiments, we analysed whether PTEN, PTP1B or SHP-2 could modulate MTF-1 activity. MTF-1 was co-expressed with individual phosphatases in CHO K1 cells and immunoprecipitated. As shown in Figure 1(A), PTEN was co-precipitated with MTF-1. Supporting experiments also demonstrated that MTF-1 was in the precipitated fraction when PTEN was immunoprecipitated from the cell extracts. However, this interaction was not found for PTP1B or SHP-2 (Figure 1B), implying that the interaction between PTEN and MTF-1 is specific.

MTF-1–PTEN-interacting regions

To analyse the region where MTF-1 interacts with PTEN, nested deletions of MTF-1 were performed by sequentially removing the serine/threonine-rich, the proline-rich and the acidic domains from the C-terminus. The constructs with a C-terminal His<sub>6</sub>-tag were co-expressed with PTEN and the truncated MTF-1s were isolated by metal-affinity resins. Figure 2(A) shows that the protein–protein interaction was abolished after deleting the acidic domain. To further demonstrate that the acidic domain of MTF-1 was sufficient for PTEN binding, a construct removing only the acidic domain (Δ317–405) was constructed. Since a linker sequence exists between the Zn-finger and acidic domains, this region was also removed (Δ317–329) to examine the PTEN-binding site. The results demonstrate clearly that deleting the linker sequence did not affect the binding with PTEN (Figure 2B). This interaction was eliminated upon the removal of the acidic domain. The acidic domain (329–405) was then fused with eGFP (enhanced green fluorescent protein) (GFP–AcD–His<sub>6</sub>), and the chimaeric protein was used to interact with PTEN. For comparison, an eGFP fused with the proline-rich domain (GFP–ProD–His<sub>6</sub>) was used as a control. As shown in Figure 2(C), the acidic domain was sufficient to bind PTEN without the presence of other structural motifs in MTF-1.

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Figure 1  Interaction of PTEN with MTF-1

(A) His6-tagged MTF-1 and FLAG-labelled PTEN were co-expressed in CHO K1 cells. The proteins were immunoprecipitated (IP) with anti-His or anti-FLAG antibodies and then immunoblotted (IB) with the designated antibodies. (B) The same experiment was conducted by co-expressing His6-tagged MTF-1 with HA-labelled PTP1B or FLAG-labelled SHP-2.

Figure 2  Analysis of the interacting regions between MTF-1 and PTEN

(A) His6-tagged mouse MTF-1 with various C-terminal deletions was co-expressed with FLAG-labelled human PTEN in CHO K1 cells. The MTF-1s were pulled down with metal-affinity resins and then immunoblotted (IB) with the designated antibodies. (B) His6-tagged MTF-1 with deletion at the linker region (Δ317–329) or linker plus acidic domain (Δ317–495) was co-expressed with FLAG-labelled PTEN. The truncated MTF-1s were immunoprecipitated (IP) with anti-His antibodies then immunoblotted (IB) with the designated antibodies. (C) GFP fused to the acidic domain (329–405; GFP-AcD-His) or the proline-rich domain (406–498; GFP-ProD-His) of MTF-1 was co-expressed with FLAG-labelled PTEN. The truncated MTF-1s were immunoprecipitated with anti-His antibodies and then immunoblotted with the designated antibodies. (D) His6-tagged MTF-1 was co-expressed with various lengths of FLAG-labelled PTEN fragments. The MTF-1 was pulled down with metal-affinity resins and then immunoblotted with the designated antibodies. (E) GFP–AcD–His6 was co-expressed with various FLAG-labelled PTEN fragments. The GFP–AcD–His6 was pulled down with metal-affinity resins and then immunoblotted with the designated antibodies.

The region where PTEN interacts with MTF-1 was also investigated. Clones with phosphatase and C2 domain (1–351), C2 and C-tail domain (186–403), and clones containing individual (phosphatase, C2 and C-tail) domains were constructed and expressed for binding analysis. Figure 2(D) shows that MTF-1 interacts with PTEN mainly at the phosphatase and C2 domains. To further identify the interacting region, GFP–AcD–His6 was co-expressed with phosphatase, C2 or C-tail fragment and pulled down by metal-affinity resins. As shown in Figure 2(E), both phosphatase and C2 fragment could interact with GFP–AcD. The results are consistent with that of Figure 2(D). Therefore these two proteins interact via the acidic domain of MTF-1 and the phosphatase/C2 domain of PTEN.

Effect of PTEN on the transcriptional activity of MTF-1

Since PTEN is associated with MTF-1, the biological significance of the interaction was investigated. First, the effect of PTEN overexpression on the MTF-1 activity was estimated. First, the effect of PTEN overexpression on the MT genes, the major target genes of MTF-1, are not expressed in CHO K1 cells, HEK-293 cells were used for the
PTEN regulates MTF-1 activity

Effect of PTEN on MT-IIA gene expression and the cellular susceptibility to Cd toxicity

The cellular PTEN level in HEK-239 cells was elevated by transfecting with the PTEN–FLAG gene (A) or suppressed with siRNA (B). Cells were then treated with or without 100 μM Zn for 6 h. The relative quantity of MT-IIA mRNA was determined by real-time PCR. The protein levels of PTEN in the samples, as estimated by immunoblotting (IB), are shown in the upper panels of each plot. Closed and open triangles indicate exogenous and endogenous PTEN respectively. (C) HEK-293 cells were transfected with control or PTEN siRNA. The transfected cells were then treated with 100 μM Zn for various time intervals. MT-IIA mRNA was quantified and compared. (D) HEK-293 cells were transfected with control or PTEN siRNA. The transfected cells were treated with various concentrations of Cd for 24 h and the tolerance to Cd toxicity was compared using the WST-1 assay. Each value represents a mean ± S.D. for three samples. Asterisks (*) denote a significant difference (P < 0.05). siPTEN, PTEN siRNA.

Effect of PTEN on protein stability, nuclear translocation and MRE-binding activity of MTF-1

The effect of PTEN on the transcriptional activity of MTF-1 may be due to an alteration in protein stability as for p53 [23]. Experiments were conducted to clarify this hypothesis. MTF-1 specific antibodies are not available. Therefore MTF-1–His6 was co-expressed with or without PTEN in CHO K1 cells and the turnover of the exogenous proteins was determined in the presence or absence of Zn after protein synthesis was blocked with cycloheximide. Figure 4(A) shows that MTF-1–His6 stability was not altered by overexpressing PTEN either in the presence or absence of Zn.

We also investigated whether nuclear translocation and MRE-binding activity of MTF-1 were altered by the quantity of PTEN. MTF-1–His6, was again co-expressed with or without PTEN in CHO K1 cells. Cytosolic and nuclear fractions of the cells were isolated after exposing the cells to Zn for various time intervals. MTF-1 in each fraction was analysed. As shown in Figure 4(B), the level of cytosolic MTF-1 reduced, whereas nuclear MTF-1 increased after Zn stimulation. However, the rate of nuclear translocation was not affected by the expression of exogenous PTEN.

To examine the effect of PTEN on MRE-binding activity of MTF-1, CHO K1 cells were expressed with or without exogenous PTEN. Whole-cell extracts were prepared and used to analyse the MRE-binding activity of endogenous MTF-1 by EMSA. A binding between MTF-1 and MRE can be observed in the absence of Zn treatment. The binding activity increased with the addition of Zn to cells. Noticeably, the expression of PTEN did not affect the MRE-binding activity (Figure 4C).

Cellular PTEN was further depleted by siRNA to analyse the effect on those properties described above. A CHO K1 cell line stably expressing His6-tagged MTF-1 was established and then transfected with PTEN siRNA. The MTF-1 stability, nuclear translocation and MRE-binding activity were evaluated in the presence or absence of Zn. The results show that these properties were not altered by depleting PTEN (Supplementary Figure S3 at http://www.BiochemJ.org/bj/441/bj4410367add.htm).

Effect of Zn on PTEN stability

Previous studies revealed that Zn induces PTEN degradation in neural and airway epithelial cells [19,24]. To investigate whether the modulation of MTF-1 activity was due to an alteration in the PTEN level with Zn treatment, the PTEN level was determined after treating cells with Zn for various time intervals. Figure 5(A) shows that the amount of PTEN was not affected by Zn treatment.
Figure 4 Effect of PTEN overexpression on MTF-1 stability, nuclear translocation and MRE-binding activity

(A) MTF-1 was co-expressed with or without PTEN in CHO K1 cells. CHX (cycloheximide, 50 μM) alone or with 100 μM Zn was added to the cells. Samples were removed at various time intervals and proteins were quantified with immunoblotting. Tubulin was used as loading control. (B) MTF-1 was co-expressed with or without PTEN in CHO K1 cells. The cells were treated with or without 100 μM Zn for various time intervals. Cytosolic and nuclear fractions were isolated and the expressed proteins were quantified by immunoblotting. Tubulin and lamin were used as loading controls for cytosolic and nuclear proteins respectively. (C) PTEN was overexpressed in CHO K1 cells. The cells were treated with or without 100 μM Zn for 2 h and whole-cell extracts were prepared for EMSA. The expression of PTEN was demonstrated by Western blotting (right-hand panel). FP, free probe; CP, non-radiolabelled MREs were added as competitors. Solid and open triangles indicate exogenous and endogenous PTEN respectively. IB, immunoblotted.

Since Zn is the key regulator of MTF-1 activity, the role of Zn on the interaction of MTF-1 and PTEN was investigated. Cells were co-transfected with MTF-1–His6 and PTEN–FLAG genes, and the interaction of the two proteins was analysed in the presence or absence of Zn. The interaction between MTF-1 and PTEN was enhanced in cells treated with Zn for 2 h (Figure 5B). We then investigated whether the Zn-enhanced binding is dose-dependent. The transfected cells were administered with different concentrations of Zn for 2 h and the interaction between MTF-1 and PTEN was estimated. The results show that the interaction increased in a dose-dependent manner (Figure 5C). A time course study of the interaction following Zn treatment was also conducted. As shown in Figure 5(D), the MTF-1–PTEN binding increased within 1 h of Zn treatment. This binding reached a maximum at 2 h of Zn exposure and declined with further treatment.

Cellular compartment for Zn-enhanced MTF-1–PTEN interaction

MTF-1 translocates into the nucleus after Zn exposure. It is not clear where the enhanced PTEN–MTF-1 binding occurs. To address this issue, MTF-1 and PTEN were co-expressed in the cells. The cytosolic and nuclear fractions were isolated from cells with and without Zn exposure. Western blot analysis showed that both proteins reside mainly in the cytoplasm. With the addition of 100 μM Zn, most of the cytosolic MTF-1 moved into the nucleus within 2 h (Figure 6A). However, PTEN resided mainly in the cytoplasm and only a small amount of PTEN was detected in the nuclear fraction. The relative quantity of PTEN in each fraction was not altered by the Zn treatment.

MTF-1 in cytosolic and nuclear fractions of the Zn-treated cells was then pulldown by metal-affinity resins and the binding
PTEN was determined. The PTEN–FLAG/MTF-1–His6 ratio of the control (Zn) cytosolic fraction with the designated antibodies. The intensity of the PTEN–FLAG and MTF-1–His6 band in each fractions with the above treatment was isolated with metal-affinity resins and immunoblotted nuclear fractions were analysed by immunoblotting (IB). (Figure 6B). Treating the cells with or without 100 μM Zn for 2 h, the proteins expressed in the cytosolic and nuclear fractions were analysed by immunoblotting (IB). (B) The MTF-1 in cytosolic and nuclear fractions with the above treatment was isolated with metal-affinity resins and immunoblotted with the designated antibodies. The intensity of the PTEN–FLAG and MTF-1–His6 band in each sample was determined. The PTEN–FLAG/MTF-1–His6 ratio of the control (Zn) cytosolic fraction was designated as 1. The relative quantities of PTEN bound with MTF-1 in both fractions were compared. CF, cytosolic fraction; NF, nuclear fraction. (C) FLAG-labelled PTEN was co-expressed with His6-tagged MTF-1 in HEK-293 cells. After treating the cells with or without 100 μM Zn for 3 h, cells were harvested for the ChIP assay (see the Materials and methods section). Antibodies as indicated were used to pull down the protein–DNA complex and the MT-IIA promoter in the samples were determined by PCR using a gene-specific primer set. NTC, no template control; Input, an aliquot of sample was prepared and used as a template for PCR to examine the level of MT-IIA promoter before immunoprecipitation (IP).

with PTEN was analysed. A distinctive profile of the MTF-1–PTEN interaction was noted when comparable amounts of MTF-1 samples were displayed on the same Western blots. After metal exposure, cytosolic MTF-1 showed an increase whereas nuclear MTF-1 exhibited a decrease in complex formation with PTEN (Figure 6B). In the absence of Zn treatment, a relatively lower amount of MTF-1 and PTEN complexes were observed for the cytosolic fractions. Upon Zn treatment, MTF-1 in the cytoplasm showed a marked increase, whereas those in the nucleus had a significant decrease in complex formation with PTEN. These results suggest that the enhancement of the PTEN–MTF-1 interaction occurs mainly in the cytoplasm at the early stage of Zn exposure. The interaction dramatically reduced once MTF-1 translocated into the nucleus.

We further investigated whether MTF-1 and PTEN interact in the nucleus after MTF-1 bound to the MT promoter. MTF-1 and PTEN were co-expressed in cells. After cross-linking the proteins with DNA, MTF-1 or PTEN was immunoprecipitated for a ChIP assay. As shown in Figure 6(C), DNA fragments covering the MT-IIA promoter region were pulled down by antibodies against MTF-1 and can be visualized clearly after PCR amplification especially in cells treated with Zn. These results could not be repeated when antibodies against PTEN were used in the immunoprecipitation. The data indicate that MTF-1 does not associate with PTEN after binding on to the target gene.

Protein but not lipid phosphatase activity of PTEN modulates MTF-1 activity

PTEN contains a dual lipid and protein phosphatase activity. We investigated whether phosphatase activity modulates the function of MTF-1. Since Zn treatment reportedly enhanced Akt activity [25,26], we examined whether the regulation of MTF-1 by PTEN was Akt-dependent. The degree of Akt phosphorylation was analysed after treating HEK-293 cells with Zn for various time intervals. Akt activity was apparently not affected by Zn treatment since the level of phosphorylated (active) Akt was not altered by up to 480 min of Zn exposure (Figure 7A). Cells were then treated with P38K (LY294002) or Akt (Akt inhibitor IV) inhibitor before Zn administration and MT-IIA gene expression was analysed. These inhibitors had no effect on MT-IIA expression (Supplementary Figure S4 available at http://www.BiochemJ.org/bj/441/bj4410367add.htm). Furthermore, Akt siRNAs were used to knockdown the Akt expression and the level of MT-IIA mRNA was analysed. Since the Akt family has three isoforms, siRNAs for these isoforms were used in combination and they did not affect MT-IIA expression in the absence or presence of Zn (Figure 7B). Since PTEN modulates P38K/Akt activity through lipid phosphatase activity, the results described above suggest that PTEN does not regulate MTF-1 through the lipid phosphatase activity.

Several human PTEN mutants without phosphatase activity have been identified. The C124S and G129R mutants are defective in both lipid and protein phosphatase activities, whereas G129E and Y138L mutants lost the lipid and protein phosphatase activity respectively [27–30]. These mutants were used to investigate the phosphatase activity of PTEN required for MTF-1 regulation. We analysed the effect of various PTEN mutants on MT expression. MT-IIA mRNA in HEK-293 cells exposed to Zn for 6 h was quantified. Zn-induced MT-IIA mRNA levels increased with the expression of exogenous PTEN (Figure 7C, Wt compared with vector). Noticeably, the enhancing effect was diminished in cells expressing C124S, G129R or Y138L. However, the level of MT-IIA mRNA was not affected in cells with G129E expression. Since G129E loses only the lipid phosphatase activity and all other mutants have a defect in the protein phosphatase activity, this result implies that the protein phosphatase activity of PTEN regulates MTF-1 activity.

Role of PTEN in modulating ZnT1 gene expression

ZnT1 is a transmembrane protein responsible for transporting cytosolic Zn out of the cell membrane [31]. Since ZnT1 expression was reportedly regulated by MTF-1 [4], the expression of ZnT1 in HEK-293 cells was examined after Zn treatment. As shown in Figure 8(A), Zn stimulates the transcription of ZnT1. To affirm that the ZnT1 gene is regulated by MTF-1, ZnT1 expression was examined after MTF-1 expression was suppressed with siRNA. ZnT1 expression declined with a decrease in MTF-1 (Figure 8B).

Since MT-IIA expression was modulated by PTEN, the role of PTEN in the expression of ZnT1 was also investigated. A time-dependent analysis of ZnT1 gene expression was conducted under PTEN-depleted conditions. HEK-293 cells were transfected with PTEN siRNA. ZnT1 expression was determined at various time intervals after Zn exposure. Figure 8(C) shows that ZnT1 expression promptly increased with Zn treatment and reached the highest level within 2–4 h of Zn exposure. The expression declined significantly at the 6 h time point. Under PTEN-depleted conditions, ZnT1 expression was reduced significantly at 2–4 h as compared with that of the control cells. The difference became insignificant at 6 h of Zn treatment.

DISCUSSION

We report in the present paper that PTEN can associate with and positively regulate the transcriptional activity of MTF-1. This is the first study to show that a non-transcriptional factor/enhancer regulates MTF-1 activity. MTF-1 activates the expression of
various genes in response to metal and oxidative stresses [32,33]. Functional MTF-1 is thus important in maintaining cell integrity when subjected to chemical insults. PTEN is another critical factor in regulating cell activity. Besides loss of growth control, marked increase of ROS (reactive oxygen species) were detected in PTEN-depleted cells [34]. MT and glutathione are critical ROS scavengers in cells whose synthesis are regulated by MTF-1 [3,5]. Since MTF-1 activity is down-regulated in PTEN-depleted cells (Figure 3), the metal resistance and antioxidative capacity can be reduced. These cells are expectedly more susceptible to various damaging agents. With decreased capacity in cell defence and growth control, occurrence and progression of tumours can be facilitated. Dysfunction or depletion of PTEN is frequently observed in various tumours [14]. Reduction of MT expression has also been correlated with the development of hepatocarcinoma [9,17]. The decline of MT expression derived from the PTEN defect may be one of the factors which participates in the tumorigenesis of cells.

MTF-1 associates with PTEN in the cytoplasm even in the absence of Zn stimulation. This is a novel finding in the regulation of MTF-1. Few proteins have been reported to interact with MTF-1. MTF-1 functions in the nucleus in the presence of stimulator(s). For instance, MTF-1 co-ordinates with HIF-1α under hypoxic conditions to bind antioxidant responsive element in MT promoters. However, whether a direct binding exists for the two proteins remains unknown [10]. Moreover, NF-κB moves into the nucleus under hypoxic conditions and binds with MTF-1 to the promoter of PGIF (placenta growth factor) for gene activation [8]. A previous study indicates that MTF-1 binds p300/CBP [CREB (cAMP-response-element-binding protein)-binding protein] to enhance the transcription of MT genes after Zn treatment [35]. The p300/CBP forms a co-activator complex with MTF-1 after Zn induction and enhances MT-I gene expression. NF-κB and p300/CBP associate with MTF-1 after stimulation, but PTEN interacts with MTF-1 in the absence of stimulators. Despite the presence of the interaction, we cannot exclude the possibility that PTEN associates with MTF-1 via additional factor(s) or they are parts of a large macromolecular complex.

PTEN interacts with a variety of proteins. However, it does not necessarily serve as a phosphatase for the interaction. PTEN physically associates with CENP-C (centromere protein C) at chromosomes to reduce chromosomal instability [36]. PTEN also associates with MSP-58 (38-kDa microspherule protein) to prevent MSP-58-driven cellular transformation [37]. Both effects are independent of phosphatase activity. Interestingly, PTEN regulates p53 stability in a phosphatase-dependent manner by blocking the Akt/Mdm2 (murine double minute 2) pathway and a phosphatase-independent manner via direct interaction with p53 [23]. As a phosphatase, the best characterized function of PTEN is to act as a tumour suppressor via regulating the PI3K signal transduction pathway [11,14]. This is the key role for the lipid phosphatase activity of PTEN. As for the protein phosphatase activity, PTEN may act on STAT3 (signal transducer and activator of transcription 3), FAK (focal adhesion kinase) or FYN kinase to inhibit tumour growth, migration and invasion [38–41]. We have shown in the present study that PTEN also exerts protein phosphatase activity to modulate MTF-1 activity.

We demonstrated in the present study that the acidic domain of MTF-1 interacts with PTEN. Although MTF-1 interacts with a few proteins under defined conditions, the interacting site is mostly uncharacterized. So far, p300 is the only protein that has been analysed and it also binds to the acidic domain of MTF-1 [35]. However, the p300–MTF-1 interaction is Zn-dependent, which differs from that of PTEN–MTF-1. The increased binding of p300–MTF-1 occurs at the nucleus upon Zn induction. The PTEN–MTF-1 interaction rises in the cytoplasm and falls in the nucleus after Zn administration. There are three HCCC-type Zn-binding clusters in p300. It is speculated that Zn may change the conformation of p300 and facilitate the binding to MTF-1 [35,42]. No characteristic of a classical or non-classical Zn-finger motif can be identified on PTEN. Although the binding of either PTEN or p300 with MTF-1 can be enhanced by Zn, the interacting mechanism is apparently different.

A previous study indicated that expression of MT genes in HCC (hepatocellular carcinoma) cells was regulated by PI3K and its downstream effectors [9]. PI3K is highly activated in HCC cells and stimulates Akt activity. However, activation of Akt negatively regulates the activity of glycogen synthase kinase-3, which is responsible for the activation of C/EBPα to enhance MT gene expression. Therefore HCC (e.g. Hep3B or primary HCC) cells have low levels of basal and metal-induced MT expression. Addition of a PI3K inhibitor significantly enhances...
MT expression in Hep3B cells. Interestingly, this phenomenon was not observed in non-HCC liver cells (e.g. THLE-2 or primary liver cells). Reduction of MT expression was thus suggested as a biomarker for the occurrence of liver cancer [9].

Akt activity is negatively regulated by PTEN. Since depletion or a defect of PTEN is frequently found in HCC cells [43–45], elevation of PI3K/Akt activity is expected in HCC cells. Consequently, a reduction in MT gene expression (as in PTEN-knockdown cells; Figure 3B) can be noted. This conclusion implies that lipid phosphatase activity of PTEN (key regulator of Akt pathway) is indirectly involved in the suppression of MT expression. In the presence of PTEN, Akt activity is low and tightly regulated, which leads to higher basal and metal-induced MT expression (as compared between THLE-2 and Hep3B cells). Addition of PI3K/Akt inhibitor did not show an effect on MT expression in THLE-2 cells since Akt activity was low originally [9]. Therefore we hypothesize that PTEN may play dual functions in regulating MT gene expression. The lipid phosphatase activity of PTEN suppresses PI3K/Akt signalling and indirectly activates CREBα to augment MT expression, whereas the protein phosphatase activity of PTEN acts directly on MTF-1 to enhance MT expression. However, whether this mechanism applies ubiquitously to different cell types remains to be investigated.

Zn treatment causes various effects on PTEN turnover in different cell types. Zn was reported to induce PTEN degradation in human airway epithelial cells through an ubiquitin–proteasome proteolytic process [24]. The same effect was observed in neural cells [19]. NEDD (neural-precursor-cell-expressed developmentally down-regulated) 4-1, an E3 ubiquitin ligase, was demonstrated to participate in the Zn-mediated PTEN degradation in neural cells. Reduction of the PTEN level can attenuate MT expression upon Zn exposure. Neural cells are very sensitive to Zn insult [46]. Reduced MT expression may attribute in part to the toxicity since Zn cannot be effectively sequestered by MTs. However, Zn-mediated PTEN degradation was not noted in HEK-293 and CHO K1 cells 6 h after Zn exposure (Figure 5A). MT expression is reported in several studies to reach a plateau at 6 h of Zn exposure and remains high for at least 12 h [47,48]. If PTEN is degraded in those cells, MT expression is expected to fall along with induction time. Therefore the Zn-mediated PTEN degradation may be cell-type-specific.

Depletion of PTEN down-regulates MT-IIA and ZnT1 gene expression (Figures 3C and 8C). This result shows a consistent effect of PTEN on the regulation of MTF-1 target genes. Differential regulation of MTF-1 target genes, especially MT and ZnT1, has been observed in several studies. Zn-induced MT expression, was suppressed in HCC cells, but ZnT1 expression was not affected [9]. Knockdown of p300 reduced MT gene expression but had no effect on the ZnT1 gene [35]. For HCC cells, the difference in gene expression may be attributed to the absence of CREBα-binding sequence in the ZnT1 promoter, but the presence in the MT promoter. Activation of CREBα enhances MT gene expression. However, this explanation is not applicable to p300 since it is not a transcription factor. MTF-1 binds with p300 and selectively enhances MT expression with Zn [49]. However, the stimulatory effect of Zn on MTF-1 phosphorylation was not observed in another study [7]. Although some kinases have been implicated in the modification of MTF-1 [50], the phosphorylation sites of MTF-1 have not been identified. It is difficult to investigate whether PTEN reverses some of the modifications on MTF-1. We showed in the present study that PTEN interacts and stimulates MT gene expression. PTEN does not alter the protein stability, nuclear translocation and MRE-binding activity of MTF-1. Noticeably, the protein phosphatase activity of PTEN participates in the regulation of MTF-1 activity. It remains unclear whether PTEN exerts the protein phosphatase activity directly on MTF-1 or through modifying other cellular protein(s) to indirectly alter MTF-1 activity.

In summary, we explored a novel mechanism that regulates the transcriptional activity of MTF-1 by PTEN. PTEN constitutively interacts with MTF-1 in the cytoplasm. This interaction...

Figure 8 Effect of PTEN on MTF-1-mediated ZnT1 expression
(A) HEK-293 cells were treated with 100 μM Zn for 6 h and ZnT1 mRNA was determined. (B) MTF-1 expression was suppressed by siRNA in HEK-293 cells and ZnT1 mRNA was determined 6 h after 100 μM Zn treatment. MTF-1 mRNA expression after siRNA transfection was detected by RT (reverse transcription)–PCR and shown in the upper panel. (C) HEK-293 cells were transfected with control or PTEN siRNA. The transfected cells were treated with 100 μM Zn for various time intervals and the ZnT1 mRNA was quantified. Each value represents a mean ± S.D. for three samples. *P < 0.05 between the paired samples. IB, immunoblotted.
increases with metal stimulation, but decreases following nuclear translocation of MTF-1. PTEN enhances MTF-1 activity through its protein phosphatase activity. Knockdown of PTEN dramatically reduces the expression of MTF-1 target genes and the cellular tolerance to heavy metal toxicity. The present study shows a regulatory mechanism of MTF-1 that is physiologically significant to the cells.

**AUTHOR CONTRIBUTION**

Meng-Chieh Lin performed and designed the research. Ya-Chuan Liu and Ming Tam provided technical support. Yu-Ju Lu established MTF-1–His6 stable-expressed CHO K1 cell line. Ya-Ting Hishe performed experiments in Figure 2(E). Meng-Chieh Lin, Ya-Chuan Liu and Lih-Yuan Lin contributed to research designation, manuscript preparation and editing.

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SUPPLEMENTARY ONLINE DATA

PTEN interacts with metal-responsive transcription factor 1 and stimulates its transcriptional activity

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Figure S1 Effect of PTEN depletion on Cd-induced MT-IIA expression

PTEN expression was suppressed by siRNA in HEK-293 cells. The cells were then treated with or without 5 μM Cd for 6 h. The relative amount of MT-IIA mRNA was determined by quantitative real-time PCR. Results are means ± S.D. for three samples. * P < 0.05 between the paired samples.

Figure S2 Effect of PTEN depletion on MT-IA expression

PTEN mRNA was depleted by siRNA in HEK-293 cells. The cells were then treated with or without 100 μM Zn for 6 h. The relative amount of MT-IA mRNA was quantified by real-time PCR. Results are means ± S.D. for three samples. * P < 0.05 between the paired samples.

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Figure S3  Effect of PTEN depletion on stability, nuclear translocation and MRE-binding activity of MTF-1

CHO K1 cells stably expressing His6-tagged MTF-1 were transfected with siRNA to suppress PTEN expression. (A) Fusidic acid (250 μM) alone or with 100 μM Zn was added to the cells. Samples were removed at various time intervals and proteins were quantified with immunoblotting (IB). Tubulin was used as a loading control. (B) Cells were treated with or without 100 μM Zn for various time intervals. Cytosolic and nuclear fractions were isolated and the expressed proteins were analysed by immunoblotting. Tubulin and lamin were used as loading controls for cytosolic and nuclear proteins, respectively. (C) Cells were treated with or without 100 μM Zn for 2 h and whole-cell extracts were prepared for EMSA. FP, free probe; CP, cold MREs as competitors. siPTEN, PTEN siRNA.

Figure S4  Effect of PI3K and Akt inhibitors on the expression of MT-IIA mRNA

HEK-293 cells were treated with various concentrations of PI3K (LY294002) or Akt (Akt inhibitor IV) inhibitor 1 h prior to the addition of 100 μM Zn. The incubation continued for a further 6 h and MT-IIA mRNA was determined. Results are means ± S.D. for three samples.

Table S1  Primer sequences for mRNA analysis by quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence (human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-IIA</td>
<td>Forward 5′-ATGGATCCCAAACGCTCCTG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-TCAGGCCACAGCAGCTGACT-3′</td>
</tr>
<tr>
<td>MT-IA</td>
<td>Forward 5′-ATGGATCCCAAACGCTCCTG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-TTCCAAGGTCCTGACGTTGAT-3′</td>
</tr>
<tr>
<td>ZnT1</td>
<td>Forward 5′-GAGATGGCTTGTGTTCAGT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-GGTCAGGGAAACATGGATTCAC-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward 5′-GAAGATGGCTTGTGTTCAGT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-GGTCAGGGAAACATGGATTCAC-3′</td>
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