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Tristetraprolin regulates the stability of HIF-1α mRNA during prolonged hypoxia

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ABSTRACT

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor involved in the cancer cell adaptation to hypoxia, a leading cause of tumor malignancy. Thus, control of HIF-1α expression may assist in treatment of cancer. The expression of HIF-1α is finely regulated *via* alterations in not only HIF-1α protein stability but also mRNA stability. However, the molecular mechanisms of regulation of HIF-1α mRNA stability have not yet been fully elucidated. Here, we show that tristetraprolin (TTP) protein, of which the mRNA expression level is downregulated in most of hepatocellular carcinoma tissues, bound directly to the 3′-UTR of HIF-1α mRNA containing eight putative TTP-binding motifs, AU-rich elements (AUUUA), to downregulate stability. Furthermore, TTP expression was induced in hypoxic cells, and overexpression of TTP repressed the hypoxic induction of HIF-1α protein. Taken together, these data suggest that TTP is a modulator of HIF-1α expression during hypoxia and may play a physiological role in regulation between cellular adaptation and apoptosis in prolonged hypoxia. In addition, cancer cells may benefit from the downregulation of TTP, which subsequently increases HIF-1α expression and assists with the adaptation of cancer cells to hypoxia.

Introduction

During tumor growth, cancer cells must adapt to the hypoxic conditions encountered in rapidly growing cancer tissue [1]. Hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor that consists of the HIF-1α and HIF-1β (aryl receptor nuclear translocator, ARNT) subunits, is necessary for cellular adaptation to hypoxia because HIF-1 transactivates the downstream genes implicated in angiogenesis, erythropoiesis, and glycolysis [2]. Hypoxic conditions can trigger cancer cell necrosis or apoptosis in the absence of adequate intracellular adaptive responses, such as the absence or malfunction of the HIF protein [3]. In addition, hypoxic conditions allow cells to become metastatic when the HIF protein induces the expression of metastasis-related genes. Therefore, inhibition of HIF function might prove an effective treatment for hypoxic and metastatic cancers [4].

It has been well established that HIF-1α levels are modulated in response to protein stability, which is regulated by the ubiquitin-proteasomal system [5]. The ubiquitination of HIF-1α only occurs in normoxic conditions after proline hydroxylation, which is destruction signal recognized by VHL protein, a component of the E3 ligase complex [6]. As cells are faced on hypoxia, HIF-1α protein is not anymore hydroxylated, but stabilized. Recent studies have reported alternative mechanisms by which HIF-1α expression is regulated. One report proposed that HuR and PTB jointly up-regulate HIF-1α translation in response to $CoCl_2$ [7]. In a recent study, HIF-1α mRNA was identified as a novel target of the miR-17-92 miRNA cluster, of which expression is induced by c-Myc, explaining the c-Myc-mediated translational repression of HIF-1α [8]. On the other hand, whereas HIF-1α protein expression increases during acute hypoxia or $CoCl_2$ treatment, HIF-1α mRNA and, in turn, protein levels gradually decrease during prolonged periods of hypoxia or during long-term $CoCl_2$ treatment of A549 lung epithelial cells [9]. A naturally occurring antisense transcript of HIF-1α (aHIF), which is complementary to the 3′-untranslated region (3′-UTR) of HIF-1α mRNA, has been discovered in clear-cell renal carcinoma samples [10]. This transcript is specifically overexpressed in nonpapillary clear-cell renal carcinomas and can be induced in lymphocytes and in a human lung epithelial cell line (A549) by hypoxia [9,10]. The sense transcripts of many genes are also regulated *via* the expression of their respective countertranscripts [11]. Thus, it has been proposed that aHIF helps to repress HIF-1α expression. A computerized prediction of the HIF-1α mRNA secondary structure revealed that a hairpin structure within the 3′-untranslated region would be disrupted by aHIF hybridization, thereby exposing binding sites (*e.g.*, AU-rich elements, or AREs) for RNA-destabilizing factors and possibly increasing mRNA degradation [9]. The molecular mechanisms regulating stability of HIF-1α mRNA, however, have not yet been fully elucidated.

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AREs are the most common RNA stability determinants found in the 3′-UTRs of many labile mRNAs. AREs can be classified into three groups, with the first containing a few copies of scattered AUUUA motifs represented by the c-Fos ARE. The second group contains multiple reiterations of the AUUU tetranucleotide. These reiterations give rise to at least two overlapping copies of the UUAUUUAUU nonamer, which is represented by the granulocyte–macrophage colony-stimulating factor (GM-CSF) ARE. The third group consists of non-AUUUA-containing AREs [12]. Tristetraprolin (TTP) is a well-known regulator of ARE-containing cytokine mRNAs, such as TNF-α, GM-CSF, c-Fos, and Cox-2, and thus plays an important role in the response to inflammation [12–14]. TTP also functions as a tumor suppressor and is often downregulated in various cancers, where it would otherwise induce cancer cell apoptosis and regulate tumor progression by targeting the mRNAs of oncogenes (*e.g.*, VEGF, COX-2, Cyclin D1, and c-Myc) [14–17]. TTP controls the stability of target mRNAs by binding to 3′-UTR-containing ARE motifs and recruiting factors that mediate decapping, deadenylation, and mRNA destruction. The 3′-UTR of the HIF-1α mRNA contains six scattered pentamer AREs and one nonamer ARE, suggesting that TTP could bind to the 3′-UTR of this mRNA and regulate translation or stability.

Here, we show that expression of TTP is downregulated in HCC, and that TTP modulates hypoxia response element (HRE)-reporter activity and directly binds to the 3′-UTR of HIF-1α mRNA. We also assessed whether the direct binding of TTP to the HIF-1α mRNA could regulate mRNA stability and modulate activity of the HIF pathway. Finally, we determined the effects of TTP on HIF-1α protein expression in hypoxic cells.

Materials and methods

Reagents. Synthetic TTP siRNA (h) (Cat. #sc-36760) was purchased from Santa Cruz Biotechnology Inc., and GFP siRNA (sense: 5′-GUUCAGCGUGUCCGGCGAGTT-3′ and antisense: 5′-CUCGCCGG ACACGCUGAACTT-3′) was purchased from Samchully Pharm Co. The primers used in this study are described in Supplementary Table S1. Anti-c-Myc (Cat. #sc-40) and anti-TTP (Cat. #sc-12563) antibodies were obtained from Santa Cruz Biotechnology Inc., and anti-HIF-1α antibody (Cat. #610958) was obtained from BD Transduction Laboratories. Anti-β-actin antibody (Cat. #A2228), actinomycin D (ActD) (Cat. #A9415) and tetracycline (Cat. #T7660) were purchased from Sigma Chemical Co.

Cell cultures, plasmids and generation of stable cell lines. HCT116 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (JBI) and 1× penicillin/streptomycin (*i.e.*, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.0085% saline; Gibco-BRL Life Technologies). Cells were grown at 37 °C in the presence of humidified air containing 5% CO_2. All other cells were maintained in Dulbecco's modified Eagle's medium with 10% FBS and 1× penicillin/streptomycin. Lipofectamine Plus reagent (Invitrogen Life Technologies) was used in all transfection experiments, according to the supplier's instructions. The primer sequences and construction strategies used to create all vectors are described in Supplementary data. To generate a TTP-inducible stable cell line (HCT116/TTPmyc^{in}), HCT116(p53$^{-/-}$) cells were co-transfected with pcDNA4/TO/TTP-myc and pcDNA6/TR (Cat. #V1025-20, Invitrogen Life Technologies), and were selected using 10 μg/ml of Zeocin and 10 μg/ml of Blasticidine S HCl (Invitrogen Life Technologies).

Reporter assays and RNA binding assays. In preparation for HRE- and UTR-reporter assays, cells were seeded at a density of 2×10^5 per well in six-well plates and incubated for 18 h before transfection. Cells were transfected with DNA [*i.e.*, each well contained 0.4 μg HRE-luciferase or 0.4 μg UTR-luciferase reporter, 0.1 μg pRL-CMV Renilla luciferase vector (Cat. #E2261, Promega Co.) and 0.5 μg of a pcDNA3.1 control or 0.5 μg of the pCMV-TTP vector] [18]. Forty-eight hours after transfection, cells were lysed in 100 μl of 1× Reporter Lysis Buffer (Cat. #E3971, Promega Co.). Luciferase activity was measured using the luciferase assay system (Cat. #E1500, Promega Co.) in a luminometer (Lumat LB9501, Berthold). Renilla luciferase was used as an internal control for the normalization of transfection efficiency. Luciferase assays were performed in duplicates, and each experiment was repeated at least twice.

The RNA binding assays were performed as previously described, with the exception that TTP proteins were obtained from HCT116/TTPmyc^{in} cells treated with 5 μg/ml tetracycline [19].

RNA procedures. Total RNA was prepared using TRIzol (Invitrogen Life Technologies), and reverse transcriptase (MBI Fermentas

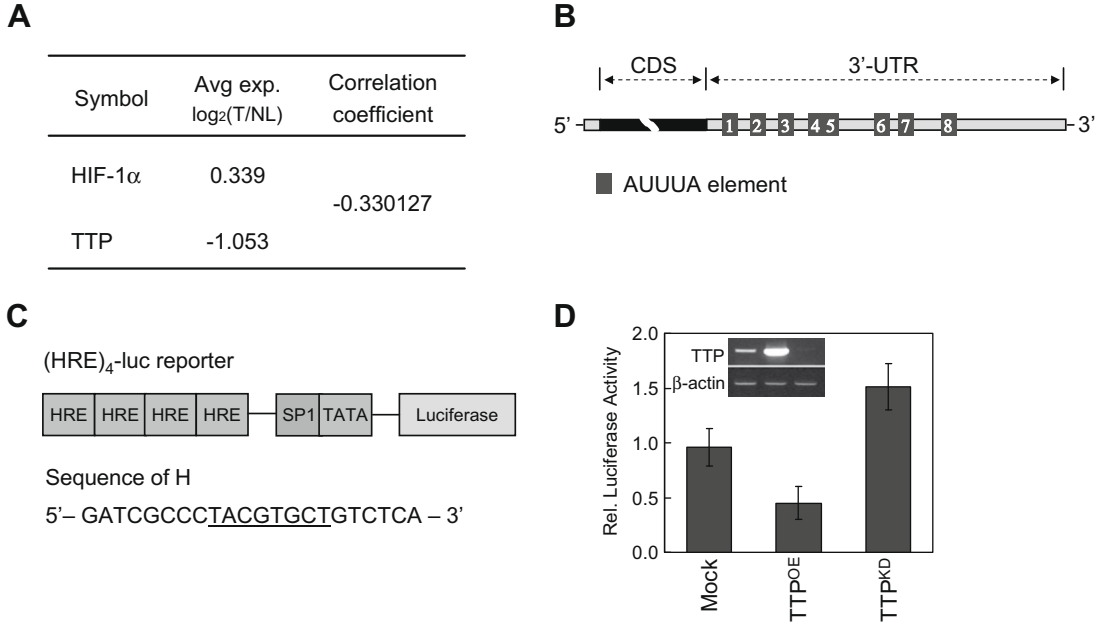

Fig. 1. HIF-1α mRNA contains putative TTP-binding AREs. (A) Expression of TTP and HIF-1α mRNA was down- and up-regulated in HCC tissues, respectively. (B) Schematic diagram of the HIF-1α mRNA. The 3′-UTR of the HIF-1α mRNA has seven pentamers (AUUUA) and one nonamer (AUUUAUUUA) AREs. (C) Schematic diagram of the HRE-luciferase reporter. (D) The luciferase activity of the HRE reporter was increased by the knockdown of TTP and decreased by the forced expression of TTP.

Inc.) was used to amplify cDNA from total RNA. The reverse transcriptase-PCR (RT-PCR) experiments were performed according to standard protocols, with the following optimized PCR conditions: 1 cycle at 95 °C for 30 s; 18–30 (for semi-quantitative RT-PCR or RT-PCR) or 45 (for quantitative real-time PCR or qRT-PCR) cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension cycle at 72 °C for 10 min. The specificity of the qRT-PCR amplification was verified by a melting curve analysis (from 50 to 90 °C) using SYBR Premix Ex Taq (TaKaRa Bio) and the Exicycler Real-Time Thermal Block (Bioneers). Relative gene expression levels were calculated as $2(\Delta C_t)$ with β-actin used for normalization, where Ct was defined as the threshold cycle number when the amplified product was first detected.

Results and discussion

TTP inhibits the hypoxia response element (HRE)-promoter activity

In cancer cells of hypoxic tumors, a variety of signals originated from environmental conditions may up-regulate HIF pathway by increasing HIF-1α protein stability or translation rate of HIF-1α mRNA [5–8]. The stability of HIF-1α mRNA is also regulated during prolonged hypoxia [9]. Our analysis of 49K-cDNA microarray chips (please see Ref. [20]) revealed that HIF-1α mRNA expression was up-regulated (average fold-change in 41 samples: $\log_2$(T/NL) = 0.339) in tumor tissues (T) compared with normal tissues (NL) (Fig. 1A). The 3′-UTR of HIF-1α mRNA contains seven pentamer (AUUUA) and one nonamer (UUAUUUAUUUAUU) ARE domains on which many RNA regulators can bind (*i.e.*, putative RNA regulator-binding motifs) (Fig. 1B). TTP has been shown to destabilize mRNAs that contain AREs within the 3′-UTRs [12–17] and TTP mRNA expression was twofold down regulated in 25 of 41 HCC tissue samples analyzed *via* DNA chips, showing negative correlation ($r = -0.330127$) with the HIF-1α expression (Fig. 1A, Supplementary Table S2). Therefore, the TTP protein may be a putative negative regulator of HIF-1α mRNA stability. In addition, previous studies showed that TTP is a tumor suppressor that induces apoptosis, reduces tumor size in xenograft assays, and regulates the mRNA stability of cancer-related genes such as VEGF, cyclin D1, and c-Myc [14–17]. Thus, it is likely that TTP acts as a negative regulator of HIF-1α mRNA stability and downregulates HIF-1α expression in cells subjected to prolonged hypoxia. However, cancer cells exposed to hypoxic conditions may require sustained HIF-1α expression and thus reduction in the expression of TTP and other negative regulator(s) of HIF expression.

Although there was no high correlation ($r = -0.330127$) between the expression of HIF-1α and TTP in the 41 HCC tissues analyzed (Fig. 1A), we cannot exclude the possibility that a relationship between the two genes may exist, because many factors contribute to regulation of gene expression, which thereby diminishes the effect of any specific factor. To determine the role of TTP in the regulation of HIF-1α expression, we first examined the effects of TTP expression on activity of the HIF pathway using the HRE-luciferase reporter (Fig. 1C). The assay results revealed that the forced expression of TTP downregulated the reporter activity, suggesting that TTP acts as a negative modulator of the HIF signaling pathway (Fig. 1D). This hypothesis was further supported by our finding that TTP siRNA treatment increased HRE-reporter activity (Fig. 1D).

TTP decreases the UTR-reporter activity via direct binding to the 3′-UTR of HIF-1α mRNA

Although a previous report showed that TTP could bind to an HIF-1α probe, any effect of such binding on HIF-1α mRNA or protein levels was not defined [21]. To further explore the involvement of TTP in regulation of HIF-1α expression, we synthesized three different HIF-1α-3′-UTR-luciferase reporter constructs using the pcDNA3.1(+) vector (*i.e.*, pcD-Luc, pcD-Luc-F, and pcD-Luc-R) (Fig. 2A, Supplementary materials and methods).

We next used the HIF-1α-3′-UTR reporter system to examine the effects of TTP on expression of the luciferase reporter gene. We co-transfected HEK293T cells with pCMV-TTP (or pcDNA3.1 as a control) and one of the three reporter constructs. As shown in Fig. 2B, the reporter activities of pcD-Luc and pcD-Luc-R, which were not expected to be affected by TTP, were similarly high in cells transfected with either pcDNA3.1 (*i.e.*, mock-transfected cells) or pCMV-TTP. However, the luciferase activity of pcD-Luc-F was significantly lower than that of pcD-Luc or pcD-Luc-R in cells co-transfected with pcDNA3.1, possibly because of regulation of the HIF-1α 3′-UTR by endogenous TTP proteins. The luciferase activity of the pcD-Luc-F reporter was further reduced by forced expression of TTP. These results suggest that TTP controls HIF-1α protein expression *via* the regulation of HIF-1α mRNA expression.

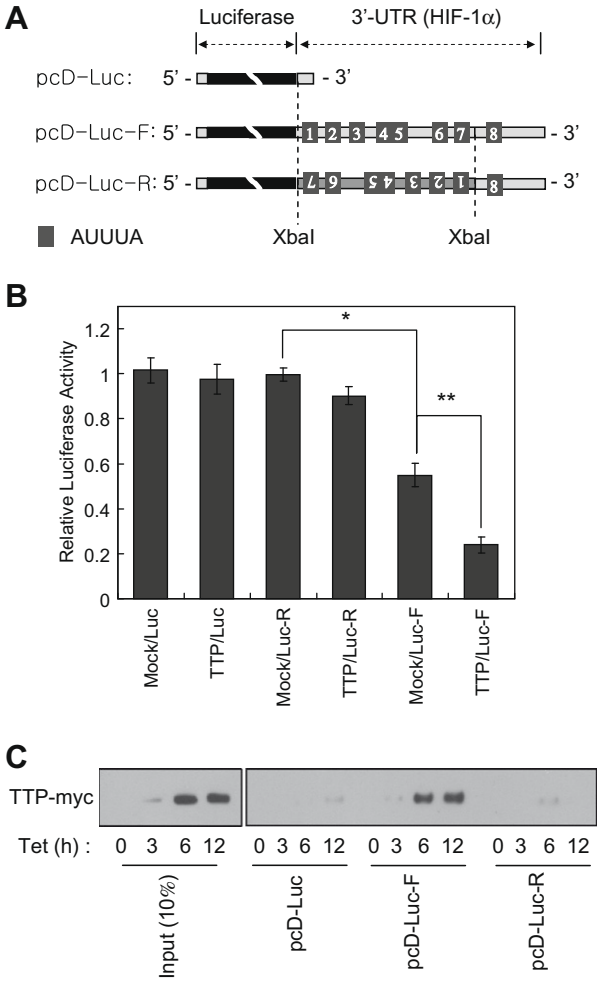

Fig. 2. TTP suppresses HIF-1α 3′-UTR-reporter activity via direct binding to the UTR. (A) Construction of the HIF-1α 3′-UTR and related reporters. Both pcD-Luc and pcD-Luc-R were used as negative controls. The inverted numbers within the XbaI fragment of pcD-Luc-R indicate the reversed sequence of the same region of the pcD-Luc-F construct. (B) The UTR-reporter assay. TTP protein down regulated the activity of pcD-Luc-F reporter, not pcD-Luc and pcD-Luc-R reporters. ($^{*}p = 0.013$, $^{**}p = 0.012$). (C) The TTP protein directly bound the HIF-1α 3′-UTR in *in vitro* RNA binding assays using biotin-labeled transcripts. The left panel indicates the amount of input TTP protein from the Tet-induced lysates.

We next examined whether TTP could bind directly to the 3′-UTR of HIF-1α mRNA. We performed a TTP binding assay using *in vitro*-transcribed RNAs from the HIF-1α-3′-UTR reporters and lysates containing the TTP-myc protein from TTP-inducible cells, HCT116/TTPmyc^{in}. The TTP-myc proteins were clearly detected in the pcD-Luc-F samples, which contained the 3′-UTR of the HIF-1α mRNA running in the forward direction (Fig. 2C). However, the TTP protein was not detected in the pcD-Luc or pcD-Luc-R samples. These results suggest that TTP, a well-known regulator of ARE-containing mRNAs, may modulate the HIF pathway *via* direct binding to the 3′-UTR of HIF-1α mRNA, which in turn promotes the degradation of HIF-1α mRNA and reduces HIF-1α protein expression.

TTP accelerates HIF-1α mRNA degradation and reduces HIF-1α protein expression

We assessed the levels of HIF-1α mRNA in TTP-overexpressing cells to determine if destabilization of HIF-1α mRNA by TTP reduced the activities of the HRE and HIF-1α 3′-UTR reporters. Compared with control cells in which HIF-1α mRNA level was initially high but gradually decreased during ActD treatment, the basal level of HIF-1α mRNA in HEK293T cells transiently transfected with the pCMV-TTP vector was initially low (Supplementary Fig. 1A). This finding was confirmed by quantitative real-time PCR analysis (Supplementary Fig. 1B). We performed a similar experiment using PLC/PRF/5 cells. While the basal levels of HIF-1α mRNA were similar in control and TTP-overexpressing cells, HIF-1α mRNA levels decreased at a faster rate in TTP-overexpressing cells than in control cells (Supplementary Fig. 1C). We also assessed changes in the HIF-1α mRNA levels of HCT116/TTPmyc^{in} cells after induction of TTP by tetracycline. HIF-1α mRNA levels gradually decreased, beginning 3 h after tetracycline treatment (Fig. 3A). To determine if the decrease in HIF-1α mRNA levels was caused by an increase in the rate of mRNA degradation because of TTP induction, we treated HCT116/TTPmyc^{in} cells with tetracycline for 3 h, followed by treatment with ActD for the indicated times. The parent cells (HCT116) were treated in the same manner, as a control. As shown in Fig. 3B (RT-PCR) and Supplementary Fig. 1D (qRT-PCR), the rate of HIF-1α mRNA degradation was clearly faster in TTP-induced cells than in parent cells, suggesting that TTP promotes the degradation of HIF-1α mRNA.

We next investigated whether the different degradation rates of HIF-1α mRNA appear as changes in expression of the HIF-1α protein in TTP-inducible cells. Differences in the amounts of mRNA may reflect variations in the level of accumulated HIF-1α protein, because the HIF-1α protein is rapidly degraded and is not usually detectable under normoxic conditions, and because proteins that are newly synthesized and stabilized after MG132 treatment, $CoCl_2$ treatment, or hypoxic conditions can be detected by immunoblot assays. Thus, we treated cells with MG132 to promote the accumulation of HIF-1α or ubiquitinated-HIF-1α (Ub-HIF-1α) proteins, and next performed immunoblot analyses. As expected, expression of HIF-1α or ub-HIF-1α proteins was significantly lower in TTP-induced cells (Fig. 3C). In addition, HIF-1α proteins were detected later in HEK293T cells transfected with the pCMV-TTP vector and treated with $CoCl_2$, compared with control cells (Supplementary Fig. 2B). The opposite result was observed in the cells treated with TTP siRNA (Supplementary Fig. 2C).

Downregulation of TTP expression in HCC may be required for the hypoxic adaptation of cancer cells

The increase of HIF-1 activity during hypoxic conditions is absolutely required for cellular adaptation to hypoxic environments. HIF-1α mRNA and protein expression, however, were gradually decreased during prolonged hypoxia [9]. Our data show how the TTP protein regulates HIF-1α mRNA expression. If TTP is a negative regulator of HIF-1α expression, TTP should be downregulated during adaptation of cancer cells to hypoxia. Supplementary Table S2 shows that expression of TTP is downregulated in a number of HCC tissues. These findings may shed light on the physiological significance of TTP downregulation in cancer cells. Previous studies have attributed the gradual decrease of HIF-1α mRNA expression in prolonged hypoxia to the increased expression of aHIF during hypoxia; however, other factors involved in the degradation of HIF-1α mRNA should be taken into account when examining the mechanism of HIF-1α regulation. We assumed that TTP expression

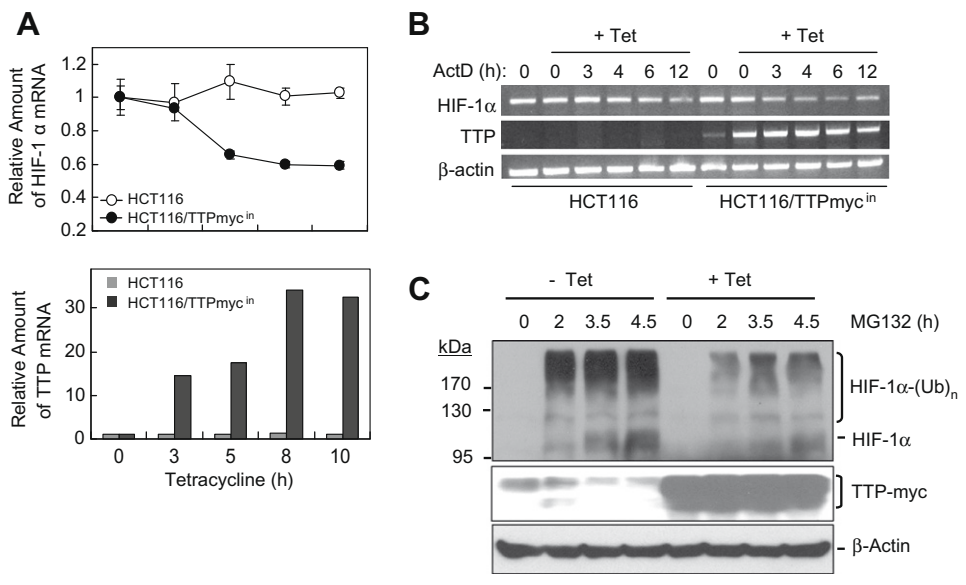

Fig. 3. TTP decreases the stability of HIF-1α mRNA and reduces the production of the HIF-1α protein. (A) TTP-inducible HCT116/TTPmyc^{in} or HCT116 cells were pretreated with tetracycline (Tet; 5 μg/ml) for 3 h, followed by actinomycin D (ActD; 10 μg/ml) for the indicated times. Total RNAs were isolated and subjected to RT-PCR to assess the HIF-1α, TTP and β-actin mRNAs. (B) In HCT116/TTPmyc^{in} cells or the HCT116 control cells, TTP was induced by Tet treatment for the indicated times, whereupon total RNAs were isolated and subjected to qRT-PCR to measure the amount of HIF-1α and TTP mRNAs. β-Actin was used as a normalizing control. (C) The HCT116/TTPmyc^{in} cells were pretreated with Tet (5 μg/ml) for 3 h, followed by MG132 (10 μg/ml) for the indicated times. Immunoblotting was performed using anti-HIF-1α and anti-Myc (to detect the TTP-myc protein) antibodies, as well as anti-β-actin antibody.

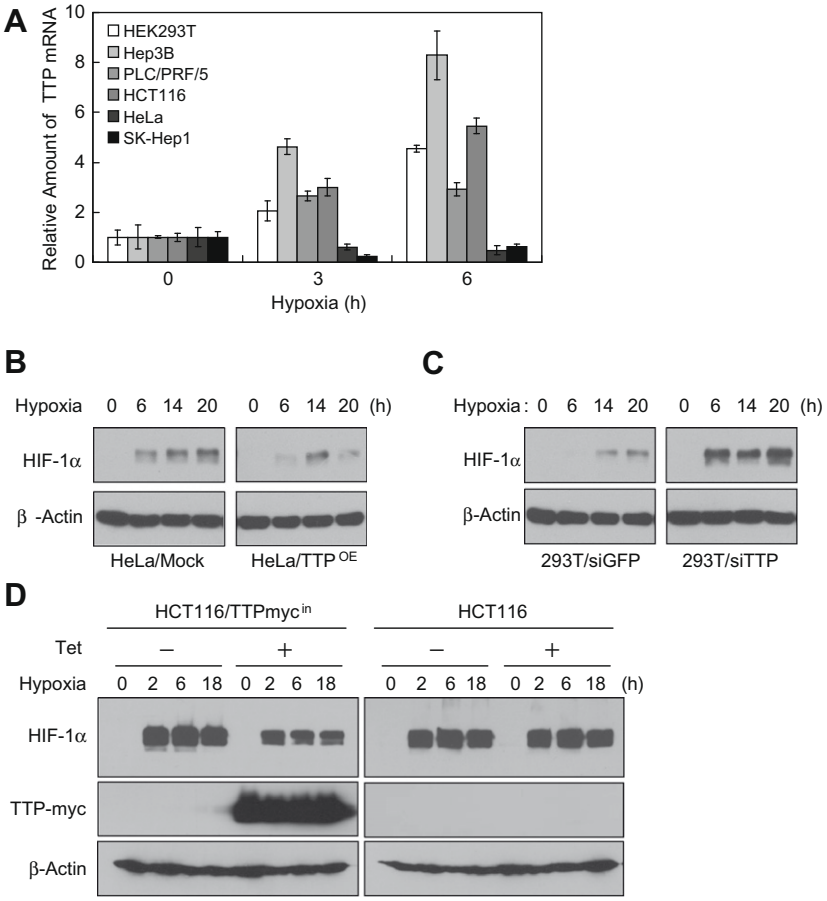

Fig. 4. TTP downregulates the accumulation of HIF-1α during hypoxia. (A) Various cell lines were exposed to 1% O_2 for 0, 3 or 6 h, and total RNAs were the isolated and subjected to qRT-PCR to detect TTP mRNA. (B–D) HeLa cells were transfected with the pCMV-TTP or pcDNA3.1 vectors, incubated for 2 days, and then exposed to 1% O_2 for the indicated times (B). The HEK293T cells were transfected with the siRNAs of TTP or GFP genes, incubated for 2 days, and then exposed to 1% O_2 for the indicated times (C). TTP-inducible HCT116/TTP-myc^{in} and the control HCT116 cells were pretreated with tetracycline (Tet; 5 μg/ml) for 5 h and then exposed to 1% O_2 for the indicated times (D). All cells (B–D) were lysed with 2× SDS–PAGE loading buffer and subjected to immunoblot analysis.

increases during hypoxia and that TTP decreases HIF-1α mRNA levels. With this assumption, we examined expression of TTP mRNA in various cell lines, under conditions of hypoxia. Expression of TTP increased in many cell lines tested except HeLa and SK-Hep1 (Fig. 4A, Supplementary Fig. 2A). Furthermore, the expressions of TTP (TIS11) and other immediate-early gene products (*e.g.*, c-fos, c-jun, and TIS 1) were induced during the immature brain response to hypoxia–ischemia (H–I) at 3 h post-hypoxia [22]. This suggests that TTP may have an important physiological role by regulating the expression of HIF-1α in the determination whether cells adapt or die in the response to prolonged hypoxia.

We next examined HIF-1α protein to determine how rapidly and highly expressed in the TTP-overexpressing or TTP-knockdowned cells under hypoxic conditions. When the pCMV-TTP vector was transiently transfected into HeLa cells in which TTP was not induced in hypoxia (see Fig. 4A), induction of HIF-1α protein expression was delayed and less than that observed in cells transfected with the pcDNA3.1 mock vector (Fig. 4B). Conversely, when HEK293T cells in which TTP was induced in hypoxia (see Fig. 4A), were transfected with TTP siRNA, HIF-1α protein was expressed more rapidly and to a higher extent than was HIF-1α protein in GFP siRNA-transfected cells under hypoxic conditions (Fig. 4C). Using TTP-inducible HCT116/TTP-myc^{in} cells, we confirmed the effects of TTP on hypoxic induction of the HIF-1α protein. Cells were pretreated with tetracycline for 5 h and next subjected to hypoxic conditions for the indicated time (Fig. 4D). The parent cell line, HCT116, was also treated with tetracycline to exclude the possibility that the observed effects stemmed from tetracycline treatment. Similar results were observed in cells treated with $CoCl_2$, a hypoxic mimetic agent (Supplementary Fig. 2B–D).

In normal cells, hypoxia-induced TTP may downregulate c-Myc mRNA expression [16]. Such regulation may be beneficial to the organism, as hypoxia is an energetically stressful condition that should slow cell proliferation. However, hypoxic cancer cells tend to increase HIF protein expression to recruit blood vessels and to maintain the expression of growth-related genes (*e.g.*, c-Myc). Therefore, inhibition of the hypoxia-mediated TTP induction, and consequent reduction in TTP function, could prove advantageous for the adaptation of cancer cells to hypoxia, and subsequent cancer growth. Our findings suggest that control of TTP expression could prove useful in cancer therapy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.174.

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