Expression of Proviral Integration Site for Moloney Murine Leukemia Virus 1 (Pim-1) Is Post-transcriptionally Regulated by Tristetraprolin in Cancer Cells

Received for publication, April 27, 2012, and in revised form, June 13, 2012 Published, JBC Papers in Press, June 14, 2012 DOI 10.1074/jbc.M112.376483

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Background: Expression of the proto-oncogene Pim-1 is post-transcriptionally controlled in cancer cells.
Results: Tristetraprolin (TTP) enhances the decay of Pim-1 mRNA by binding to the AU-rich element in its 3′-UTR.
Conclusion: TTP contributes to tumor suppression in part by down-regulating Pim-1 expression.
Significance: This work explains the mechanisms by which Pim-1 expression is regulated in cancer cells.

The proviral integration site for Moloney murine leukemia virus 1 (Pim-1) is an oncogenic serine/threonine kinase that is up-regulated in several human cancers, facilitates cell cycle progression, and suppresses apoptosis. Previously, it has been reported that the Pim-1 3′-UTR plays important roles in the regulation of Pim-1 mRNA stability. However, the mechanisms explaining how Pim-1 mRNA stability is determined by its 3′-UTR are not well known. Here, we demonstrate that tristetraprolin (TTP) plays a critical role in the regulation of Pim-1 mRNA stability. Our results show that the level of Pim-1 expression is inversely correlated with TTP expression in human cancer cells. Pim-1 mRNA contains two AU-rich elements (ARE1 and ARE2) in the 3′-UTR. TTP bound to ARE2 and enhanced the decay of Pim-1 mRNA. Overexpression of TTP decreased Pim-1 expression and p21 and p27 phosphorylation and inhibited cell growth. Overexpression of Pim-1 cDNA without the 3′-UTR attenuated the inhibitory effects of TTP on p21 phosphorylation and cell growth. In addition, inhibition of p21 by siRNA attenuated the inhibitory effect of TTP on cell growth. Our results suggest that TTP post-transcriptionally down-regulates Pim-1 expression and that the overexpression of TTP may contribute to tumor suppression in part by down-regulating Pim-1 expression.

The proto-oncogene Pim-1 is a serine/threonine Pim-1 kinase with multiple cellular functions, including roles in cell survival, proliferation, differentiation, apoptosis, and tumorigenesis (1, 2). Pim-1 kinase accomplishes its physiological activity through the phosphorylation of a wide range of cellular substrates (3), including Myc (4), p21Cip1/WAF1 (5, 6), and p27KIP1 (7). Pim-1 also synergizes with c-Myc in cell growth and transformation (8, 9). The expression of Pim-1 is induced by multiple cytokines, including SCF, G-CSF, IFN-γ, GM-CSF, IL-2, IL-3, IL-6, IL-7, and prolactin, through the activation of jAK/STAT signaling pathways (2), indicating that mutations in the signal transduction pathway through these receptors are responsible for the induction of Pim-1. It has been reported that Pim-1 is up-regulated in cells expressing constitutively active mutant STAT5 (10) or FLT3 (11). Overexpression of Pim-1 is linked to the development and progression of several types of cancer, including myeloid and hematopoietic malignancies (12), prostate cancer (13), squamous cell carcinomas (14), gastric and colorectal carcinomas (15), pancreatic ductal adenocarcinoma (16), and bladder cancer (17). Recent studies demonstrated that knockdown of Pim (18), decreasing Pim-1 levels by use of a monoclonal antibody (19), and Pim-1 kinase inhibitors (20, 21) induced antiproliferative activity in tumor cells, supporting the idea that Pim-1 is a potential tumor target in the development of therapeutic agents (22). Pim-1 was originally identified as a preferential proviral integration site for Moloney murine leukemia virus 1 (23). Proviral insertion in the 3′-UTR of Pim-1 causes a significant increase in the Pim-1 mRNA level (24). It has been reported that the Pim-1 transcript contains the AUUUA destabilizing motif and has a short half-life due to this motif (25). The destabilizing function of the AU-rich element (AREs) is believed to be regulated by ARE-binding proteins (26). Tristetraprolin (TTP) is an ARE-binding protein that can recognize AREs and promote the decay of the transcripts (27, 28). TTP expression is diminished in many cancers (29–31), which may contribute to an increase in the level of the transcripts containing AUUUA motifs in their 3′-UTRs.

In this study, we investigated the role of TTP in the post-transcriptional regulation of Pim-1 gene expression in human prostate cancer cells. Here, we report that the overexpression of TTP decreased the Pim-1 expression levels in LNCaP cells. TTP bound to the AUUUA motif in the Pim-1 mRNA and

The abbreviations used are: ARE, AU-rich element; TTP, tristetraprolin; qRT-PCR, quantitative real-time PCR; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
promoted the AUUUA motif-mediated degradation of the Pim-1 mRNA. Mutation in the ARE motif prevented the binding of TTP to this motif and the degradation of the luciferase gene containing the Pim-1 3’-UTR. Overexpression of TTP in LNCaP cells decreased the phosphorylation of Pim-1 substrates p21 and p27 and significantly inhibited the growth of cells, and transfection of Pim-1 cDNA without the 3’-UTR restored the phosphorylation of p21 and p27 and cell growth. Together, our findings suggest that TTP inhibits the expression of Pim-1 through interaction with the ARE motif in the Pim-1 3’-UTR and that limiting cellular TTP levels or mutation in the ARE motif can enhance the Pim-1 level, which may contribute to the development and progression of several types of cancers.

EXPERIMENTAL PROCEDURES

Cell Lines—The human cancer cell lines DU145, LNCaP (prostate carcinoma), and HeLa were purchased from the Korean Cell Line Bank (Seoul, Korea). A549, DU145, and LNCaP cells were cultured in RPMI 1640 medium, and HeLa cells were cultured in DMEM. All cell lines were supplemented with 10% FBS (WelGENE, Inc., Daegu, Korea) and maintained at 37 °C in a humidified atmosphere of 5% CO2.

Plasmids, siRNA, Transfections, and Dual-Luciferase Assay—The pcDNA6/V5-TTP construct has been described previously (32). Full-length human Pim-1 was amplified from the cDNA of LNCaP cells using PCR primers 5’-GGATCCACGATGCTCTTTGTCCTTGTCCTGTGCAAGTTGACCCGCA-3’ and 5’-CTCGAGAAGGAGGTCTATTTGCTAGTTG-3’ (with restriction enzyme sites underlined). The PCR product was ligated into the BamHI/XhoI sites of psiCHECK2 (Promega, Madison, WI). Oligonucleotide probe pairs for luciferase expression vector (Promega). For luciferase assays, LNCaP and HeLa cells were cotransfected with various types of psiCHECK2-Pim-1 3’-UTR constructs and pcDNA6/V5-TTP using TurboFect in vitro transfection reagent. Transfected cells were lysed with lysis buffer (Promega) and mixed with luciferase assay reagent (Promega), and the chemiluminescence signal was measured in a SpectraMax L microplate (Molecular Devices). Firefly luciferase was normalized to Renilla luciferase in each sample.

Cell Cycle Analyses—Cells were harvested, washed twice in PBS at room temperature, and resuspended at 2 × 106 cells/ml in PBS. For propidium iodide staining, the washed cells were fixed overnight in pure ethanol at −20 °C and then treated according to the manufacturer’s procedure (Molecular Probes). Cells were washed twice in PBS, resuspended in FACS buffer (PBS, 0.2% BSA, and 1% sodium azide), and analyzed using a FACScalibur cytometer (BD Biosciences).

Semiquantitative RT-PCR—DNase I-treated total RNA (3 μg) was reverse-transcribed using oligo(dT) and SuperScript II reverse transcriptase (Promega) according to the manufacturer’s instructions. Semiquantitative RT-PCR was performed using the following primer sets: TTP, CGCTACAAAGCTGATGAGG; GGTATGAGG; and GAGGTAGAACTTGTGACAGA; Pim-1, TCTCATGATGGTGTTGGCCCT and TGTCGACTGTCATACATG-CCC; and ATCAATGGTTGTCCAGCACC; Pim-3, TGACCGCAGTAAAGGAGGAAA and ACACCATATCGTGAAGAGCAGGC; and GAPDH, ATCTTCAGGCTATCGTGTGC and TGCCGGTCTCACATTTTCTTG.

SDS-PAGE Analysis and Western Blotting—Proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membranes (Whatman), and probed with the appropriate dilutions of the following antibodies: anti-human TTP (ab36558, Abcam); anti-V5 (20-783-70389, GenWay Biotech, Inc.); and anti-human Pim-1 (2409-1, anti-p21 (2990-1), anti-phospho-p21 (T3753), anti-p27 (2814-1), and anti-phospho-p27 (T7375) (Epitomics, Inc.). Immunoreactivity was detected using the BioRad ChemiDoc XRS+ imaging system. Films were exposed at multiple time points to ensure that the images were not saturated.

RNA Kinetics—For the RNA kinetic analysis, we used actinomycin D and assessed expression of Pim-1 and luciferase mRNAs by quantitative real-time PCR (qRT-PCR). qRT-PCR was performed using the ABI StepOnePlus system by monitoring in real time the increase in fluorescence of Power SYBR Green Master Mix (Applied Biosystems). The specificity of each primer pair was confirmed by melting curve analysis and agarose gel electrophoresis. The same Pim-1 and GAPDH primer pairs as described above were used, with the following luciferase primer pair: qLuc, ACGTGCTGGACTCCTTCATC and GACCTCCT GGAGAAGGAGGAAA and ACACCATATCGTGAAGAGCAGGC; and GAPDH, ATCTTCAGGCTATCGTGTGC and TGCCGGTCTCACATTTTCTTG.

EMSA—The biotinylated RNA probes for wild-type (5’-CCUGGAGGAAGAUGUUAUAAUUAUUAUUAUUAUUAUUA-5’-GGCCGCGGAATAGGAGGAAAACCCAGCTGCTGTGATACATAACATTGACCTCCAGGC-3’ with restriction enzyme sites underlined) in the Pim-1 mRNA 3’-UTR were synthesized at Integrated DNA Technologies (Coralville, IA). The oligonucleotides were ligated into the XhoI/NotI sites of the psiCHECK2 vector.

For luciferase assays, LNCaP and HeLa cells were cotransfected with the following primer sets: TTP, CGCTACAAAGCTGATGAGG; GGTATGAGG; and GAGGTAGAACTTGTGACAGA; Pim-1, TCTCATGATGGTGTTGGCCCT and TGTCGACTGTCATACATG-CCC; and ATCAATGGTTGTCCAGCACC; Pim-3, TGACCGCAGTAAAGGAGGAAA and ACACCATATCGTGAAGAGCAGGC; and GAPDH, ATCTTCAGGCTATCGTGTGC and TGCCGGTCTCACATTTTCTTG.
TTP Inhibits Pim-1 Expression and Cell Growth

UUGG GUUCCCUUCCAUUCC-3’ and mutant (5’-CCUG-GAGGUCAAUGUUAGUAGCAGCAGCAUUUGGU-UCCCUUCCAUUCC-3’) Pim-1 ARE2 were synthesized by ST Pharm Co., Ltd. (Seoul, Korea). A mutant RNA probe in which four AUUUA petamers were each substituted with AGCAG was used as a negative control. Cytoplasmic extracts were prepared from LNCaP and HeLa cells non-transfected or transfected with pcDNA6/V5-TTP using NE-PER nuclear and cytoplasmic extraction reagent (Pierce). RNA EMSA was performed using the LightShift chemiluminescent EMSA kit (Pierce) according to the manufacturer’s instructions. In brief, 50 fmol of biotinylated RNA was combined with 3 μg of cytoplasmic protein extract in binding buffer. For competition EMSA, a 50-fold excess of unlabeled wild-type or mutant RNA probe was added to the binding reaction. For the supershift EMSA, anti-V5 antibody, anti-human TTP antibody, or control antibody (I-5381, Sigma) was added to the reaction mixture. After the addition of antibodies, reaction mixtures were incubated overnight at 4 °C. The reaction mixtures were resolved on 5% nondenaturing polyacrylamide gels in 0.5 X Tris borate/EDTA buffer. Gels were transferred to a nylon membrane (HybondTM-N+) in 0.5 X Tris borate/EDTA at 70 V for 1 h at 4 °C. The RNAs were UV light-cross-linked to the membrane and detected using streptavidin-horseradish peroxidase binding and chemiluminescence.

For ribonucleoprotein precipitation assay, the biotinylated RNA probe-protein complexes in the reaction mixture were precipitated using streptavidin magnetic beads (656-01, Invitrogen). TTP proteins in the precipitated samples were detected by Western blot analysis using anti-V5 antibody.

Cell Proliferation—For the MTS cell proliferation assay, LNCaP cells were plated in triplicate at 5.0 x 10^4 cells/well in 96-well culture plates in RPMI 1640 medium. At the indicated times, CellTiter 96® AQueous One solution reagent (Promega) was added to each well according to the manufacturer’s instructions. Absorbance at 490 nm was determined for each well using a Wallac 1420 VICTOR multilabel counter.

Statistics—For statistical comparisons, p values were determined using Student’s t test

RESULTS

Pim-1 Expression Is Inversely Correlated with TTP Expression in Human Cancer Cell Lines—It has previously been reported that Pim-1 mRNA has a short half-life due to the presence of ARE motifs in the 3’-UTR (24, 33). TTP is an ARE-binding protein and enhances the degradation of ARE-containing transcripts (27, 28). Therefore, it is possible to speculate that the expression of Pim-1 is post-transcriptionally regulated by TTP. To confirm this hypothesis, we first examined TTP and Pim-1 expression levels by Western blotting in four human cancer cell lines: DU145, LNCaP, A549, and HeLa. The expression level of TTP was high in DU145 and A549 cells but extremely low in LNCaP and HeLa cells. In contrast, the level of Pim-1 expression in A549 and DU145 cells was low compared with that in LNCaP and HeLa cells (Fig. 1A). We next determined whether the overexpression of TTP could reduce the Pim-1 expression in LNCaP and HeLa cells. Cells were transfected with the TTP expression vector (pcDNA6/V5-TTP) or the pcDNA6/V5 empty vector as a negative control. Overexpression of TTP in LNCaP and HeLa cells was confirmed by Western blot analysis (Fig. 1B and supplemental Fig. 1A). Overexpression of TTP significantly inhibited the level of Pim-1 mRNA expression (Fig. 1C and supplemental Fig. 1B). It has been reported that the Pim-1 gene encodes two isoforms of 34 and 44 kDa through the use of alternative translation initiation codons of the Pim-1 mRNA (34). Overexpression of TTP inhibited the levels of both Pim-1 isoforms (supplemental Fig. 1C). The Pim kinase family consists of three members: Pim-1, Pim-2, and Pim-3. To determine whether the expression of Pim-2 and Pim-3 is affected by TTP, we analyzed their expression levels by RT-PCR in LNCaP and HeLa cells transfected with pcDNA6/V5-TTP or pcDNA6/V5. overexpression of TTP did not affect the expression levels of both Pim-2 and Pim-3 (supplemental Fig. 1, D and E).

We also tested whether the down-regulation of TTP affects Pim-1 expression. For this purpose, we used A549 cells expressing high levels of TTP. A549 cells were transfected with siRNA against TTP to reduce the expression level of TTP. Although treatment with nonspecific siRNA (scrambled siRNA) did not induce a change in Pim-1 expression, down-regulation of TTP indicates in A549 and DU145 cells was low compared with that in LNCaP and HeLa cells. In contrast, the level of Pim-1 expression in A549 and DU145 cells but extremely low in LNCaP and HeLa cells (Fig. 1A). We next determined whether the overexpression of TTP could reduce the Pim-1 expression in LNCaP and HeLa cells. Cells were transfected with the TTP expression vector (pcDNA6/V5-TTP) or the pcDNA6/V5 empty vector as a negative control. Overexpression of TTP in LNCaP and HeLa cells was confirmed by RT-PCR and Western blot analysis (Fig. 1B and supplemental Fig. 1A). Overexpression of TTP significantly inhibited the level of Pim-1 mRNA expression (Fig. 1C and supplemental Fig. 1B). It has been reported that the Pim-1 gene encodes two isoforms of 34 and 44 kDa through the use of alternative translation initiation codons of the Pim-1 mRNA (34). Overexpression of TTP inhibited the levels of both Pim-1 isoforms (supplemental Fig. 1C). The Pim kinase family consists of three members: Pim-1, Pim-2, and Pim-3. To determine whether the expression of Pim-2 and Pim-3 is affected by TTP, we analyzed their expression levels by RT-PCR in LNCaP and HeLa cells transfected with pcDNA6/V5-TTP or pcDNA6/V5. overexpression of TTP did not affect the expression levels of both Pim-2 and Pim-3 (supplemental Fig. 1, D and E).

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by siRNA significantly increased the level of Pim-1 expression (Fig. 1D). However, the inhibition of TTP by siRNA did not affect the levels of Pim-2 and Pim-3 (supplemental Fig. 1F). These results indicate that TTP down-regulates Pim-1 expression in human cancer cells.

**TTP Destabilizes Pim-1 mRNA**—To determine whether TTP-induced inhibition of Pim-1 expression resulted from changes in the stability of Pim-1 mRNA, the half-life of this mRNA was measured by qRT-PCR in LNCaP and HeLa cells transfected with pcDNA6/V5-TTP (LNCaP/TTP and HeLa/TTP, respectively) or the pcDNA6/V5 control vector (LNCaP/pCNA and HeLa/pCNA, respectively). In the control LNCaP/pCNA and HeLa/pCNA cells, the half-life of Pim-1 mRNA was >1 h after actinomycin D treatment. However, in the TTP-overexpressing cells, the half-life was reduced to <30 min in LNCaP/TTP cells (Fig. 2A) and to <1 h in HeLa cells (supplemental Fig. 2A). These results indicate that the overexpression of TTP contributes to a decrease in Pim-1 levels through the destabilization of Pim-1 mRNA.

**ARE2 in the Pim-1 3′-UTR Is Essential for the TTP Inhibitory Effect**—The TTP protein regulates mRNA stability through binding to the AREs in the mRNA 3′-UTR (27, 31, 35). Consistent with a previous study (25), analysis of the 1335-bp-long human Pim-1 3′-UTR revealed the presence of two ARE motifs: ARE1 (AUUUUA) and ARE2 (AUUUUAUUAUUUAUUUAUUA) (Fig. 2B). To determine whether they are necessary for the TTP inhibitory effect, we used a luciferase reporter gene linked to the Pim-1 3′-UTR fragments containing the AREs (Frag-ARE1 or Frag-ARE2) in plasmid psiCHECK. When LNCaP cells were transfected to overexpress TTP (Fig. 2A), the half-life of the reporter gene linked to Frag-ARE2 was significantly decreased (<1 h) compared with the original reporter gene (>1 h) (Fig. 2E), and the luciferase activity of Frag-ARE2 was dramatically inhibited by 84% (Fig. 2C). However, Frag-ARE1 abrogated the effects of TTP on the half-life (>1 h) and activity (6% inhibition) of the reporter gene in LNCaP cells (Fig. 2, C and E). To determine whether the ARE2 motif in Frag-ARE2 is essential for the TTP inhibitory effect, we prepared the oligonucleotide Oligo-ARE2 WT (containing ARE2) and its deletion mutant, Oligo-ARE2 MUT (Fig. 2B). The ARE2 mutant prevented the TTP effects on the half-life and activity of the reporter gene (Fig. 2, D and E), strongly suggesting that Pim-1 ARE2 is involved in the TTP inhibitory activity. ARE2 of Pim-1 was also responsible for the TTP inhibitory effect in HeLa cells (supplemental Fig. 2, B and C).

**TTP Interacts with ARE2 in the 3′-UTR of Pim-1**—To determine whether TTP interacts with the ARE2 in the Pim-1 3′-UTR, cytoplasmic extracts were prepared from LNCaP or HeLa cells transfected with pcDNA6/V5-TTP to overexpress TTP and incubated with a biotinylated RNA probe containing wild-type or mutant ARE2 of Pim-1. The biotinylated RNA probe-protein complexes were precipitated with streptavidin-agarose, and the presence of TTP in the precipitates was determined by Western blotting using anti-V5 antibody. Although a TTP band was observed in the precipitated samples obtained using wild-type Pim-1 ARE2, it was not detected in the samples using mutant Pim-1 ARE2 (supplemental Fig. 3, A and B). These results demonstrate that TTP interacts specifically with Pim-1 ARE2. To confirm the interaction between TTP with Pim-1 ARE2, RNA EMSA was conducted. When RNA EMSA was conducted using the wild-type Pim-1 ARE2 probe, a dominant probe-protein complex was observed. However, mutation of Pim-1 ARE2 prevented the formation of this complex. The complex was reduced, and a high molecular weight antibody complex was formed by preincubation of the reaction mixture with anti-V5 antibody but not with control antibody (Fig. 2F and supplemental Fig. 4A). To demonstrate the association between endogenous TTP and Pim-1 ARE2, cytoplasmic extracts were prepared from non-transfected LNCaP or HeLa cells. When wild-type Pim-1 ARE2 was mixed with cytoplasmic extracts from LNCaP or HeLa cells, a dominant probe-protein complex was observed (Fig. 2G and supplemental Fig. 4B). However, the mutant Pim-1 ARE2 probe failed to form the complex. In addition, the complex did not compete with an excess of the unlabeled wild-type Pim-1 ARE probe, demonstrating that the complex is Pim-1 ARE2-specific (supplemental Fig. 4, C and D). Formation of the Pim-1 ARE2 probe-protein complex was reduced by preincubation of the reaction mixture with anti-TTP antibody but not with control antibody (Fig. 2G and supplemental Fig. 4, B–D). These data suggest that the inhibition of Pim-1 expression requires the formation of a Pim-1 ARE2-dependent complex containing TTP.

**Pim-1 Mediates the Inhibitory Effect of TTP on the Growth and Cell Cycle of LNCaP Cells**—We next determined the effect of TTP overexpression on the proliferation of LNCaP cells. We transfected LNCaP cells with pcDNA6/V5-TTP (LNCaP/TTP or pcDNA6/V5) or pcDNA6/V5 (LNCaP/pCNA), and the cell cycle and the growth rates were compared. Overexpression of TTP and down-regulation of Pim-1 were confirmed by Western blotting (Fig. 3A, upper panel). The cell cycle of LNCaP cells was arrested at G1 phase (supplemental Fig. 5), and the growth of LNCaP cells was significantly suppressed by the overexpression of TTP (Fig. 3A, lower panel). The inhibition of Pim-1 by siRNA treatment (Fig. 3B, upper panel) resulted in suppression of cell growth to a level similar to that achieved by TTP overexpression (Fig. 3B, lower panel). To determine whether the TTP-induced inhibition of cell growth was mediated by a reduction in Pim-1 levels, we transfected LNCaP cells with the Pim-1 cDNA, in which expression is not affected by TTP because the transcript does not contain the Pim-1 3′-UTR. Overexpression of Pim-1 (Fig. 3A, upper panel) increased the growth of LNCaP/TTP cells, which exceeded that of LNCaP/pCNA control cells (Fig. 3A, lower panel). It has previously been reported that the ectopic expression of Pim-1 increases cell mitogenesis, independent of growth factors (36). We also found that the overexpression of Pim-1 increased the growth of LNCaP cells (Fig. 3C). These results show that the level of Pim-1 expression correlates with the growth of LNCaP cells and that TTP suppresses cell growth through the down-regulation of Pim-1.

**Inhibition of Pim-1 Target Protein p21 by siRNA Attenuates the Inhibitory Effect of TTP on Cell Growth**—It has been reported that Pim-1 suppresses cell growth through the
phosphorylation of p21 (5, 6). To determine the effects of TTP overexpression on the phosphorylation of p21, we analyzed the expression level and phosphorylation of p21 in LNCaP/TTP and LNCaP/pcDNA cells. Although the overexpression of TTP increased the total protein level of p21, it decreased phosphorylation (Fig. 4A). Overexpression of Pim-1 cDNA in LNCaP/TTP cells decreased the total protein level of p21 and increased its phosphorylation (Fig. 4B), indicating that changes in the levels of total protein and phosphorylation of p21 in LNCaP cells are caused by the reduced expression of Pim-1. p27 is another Pim-1 target (7), and we found that TTP overexpression decreased its phosphorylation (Fig. 4A), but the overexpression of Pim-1 cDNA increased its phosphorylation (Fig. 4B). In the case of p27, the overexpression of TTP or Pim-1

FIGURE 2. TTP enhances the decay of Pim-1 by binding to the second ARE of the Pim-1 3′-UTR. A, TTP destabilizes Pim-1 mRNA. LNCaP cells were transfected with pcDNA6/V5-TTP or the pcDNA6/V5 control vector. Upper panel, the expression of TTP was determined by Western blotting (WB) using anti-TTP antibody. Lower panel, the expression of Pim-1 mRNA in LNCaP cells was determined by qRT-PCR at the indicated times after the addition of 5 μg/ml actinomycin D. The results represent means ± S.D. of three independent experiments. **, p < 0.01. Rel., relative. B, schematic representation of the PCR primers (arrows) and luciferase reporter constructs used in this study. Fragments (Frag) and oligonucleotides (Oligo) derived from the 1335-bp-long Pim-1 mRNA 3′-UTR were cloned downstream of the luciferase reporter gene in the psiCHECK2 luciferase expression vector. White circles represent the WT pentameric motif AUUUA, and gray circles represent the mutants (MUT) of motif AGCA. C–E, LNCaP cells were cotransfected with the psiCHECK2 luciferase reporter construct containing ARE1 (Frag-ARE1) or ARE2 (Frag-ARE2) (C and E), Oligo-ARE2 WT or Oligo-ARE2 MUT and pcDNA6/V5-TTP or the pcDNA6/V5 control vector (D and E). C and D, cells were harvested, and luciferase activity was normalized to firefly activity. The TTP-induced inhibition of luciferase activity observed with each construct was compared with that obtained with the pcDNA6/V5 empty vector. Results represent means ± S.D. of three independent experiments. ***, p < 0.001. E, the expression of luciferase mRNA in LNCaP cells was determined by qRT-PCR at the indicated times after the addition of 5 μg/ml actinomycin D. Results represent means ± S.D. of three independent experiments. ***, p < 0.001. F and G, an RNA EMSA was performed by mixing cytoplasmic extracts containing 3 μg of total protein from pcDNA6/V5-TTP-transfected LNCaP cells (F) or LNCaP cells with 50 fmol of biotinylated wild-type (ARE2 WT) or mutant (ARE2 MUT) probe (G). Anti-V5 (F), anti-TTP (G), or control antibody was added to the reaction mixtures. The binding reactions were then separated by electrophoresis on a 5% polyacrylamide gel under non-denaturing conditions. Arrows indicate positions of the TTP-containing band.
cDNA did not affect the total protein level of p27 (Fig. 4, A and B). To determine whether the TTP-induced inhibition of cell growth is mediated by the increase in p21 levels, we analyzed the growth of LNCaP/TTP cells after the knockdown of p21 by siRNA treatment. The inhibition of p21 by siRNA treatment (Fig. 4C) restored the growth of LNCaP/TTP cells to a level similar to that of LNCaP/pcDNA control cells (Fig. 4D). These results suggest that the inhibitory effect of TTP on LNCaP cell growth is mediated by the Pim-1/p21 pathway.

DISCUSSION

The expression of Pim-1 is up-regulated in many human tumors and is correlated with the enhanced metastatic potential of the tumor (37). Hence, the mechanisms for the down-regulation of Pim-1 gene expression have been the subject of substantial interest. It has previously been reported that the AUUUA motif in the Pim-1 3′/H11032-UTR plays an important role in the destabilization of Pim-1 mRNA (25) and that proviral insertion in the Pim-1 3′-UTR results in high levels of Pim-1 mRNA (24). In this study, we demonstrated that an ARE-binding protein (TTP) binds to the ARE motif and enhances the decay of the Pim-1 transcript. Mutation of the AUUUA motif in the Pim-1 3′-UTR abrogates the binding of TTP to the Pim-1 3′-UTR and the TTP-induced decay of the Pim-1 3′-UTR-containing luciferase gene. Based on our results, it is possible to speculate that proviral insertion in the Pim-1 3′-UTR may disrupt the AUUUA motif, resulting in the inhibition of TTP binding to the Pim-1 3′-UTR and an increase in the level of Pim-1.

TTP is an ARE-binding protein that promotes the degradation of ARE-containing mRNA (27, 31, 35). Given previous reports that TTP expression is significantly decreased in many cancers (29), it is possible to speculate that the reason for the enhanced level of Pim-1 in tumor cells, which gives cells a competitive growth advantage, may result from the low level expression of TTP. In support of this hypothesis, we found that the inhibition of TTP by siRNA significantly increased the Pim-1 expression levels in cancer cells in the absence of added growth factors. In addition, restoring TTP expression suppressed the expression of Pim-1 and cell proliferation. The expression of Pim-1 cDNA without the 3′-UTR restored the growth of cells overexpressing TTP, indicating that the inhibitory effect of TTP on cell growth is mediated by the down-regulation of Pim-1.

It is believed that the oncogenic and prosurvival potential of the Pim-1 kinase contributes to tumorigenesis and promotes drug resistance in prostate cancer (1, 2). These findings are important because they provide evidence that the development of effective cancer treatments may be possible by targeting Pim-1 kinase (22). Several small compounds inhibiting Pim-1 activity have been reported to exhibit antiproliferative activity in human tumor cells (38–40). In addition, the inhibition of Pim-1 expression by Pim-1 mAb treatment has been reported to suppress tumor growth (19). In this study, we found that the overexpression of TTP significantly reduced the Pim-1 expression levels and growth of cancer cells. Our results indicate that an inducer of TTP expression could be a candidate for a chemotherapeutic drug. Such molecules may potentially enable the inhibition of prostate cancer growth mediated by Pim-1 without severe physiological side effects because normal cells already express a high level of TTP.

Cell cycle regulatory proteins p21 and p27 have been reported to be substrates of Pim-1 (5–7). Consistent with previous reports, we found that the down-regulation of Pim-1 by TTP led to decreased p21 and p27 phosphorylation. However,
although the phosphorylation of p21 by Pim-1 has been reported to stabilize the p21 protein and to increase p21 protein levels (5, 6), we were surprised to find that the TTP-mediated reduction in p21 phosphorylation increased p21 protein levels. The inhibition of p21 by siRNA restored the growth of TTP-overexpressing cells, indicating that the inhibitory effect of TTP on tumor cell growth is mediated by an increase in the level of p21. There has been an ongoing controversy about whether the phosphorylation of Thr-145 or Ser-146 influences the stability of p21 (41, 42). It was found that PKC and Akt can phosphorylate and promote p21 degradation (41). However, it was also found that Akt phosphorylation of the same site results in the stabilization of p21 (42). Besides phosphorylation, association with other cellular proteins is also an important factor that regulates p21 protein stability and, therefore, protein levels. For example, the binding of cyclin D1 (43) or the chaperone Hsp90 and WISp3 (44) to p21 dramatically stabilizes it. Further studies are needed to determine whether such interacting proteins are required for the stabilization of p21 protein phosphorylated by Pim-1.

In conclusion, we have demonstrated that TTP plays an important role in the post-transcriptional regulation of Pim-1 gene expression. We determined that Pim-1 contains an ARE in its mRNA 3′-UTR and that TTP destabilizes its mRNA by binding to the Pim-1 mRNA ARE. As a result, TTP-induced down-regulation of Pim-1 leads to a decrease in the phosphorylation of p21 and p27 but increases the protein level of p21, which contributes to the suppression of cancer cell growth. This study therefore provides a molecular mechanism for the post-transcriptional regulation of Pim-1 gene expression. Because down-regulation of TTP is among the key factors that increase Pim-1 expression in many cancer cells, it is conceivable that increasing TTP expression through a specific drug might represent a method for the treatment of cancer in humans.
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