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Production and Characterization of ZFP36L1 Antiserum against Recombinant Protein from *Escherichia coli*

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Abstract

Tristetraprolin/zinc finger protein 36 (TTP/ZFP36) family proteins are anti-inflammatory. They bind and destabilize some AU-rich element-containing mRNAs such as tumor necrosis factor mRNA. In this study, recombinant ZFP36L1/TIS11B (a TTP homologue) was over-expressed in *E. coli*, purified, and used for polyclonal antibody production in rabbits. The antiserum recognized nanograms of the antigen on immunoblots. This antiserum and another antiserum developed against recombinant mouse TTP were used to detect ZFP36L1 and TTP in mouse 3T3-L1 adipocytes and RAW264.7 macrophages. Immunoblotting showed that ZFP36L1 was stably expressed with a size corresponding to the lower mass size of ZFP36L1 expressed in transfected human embryonic kidney 293 cells, but TTP was induced by cinnamon extract and not by lipopolysaccharide (LPS) in adipocytes. In contrast, ZFP36L1 was undetectable but TTP was strongly induced in LPS-stimulated RAW cells. Quantitative real-time polymerase chain reaction confirmed the higher levels of ZFP36L1 mRNA in adipocytes and TTP mRNA in RAW cells. Low levels of ZFP36L1 expression were also confirmed by northern blotting in mouse embryonic fibroblasts. These results demonstrate that ZFP36L1 antiserum is useful in the detection of this protein and that TTP and ZFP36L1 are differentially expressed and regulated at the mRNA and protein levels in mouse adipocytes and macrophages.

Keywords

antibody production; cinnamon; insulin; lipopolysaccharide; mouse cell; protein expression and purification; real-time PCR; tristetraprolin; ZFP36L1

Introduction

The tristetraprolin (TTP) family of CCHH tandem zinc finger proteins (ZFPs) consists of three known members in mammals (ZFP36 or TTP or TIS11, ZFP36L1 or TIS11B, and ZFP36L2 or TIS11D) and another recently identified fourth member in mouse and rat X chromosome

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but not in humans (ZFP36L3) (1–3). TTP, the best-studied family member, is the product of the immediate-early response gene *Zfp36* in the mouse and *ZFP36* in humans (4). TTP binds to AU-rich elements (AREs) in some mRNAs and destabilizes those transcripts (5–9). The mRNAs encoding tumor necrosis factor- α (TNF α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 2, and immediate-early response gene 3 are stabilized in TTP knockout mice and in cells derived from them (7,8,10,11). Excessive secretion of these cytokines results in a severe systemic inflammatory response in the TTP knockout mice (12, 13). In addition to its association with autoimmune diseases (14,15), other human studies show that TTP gene expression is reduced in fats of obese people with metabolic syndrome (16,17) and brains of suicide victims (18). TTP gene expression is modestly induced by insulin and other growth factors (4), as well as nutritional products such as cinnamon (19) and green tea (20). These studies demonstrate the potential beneficial effects of nutritional intervention for the prevention of such negative conditions.

ZFP36L1 is also known as TIS11B, cMG1, BRF1, ERF1, and Berg36 (1). The tandem zinc finger domains are highly conserved between ZFP36L1 and TTP, but the N- and C-terminal regions are highly divergent (3). ZFP36L1 has similar biochemical effects of TTP in various assays (21–24). Over-expression of ZFP36L1/TIS11B induces myeloid cell proliferation in response to granulocyte colony-stimulating factor (25). Mice deficient in ZFP36L1 develop chorioallantoic fusion defects and died in utero before embryonic day 11 (26). By analogy with TTP, the phenotype suggests that ZFP36L1 may also destabilize some mRNAs whose protein products accumulate higher than normal levels in the fetoplacental unit, leading to abnormalities of placentation and the death of the embryo in ZFP36L1 knockout mice (26). Genetic knockout studies also show that ZFP36L1 is required for normal vascularisation and regulates vascular endothelial growth factor expression at the posttranscriptional level (27), whose mRNA stability is also down-regulated by ZFP36L1/TIS11B in cultured cells (28). The destabilizing effect of ZFP36L1/BRF1 on its mRNA targets was shown to be regulated by protein kinase B (29,30).

ZFP36L1 mRNA can be induced by mitogens and growth factors with induction kinetics different from those of TTP (31,32). Since ZFP36L1 can act like TTP in cell-free RNA binding and cellular transfection assays as well as in a cell-free deadenylation assay, ZFP36L1 could play a role in normal physiology similar to that of TTP. However, ZFP36L1 protein has not been adequately characterized, partly due to lack of high-titer antibodies and purified protein.

In this study, recombinant ZFP36L1 was over-expressed as a maltose-binding protein (MBP) fusion protein in *E. coli*, purified, and used for polyclonal antibody production in rabbits. We used ZFP36L1 antiserum and the MBP-TTP antiserum for the detection of these two proteins in mouse 3T3-L1 adipocytes and RAW264.7 cells following treatments with insulin, lipopolysaccharide (LPS), and cinnamon extracts and cinnamon polyphenols (which are proposed to enhance insulin action (33)). Real-time polymerase chain reaction (PCR) was used to estimate the relative abundance of both mRNA levels in adipocytes and RAW cells. TTP expression is highly inducible in both cell types and its mRNA and protein levels are much higher in RAW cells than adipocytes. However, ZFP36L1 gene expression is insensitive to the same stimuli in adipocytes and its mRNA and protein levels are much higher in adipocytes than RAW cells.

Materials and Methods

Expression of MBP-ZFP36L1 in *E. coli*

Plasmid pMBP-ZFP36L1 was constructed by subcloning the cDNA fragment coding for the full-length mouse ZFP36L1 (GenBank Accession No. [NM_007564](#)) into pMAL-c2 vector (23). The plasmid was transformed into *E. coli* BL21(DE3) cells. A single colony was

inoculated into LB-Amp medium and grown overnight at 37°C. The overnight culture was inoculated in fresh medium and grown for 2 h at 37°C to reach 0.6–1.0 OD at 600 nm. Isopropylthio- β -D-galactoside (IPTG) was then added to the culture (0.3 mM final concentration) and protein was induced at 25°C for 4 h.

Purification of MBP-ZFP36L1 from *E. coli*

E. coli cells were sonicated in a buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 10 mM β -mercaptoethanol, 1 mM PMSF, 2 μ M leupeptin, and 1 mM ZnCl₂) and the homogenate was centrifuged at 10,000g for 10 min. The supernatant was applied onto an amylose resin column followed by wash and elution as described (34). Fractions containing MBP-ZFP36L1 were centrifuged as above before being loaded onto a Superose 12 HR 10/30 column and eluted with Buffer A (20 mM ethanolamine, 5 mM EDTA, 10 mM β -mercaptoethanol, pH 9.0). Fractions containing MBP-ZFP36L1 were centrifuged as above and the supernatant was applied to a Mono Q HR 5/5 column. The column was washed with Buffer A and eluted with a linear gradient from 0 to 100% of Buffer B (1 M NaCl in M Buffer A). MBP-ZFP36L1 fractions with the highest purity were concentrated with Centricon-10.

Production of MBP-ZFP36L1 Antiserum

Anti-MBP-ZFP36L1 serum was produced according to standard procedures (Covance Research Products, Denver, PA). Briefly, 250 μ g of MBP-ZFP36L1 was diluted into 0.5 mL in PBS, mixed with 0.5 mL of Freund's complete adjuvant, and injected into a female New Zealand white rabbit. Three boosts of 125 μ g each of the antigen in Freund's incomplete adjuvant were performed every 4 weeks following the primary injection.

Expression and Purification of His-ZFP36L1 Protein from Transfected Human Cells

Human embryonic kidney (HEK) 293 cells were transfected with the calcium-phosphate precipitation method (6) using plasmids containing DNA sequence encoding six consecutive histidine residues and the full-length mouse ZFP36L1 protein. The transfected cells were lysed in lysis buffer (10 mM Hepes, pH 7.6, 3 mM MgCl₂, 40 mM KCl, 0.5 % Nonidet P-40 (v/v), 8 μ g/mL leupeptin, and 0.5 mM PMSF) as described previously (6). The cell lysate was centrifuged at 1000g for 5 min and the supernatant was further centrifuged at 10,000g for 10 min. Histidine-tagged ZFP36L1 from the 10,000g supernatant was bound to Ni-NTA beads (Qiagen, Madison, WI), washed with 10 mM imidazole buffer six times, and eluted with elution buffer (100, 200, or 250 mM imidazole, 50 mM NaH₂PO₄, 300 mM NaCl, 0.05 % Tween 20, pH 8.0) as described previously (6).

Cell Culture

Mouse 3T3-L1 fibroblasts were maintained and the differentiation of adipocytes were induced as described (35). Adipocytes were serum-starved in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD) for 3–4 h before being treated with chemicals for various times. Water-soluble cinnamon extract and cinnamon polyphenols were prepared as described (33). Mouse RAW 264.7 cells were cultured and treated with 0.1 μ g/mL LPS as described (35). Mouse embryonic fibroblasts were derived from day 14.5 embryos (11). During passages, trypsinized cells were seeded at 10⁶ cells per 100 mm Petri dishes in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μ g/mL streptomycin. At nearly 70% confluence, the cultures were serum starved with DMEM containing 0.5% FCS (v/v) for 16 h to synchronize the culture. Cell extracts were prepared as described above.

Protein Concentration Determination, SDS-Polyacrylamide Gel Electrophoresis (PAGE), and Immunoblotting

Protein concentrations were determined using the Protein Assay Dye Reagent Concentrate following NaOH treatment (6). The absorbance was measured using Bio-Tek Spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). Bovine serum albumin was the protein standard. SDS-PAGE and immunoblotting procedures were performed as described (6). The blot was incubated with the primary antibodies (1:1000–2000 dilution) overnight and the secondary antibodies (1:10000 dilution) for 4 h. Proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) followed by imaging with BioChem Image Acquisition and Analysis System (UVP BioImaging Systems, UVP Inc, Upland, CA). The primary antibodies were anti-MBP serum (New England BioLab), anti-MBP-TTP serum raised against the recombinant full-length mouse TTP (6) and anti-MBP-ZFP36L1 serum as described above. The secondary antibodies were affinity-purified goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate (Bio-Rad Laboratory).

RNA Isolation and cDNA Synthesis

RNA was isolated according to the manufacturer's instructions using TRIzol reagent (Invitrogen) or using RNeasy miniprep kit (Qiagen, Valencia, CA). RNA concentrations and integrity were determined using RNA 6000 Nano Assay Kit and the Bioanalyzer 2100 according to the manufacturer's instructions (Agilent Technologies, Palo Alto, CA, USA) with RNA 6000 Ladder as the standards (Ambion, Inc., Austin, TX, USA). cDNAs were synthesized in the reaction mixture (20 µL) contained 5 µg total RNA, 2.4 µg oligo(dT)_{12–18} primer, 0.1 µg random primers, 500 µM dNTPs, 10 mM DTT, 40 u RNaseOUT, 200 u SuperScript II reverse transcriptase (Invitrogen) (19) or using High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA).

PCR Primers and TaqMan Probes

The primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA) and were synthesized by Biosearch Technologies, Inc. (Novato, CA, USA). The mRNA names, GenBank accession numbers, amplicon sizes, and the sequences (5' to 3') of the forward primers, TaqMan probes (TET-BHQ1) and reverse primers, respectively, are described as below: Rpl32 (NM_172086, 66 bp, aaccgaaaagccattgtagaaa, agcagcacagctggccatcagagtc, cctggcgcttgggattgg); Ttp (Zfp36) (NM_011756, 70 bp, ggtaccccaggctgcttt, aactcaatataatcctgccttagcctt, acctgaaccccagaactgga); and Tis11b (Zfp3611) (NM_007564, 63 bp, tgcgaacgccacgat, accaccacctcgtgtccgcc, cttcgctcaagtcaaaaatgg).

Real-time PCR Analysis

TaqMan reaction mixture (25 µL) contained 25 ng of RNA-derived cDNAs, 200 nM each of the forward primer, reverse primer, and TaqMan probe, and 12.5 µL of 2X Absolute QPCR Mix (ABgene House, Epsom, Surrey, UK). The reactions were performed in 96-well plates in a ABI Prism 7700 real time PCR instrument or in a ABI Prism 7900HT Sequence Detector System (Applied Biosystems). The thermal cycle conditions were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 50 cycles at 95 °C for 15 s each and 60 °C for 60 s. The double C_T method of relative quantification was used to determine the fold change in expression (19).

Northern Blotting

Mouse embryonic fibroblasts that had been serum-starved for 16 h were treated with 10% FCS or with 1 µM phorbol myristyl acetate (PMA) (Sigma, St. Louis, MO) for indicated periods. Ten µg of RNA was used for northern blot analysis (4). Blots were hybridized to randomly

primed, a ^{32}P -labeled ZFP36L1 cDNA probe (26) and rehybridized with a glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA probe (7). The blot was exposed to x-ray film.

Results and Discussion

Expression of Recombinant ZFP36L1 in *E. coli*

It was difficult to express full-length proteins in the TTP family (5). Previous studies showed that soluble TTP fused to MBP was readily expressed in *E. coli* (5). We therefore used the MBP fusion system for expressing ZFP36L1 in *E. coli*. A protein band with the predicted molecular mass of the full-length MBP-ZFP36L1 (calculated M_r 75,265) was detected in the 10,000g supernatant of IPTG-induced cells (Figure 1A). More soluble MBP-ZFP36L1 accumulated in *E. coli* grown at 25°C and 30°C than those at 37°C. MBP-ZFP36L1 was expressed more in cells treated with IPTG for 3–4 h at 25 and 30°C and little recombinant protein was detected in cells induced for 18 h (Figure 1A). The immuno-reactive bands with smaller sizes than the full-length MBP-ZFP36L1 on the immunoblots suggest some degradation of the recombinant ZFP36L1 in the bacterial cells.

Purification of Recombinant ZFP36L1 from *E. coli*

We previously reported a successful procedure for purifying recombinant MBP-TTP from *E. coli* (5). Since the amino acid sequences of TTP and ZFP36L1 are highly divergent except for the CCCH zinc finger domains (3), it is necessary to test the purification procedure for ZFP36L1. Therefore, a similar procedure was used to purify MBP-ZFP36L1 by amylose resin affinity column, Superose 12 size column, and Mono Q anion exchange column (Figure 1B–D). About 30 mg of total protein was eluted from the amylose resin affinity column using a cell extract from one liter of *E. coli* transformed with pMBP-ZFP36L1. The majority of the eluted protein was MBP-ZFP36L1, although the eluted fractions contained other proteins (Figure 1B, upper panel). Some degradation products of the recombinant protein were presented in the partially purified protein fractions, as shown by immunoblotting with anti-MBP antibodies (Figure 1B, bottom panel). Fractions containing MBP-ZFP36L1 were desalted by Superose 12 size column. The recombinant protein was recovered in fractions 9–12 (Figure 1C). The size exclusion chromatograph did not appear to be very effective for the purification itself because gel staining did not show significant improvement of the purity of the recombinant protein (Fig. 1B vs. 1C, upper panels). Fractions containing MBP-ZFP36L1 were pooled and further purified by Mono Q anion exchange column (Figure 1D). Coomassie blue staining of the gel showed that the recombinant protein was purified to near homogeneity by Mono Q column (Figure 1D). These results indicate that the procedure developed previously to purify TTP (5) is effective in purifying members of TTP family proteins and is probably applicable to purification of other zinc finger proteins. More experiments will be needed, however, to generalize the utility of this procedure for purifying other zinc finger proteins.

Production and Characterization of MBP-ZFP36L1 Antiserum

There was a lack of quality antibodies for the detection of ZFP36L1 in cells and tissues. We therefore raised ZFP36L1 antiserum against the recombinant MBP-ZFP36L1 in rabbits using Mono Q fractions with the highest purity of the protein (Figure 1D). The anti-MBP-ZFP36L1 serum at a 1:10,000 dilution readily detected the recombinant protein in *E. coli* extracts (Figure 2A). This serum was able to detect as little as 5 ng of the partially purified MBP-ZFP36L1 from amylose resin fraction (Figure 2B). This immunoblot also showed that MBP-ZFP36L1 was partially degraded after expression in *E. coli* and purified by amylose resin affinity column (Figure 2B). The antiserum at 1:1000 dilution was able to detect the full-length protein in the 10,000g supernatant from the transfected HEK293 cells when micrograms of proteins were loaded into the gel (Figure 2C). The titer of ZFP36L1 antiserum was less than those of TTP

antisera against recombinant human and mouse TTP which could detect 1 ng of the antigen (6, 35).

Stable Expression of ZFP36L1 in Mouse 3T3-L1 Cells

ZFP36L1 mRNA has been detected in mouse tissues (26); however, limited information is available about the expression of ZFP36L1 protein. Figure 3A shows that an immuno-reactive band was clearly detected with a molecular size similar to that predicted for mouse ZFP36L1 in the soluble extracts of 3T3-L1 adipocytes. The level of ZFP36L1 was similar in the control (lane 3) and in the adipocytes treated with 100 nM insulin (lane 4), 10 µg/mL waster-soluble cinnamon extract dissolved in 0.1 N NH₄OH (lane 5) or DMSO (lane 6), or 1 µg/mL fractions 1–7 of HPLC-purified cinnamon polyphenols (lanes 7–14), or 0.1 µg/mL LPS (data not shown). No similar protein band was detected in mouse RAW264.7 cells following 0.1 µg/mL LPS stimulation (lanes 2 and 15). To provide a positive control for ZFP36L1 detected in adipocytes, HEK293 cells were transfected with a DNA construct coding for His-tagged ZFP36L1. The sizes of immuno-reactive bands recognized by ZFP36L1 antiserum in adipocytes (Figure 3B, lanes 2–7) corresponded to the lower mass band of ZFP36L1 expressed in transfected HEK293 cells (Figure 3B, lanes 9–11). Like TTP antiserum, ZFP36L1 antiserum was able to detect only a sharp band when the expression of the protein was greatly increased in adipocytes as reported here for ZFP36L1 (Figure 3A) and previously for ZFP36L1 (36) and in LPS-stimulated RAW264.7 macrophages as reported previously for TTP (35). These results suggest that ZFP36L1 protein is expressed in mouse 3T3-L1 adipocytes but not detectable in LPS-stimulated mouse RAW264.7 cells under these immunoblotting procedures, and that ZFP36L1 antiserum is useful in the detection of the protein in highly expressed cells and tissues.

Differential Expression of TTP and ZFP36L1 Proteins in Mouse Cells

The effects of LPS and cinnamon on TTP and ZFP36L1 in RAW264.7 cells and 3T3-L1 adipocytes were shown in Figure 3C and 3D, respectively. TTP in RAW264.7 cells was dramatically induced by 0.1 µg/mL LPS (Figure 3C, lane 2). TTP was also clearly induced but much weaker in 3T3-L1 adipocytes by 100 µg/mL of cinnamon extract suspended in DMSO (lanes 3–5), water (lanes 6–8), or 0.1 M NH₄OH (lanes 9–11). Furthermore, TTP migrated on SDS-PAGE gel with a molecular mass bigger than the predicted size. In contrast, ZFP36L1 was not detected in RAW264.7 cells treated with 0.1 µg/mL LPS (Figure 3C, lane 2), but was detected as a relatively sharp band on immunoblots in 3T3-L1 adipocytes treated with 0–100 µg/mL of cinnamon extract suspended in DMSO (lanes 3–5), water (lanes 6–8), or 0.1 M NH₄OH (lanes 9–11). The multiple bands detected above the TTP bands in the adipocytes (Figure 3C) were non-specifically cross-reacted bands since the intensities of these bands were similar between the controls (Figure 3C, lanes 3, 6, 9) and the different treatments (Figure 3C, lanes 4, 5, 7, 8, 10, 11). These immunoblotting results are in agreement with previous report in which the TTP antiserum reacts with non-specific proteins when large amounts of protein and extended exposure conditions are used for the detection of this extremely low-abundance protein in endogenous cells and tissues (35). Previous results have shown that TTP is a hyperphosphorylated protein (37, 38). The differences between these two proteins suggest that ZFP36L1 is probably phosphorylated to a much less extent than TTP, in agreement with the fact that less potential phosphorylation sites are conserved in ZFP36L1 than those in TTP (3).

Differential Expression of TTP and ZFP36L1 mRNAs in Mouse Cells

The differential intensity of the immuno-reactive bands on the immunoblots described above suggested that the mRNA levels of TTP and ZFP36L1 might also be different in the RAW cells and adipocytes. Quantitative real-time PCR analysis showed that TTP mRNA level was approximately twice that of ZFP36L1 mRNA level in RAW264.7 cells (Table 1). On the contrary, TTP mRNA level was less than 15% of ZFP36L1 mRNA level in the adipocytes

(Table 1). Between these two cell lines, TTP mRNA level in RAW264.7 cells was close to 2 fold of that in the adipocytes, but ZFP36L1 mRNA level in RAW264.7 cells was only 10% of that in the adipocytes (Table 1). The higher levels of ZFP36L1 mRNA in adipocytes than in RAW cells might contribute to the readily detectable ZFP36L1 protein in the adipocytes.

The inability to detect ZFP36L1 protein in RAW cells might be due to low levels of ZFP36L1 gene expression in these cells. We therefore analyzed ZFP36L1 gene expression in another type of cells. RT-PCR analysis showed that ZFP36L1 mRNA levels were readily detected in serum-starved mouse embryonic fibroblasts isolated from embryos collected at embryonic day 14.5 (data not shown). Northern blotting showed that ZFP36L1 mRNA levels in these cells were slightly increased by addition of 10% serum in culture (Figure 4A), but markedly increased by PMA treatment that peaked at 60 min and returned to basal levels at 180 min (Figure 4B). The utility of ZFP36L1 antiserum was tested previously using fibroblasts derived from wild-type and ZFP36L1 knockout mice. ZFP36L1 antiserum produced here was able to detect endogenous ZFP36L1 as a minimally regulated protein in serum-treated wild-type fibroblasts but was absent in ZFP36L1-deficient fibroblasts (11). However, this antiserum also cross-reacts with non-specific bands in the wild-type fibroblasts (11). A similar cross-reactivity was noted with the TTP antiserum because large amounts of proteins and extended exposure conditions are required for the detection of this extremely low-abundance protein in endogenous cells and tissues (11, 35). Like TTP antiserum, ZFP36L1 antiserum was able to detect only a sharp band when the expression of the protein was relatively abundant in adipocytes (36) and in LPS-stimulated RAW264.7 macrophages reported previously (35). These results demonstrate the utility of the antiserum for detecting endogenous ZFP36L1 in mouse cells, and as a tool for its regulation and expression in different cell types.

The above results suggest that both TTP and ZFP36L1 genes are differentially expressed in mouse cells. Previous studies have shown that both TTP and ZFP36L1 mRNAs are readily detected in heart, kidney, lung, spleen, and thymus but are less in brain, liver, or testis (26). The major difference of mRNA expression between these two genes is that ZFP36L1 mRNA, but not TTP mRNA, is readily detected in embryonic stem cells, 12.5–14.5 day old embryo and adult ovary (26). It was also reported that the gene expression of ZFP36L1/TIS11B but not TTP or ZFP36L2/TIS11D is induced during myogenic differentiation in murine myoblast cell line C2C12 and primary mouse myoblasts (39). Another example of differential expression of TTP and ZFP36L1 mRNAs is described using human monocytes following LPS stimulation. TTP mRNA induction peaks at 60 min and declines to the baseline in about 2 h with a peak induction of about 16-fold; however, ZFP36L1 mRNA induction peaks at 90 min and declines slowly with the peak of induction at approximately 4.4-fold (32). The induction patterns of these two mRNAs are different from that observed in a rat intestinal epithelial (RIE-1) cell line (31), whereas, both TTP and ZFP36L1 (cMG1) mRNAs were induced by epidermal growth factor, angiotensin II and 12-O-tetradecanoyl phool-13-acetate. However, ZFP36L1, but not TTP, was induced by insulin and insulin-like growth factor I in RIE-1 cells (31). Clearly, the expression and regulation of TTP and ZFP36L1 gene expression is determined by the types of cells in responding to different kinds of stimuli.

Conclusion

This study describes a procedure for the expression and purification of recombinant ZFP36L1 protein and reports a high titer antiserum against the purified recombinant protein. Immunoblotting, real-time PCR, and northern blotting analyses show that both TTP and ZFP36L1 genes are differentially expressed and regulated in mouse cells. TTP gene expression is highly inducible in both RAW macrophages and 3T3-L1 adipocytes and its mRNA and protein levels are much higher in RAW cells than adipocytes. ZFP36L1 gene expression is insensitive to the same stimuli tested in 3T3-L1 adipocytes and its mRNA and protein levels

are much higher in adipocytes than RAW cells. These results demonstrate that ZFP36L1 antiserum is useful in the detection of this protein in certain cell types and support the observation that TTP, and its homologue, ZFP36L1, are differentially expressed and regulated in mouse cells.

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Abbreviations

TTP	tristetraprolin
ZFP36L1	zinc finger protein 36-like 1
ARE	AU-rich element
DMEM	Dulbecco's modified Eagle's medium
FCS	fetal calf serum
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
HEK	human embryonic kidney
IPTG	isopropylthio- β -D-galactoside
LPS	lipopolysaccharide
MBP	maltose-binding protein
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMA	phorbol myristyl acetate
RPL32	ribosomal protein L32

TNF

tumor necrosis factor

ZFP

zinc finger protein

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Figure 1.

Expression and purification of recombinant ZFP36L1 from *E. coli*. (A) MBP-ZFP36L1 expression. MBP-ZFP36L1 was induced by 0.3 mM IPTG at 25°C, 30°C, and 37°C for various times as indicated. MBP-ZFP36L1 protein was detected by Anti-MBP antibodies. (B-D) MBP-ZFP36L1 purification. MBP-ZFP36L1 was affinity-purified by an amylose resin column. The fractions were analyzed by SDS-PAGE and detected by Coomassie blue staining (B, upper) and anti-MBP antibodies (B, bottom). Fractions with MBP-ZFP36L1 were further passed through a Superose 12 size column and detected with Coomassie blue staining (C, upper) and anti-MBP antibodies (C, bottom). Proteins from the size column were finally purified by a Mono Q column and detected by Coomassie blue staining (D). The arrows point to the full-length MBP-ZFP36L1 protein.

Figure 2.

Characterization of MBP-ZFP36L1 antiserum. (A) Detection of MBP-ZFP36L1 in transformed BL21(DE3) cells. MBP-ZFP36L1 was induced by 0.3 mM IPTG at 30°C for 3 h. MBP-ZFP36L1 protein was detected by anti-MBP-ZFP36L1 serum (1:10000 dilution, 1 h) followed by incubation of the secondary antibodies (1:10000 dilution, 30 min). Immuno-reactive bands were appeared after being exposed to X-ray film for one second. Lane 1, BL21 (DE3) cells; lane 2, BL21(DE3) cells transformed with plasmid pMBP-ZFP36L1 without IPTG induction; lanes 3–4, BL21(DE3) cells transformed with the plasmid with IPTG induction. (B) Detection limit of MBP-ZFP36L1 antiserum. MBP-ZFP36L1 was affinity-purified by an amylose resin column and detected by immunoblotting using anti-MBP-ZFP36L1 serum

(1:10000 dilution, 1 h). (C). Detection of ZFP36L1 purified from transfected HEK293 cells. Human cells were transfected with a plasmid coding for histidine-tagged mouse ZFP36L1. His-ZFP36L1 was purified by Ni-NTA beads following extensive washes with 10 mM imidazole buffer and successively eluted with 100, 200, 1st 250, and 2nd 250 mM imidazole buffer. Proteins in the 10,000g supernatant, unbound pass through, six washes, four elutions, and the final beads were separated by SDS-PAGE. His-ZFP36L1 was detected by anti-MBP-ZFP36L1 serum (1:1000 dilution, 1 h).

Figure 3.

Expression of ZFP36L1 and TTP in mouse 3T3-L1 adipocytes and macrophages. (A) Stable expression of ZFP36L1 in adipocytes. ZFP36L1 was detected with anti-MBP-ZFP36L1 antibodies. Each lane was loaded with 100 μ g of protein in the supernatant except that lane 2 was loaded with 20 μ g protein. Lane 1, protein size standard; lanes 2 and 15, RAW264.7 cells treated with LPS (0.1 μ g/mL); lanes 3–4, 3T3-L1 cells treated with 0 and 100 nM insulin; lanes 5–6, 3T3-L1 adipocytes treated with 10 μ g/mL cinnamon extract; lanes 7–14, 3T3-L1 adipocytes treated with 1 μ g/mL cinnamon polyphenols 1A, 1B, 2, 3, 4, 5, 6 and 7, respectively. (B) Comparison of ZFP36L1 expressed in mouse 3T3-L1 adipocytes and transfected human HEK293 cells. ZFP36L1 was detected with anti-MBP-ZFP36L1 antibodies. Lane 1, protein

size standard; lane 2, 3T3-L1 adipocytes treated with 0.1% DMSO; lanes 3 and 4, 3T3-L1 adipocytes treated with 10 and 100 nM insulin; lane 5, 3T3-L1 adipocytes treated with 10 µg/mL cinnamon extract; lanes 6 and 7, 3T3-L1 adipocytes treated with 1 µg/mL cinnamon polyphenol fractions 3 and 8, respectively; lanes 8–11, 1, 5, 10, and 15 µL of the transfected HEK293 cell extract, respectively. Lanes 2–7 were loaded with 100 µg of protein in the supernatant. (C, D) Differential expression of TTP/ZFP36 and ZFP36L1 proteins in mouse cells. TTP and ZFP36L1 were detected with anti-MBP-TTP and anti-MBP-ZFP36L1 antibodies, respectively. Each lane was loaded with 100 µg of protein in the supernatant except that lane 2 was loaded with 20 µg protein. Lane 1, protein size standards; lane 2, RAW264.7 cells treated with LPS (0.1 µg/mL); lanes 3–5, 3T3-L1 adipocytes treated with 0, 10, and 100 µg/mL cinnamon extract dissolved in 100% DMSO; lanes 6–8, 3T3-L1 cells treated with 0, 10, and 100 µg/mL cinnamon extract dissolved with water in neutral pH; lanes 9–11, 3T3-L1 cells treated with 0, 10, and 100 µg/mL cinnamon extract dissolved in 0.1 M NH₄OH. The arrows point to the full-length MBP-ZFP36L1, His-ZFP36L1, and TTP/ZFP36 proteins.

Figure 4.

Northern blotting analysis of ZFP36L1 mRNA levels in mouse embryonic fibroblasts. Mouse embryonic fibroblasts that had been serum-starved for 16 h were treated with 10% FCS or with 1 μ M PMA for 0, 30, 60, 120, and 180 min. Ten μ g of RNA was used for northern blot analysis.

Table 1

Real-time PCR analysis of TTP and ZFP36L1 mRNA levels in mouse cells

Mouse cells	mRNA	Cycle of threshold ($C_T \pm SD$)	Expression ratio (Relative to Ttp) (Fold)	Expression ratio (3T3-L1/RAW) (Fold)
RAW264.7 cells	Rpl32	18.19 ± 0.04	Internal control	1.00
	Ttp/Tis11/Zfp36	23.64 ± 0.20	1.00	1.00
	Tis11b/Zfp36l1	24.76 ± 0.08	0.46	1.00
3T3-L1 adipocytes	Rpl32	18.07 ± 0.13	Internal control	1.09
	Ttp/Tis11/Zfp36	24.21 ± 0.30	1.00	0.62
	Tis11b/Zfp36l1	21.31 ± 0.60	7.45	10.06

Twenty-five ng of RNA-derived cDNAs were used for the quantitation of mRNA levels using 50 cycles of real-time PCR program. The mean C_T (cycle of threshold) values and standard deviations (n=4) are shown in the table. The relative ratios of mRNA levels were calculated using the double delta C_T method normalized with Rpl32 C_T value as the internal control and Ttp C_T value or RAW264.7 C_T value as the calibrator.