Tristetraprolin Inhibits Ras-dependent Tumor Vascularization by Inducing Vascular Endothelial Growth Factor mRNA Degradation[□]

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Vascular endothelial growth factor (VEGF) is one of the most important regulators of physiological and pathological angiogenesis. Constitutive activation of the extracellular signal-regulated kinase (ERK) pathway and overexpression of VEGF are common denominators of tumors from different origins. We have established a new link between these two fundamental observations converging on VEGF mRNA stability. In this complex phenomenon, tristetraprolin (TTP), an adenylate and uridylylate-rich element-associated protein that binds to VEGF mRNA 3'-untranslated region, plays a key role by inducing VEGF mRNA degradation, thus maintaining basal VEGF mRNA amounts in normal cells. ERKs activation results in the accumulation of TTP mRNA. However, ERKs reduce the VEGF mRNA-destabilizing effect of TTP, leading to an increase in VEGF expression that favors the angiogenic switch. Moreover, TTP decreases RasVal12-dependent VEGF expression and development of vascularized tumors in nude mice. As a consequence, TTP might represent a novel antiangiogenic and antitumor agent acting through its destabilizing activity on VEGF mRNA. Determination of TTP and ERKs status would provide useful information for the evaluation of the angiogenic potential in human tumors.

INTRODUCTION

The formation of new blood vessels is essential for tissue growth, expansion, and metastasis (Risau, 1996). Vascular endothelial growth factor (VEGF) is a key mediator of physiological and pathological angiogenesis (Ferrara, 2002). Increased production of VEGF has been shown to occur by both transcriptional and posttranscriptional mechanisms (Ferrara, 1999). Post-transcriptional regulation is emerging as an important control point for gene expression in tumors (Nabors *et al.*, 2001). This regulation involves mRNA binding proteins that act on *cis* elements located in the 5'- or

3'-untranslated regions (UTRs) of mRNAs to alter mRNA stability or the efficiency of translation (Tourrière *et al.*, 2002). VEGF mRNA-binding proteins have been described previously as stabilizing or destabilizing factors. Some of them are heterogeneous nuclear ribonucleoprotein (RNP) L (Shih and Claffey, 1999), Elav protein HuR (Levy *et al.*, 1998), poly(A)-binding protein-interacting protein 2 (Onesto *et al.*, 2004), and tetradecanoyl phorbol acetate-inducible-sequence 11b (TIS11b) (Ciais *et al.*, 2004). Another member of the TIS11 family, tristetraprolin (TTP), which presents with TIS11b 70% homology in the RNA binding domain but only an average of 20% homology in the rest of the protein, is also involved in the regulation of labile mRNA turnover. TTP has been described as an mRNA-destabilizing protein that negatively regulates the expression of proinflammatory mediators such as tumor necrosis factor α , granulocyte-macrophage colony-stimulating factor (Carballo *et al.*, 2000), interleukin (IL)-2 (Raghavan *et al.*, 2001), cyclooxygenase 2 (Sully *et al.*, 2004), and inducible nitric-oxide synthase (Fechir *et al.*, 2005). TTP exerts its action by binding to *cis*-acting elements that are adenylate and uridylylate-rich elements (AREs) (Shaw and Kamen, 1986). The mitogen-activated protein kinase p38 regulates the stability of TTP protein in myeloid cells and synergizes with the extracellular signal-regulated kinases (ERKs) to regulate expression of TTP at a posttranslational level (Brook *et al.*, 2006). TTP acts as a potent tumor suppressor when overexpressed in a v-H-Ras-dependent mast cell tumor model (Stoecklin *et al.*, 2003). One of the best-characterized signaling pathways directly activated by Ras involves Raf-1, mitogen-activated protein kinase kinase

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Abbreviations used: ARE, AU-rich element; DRB, 5,6-dichlorobenzimidazole riboside; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; GST, glutathione transferase; PBS, phosphate-buffered saline; REMSA, RNA electrophoretic mobility shift assay; RNP, ribonucleoprotein; siRNA, small interfering RNA; UTR, untranslated region; VEGF, vascular endothelial growth factor.

(MEK), and ERKs. The ERK pathway is central to a variety of cellular processes, including proliferation, differentiation, and survival. The ERK pathway plays a critical role in the regulation of VEGF expression (Pages and Pouyssegur, 2005). Constitutive activation of either Ras or Raf, which leads to constitutive activation of ERKs, allows tumor cells to evade from both normal growth and apoptosis. The link among ERK activity, the angiogenic switch, and VEGF mRNA regulation deserved to be carefully investigated. In this regard, we showed that TTP regulates VEGF mRNA expression at the posttranscriptional level and blocks VEGF expression in Ras-transformed cells *in vivo*. Our study establishes that ERKs control the degradative potential of TTP in the context of regulation of VEGF mRNA stability.

MATERIALS AND METHODS

Plasmid Constructs

The VEGF 3'-UTR/Luciferase constructs (Ciais *et al.*, 2004) and the plasmids used to prepare NsiI, and Δ NsiI probes (Onesto *et al.*, 2004) have been described previously. pCDNA4/TO/mycTTP vector was generated by inserting a 1-kb DNA fragment corresponding to the coding region of the TTP cDNA (Stoecklin *et al.*, 2003) within EcoRI and AgeI sites of the pCDNA4/TO. The pGEX6P1/TTP vector was constructed by inserting an EcoRI/EcoRI 2-kb fragment corresponding to the TTP coding region plus a portion of the initial hphMX-TTP vector (Stoecklin *et al.*, 2003) within the EcoRI sites of the pGEX6P1 vector (GE Healthcare, Chalfont St. Giles, United Kingdom).

Antibodies

Monoclonal antibodies (anti-myc, 9E10 and anti-phospho ERKs) were from Sigma (L'Isle d'Abeau, Chânes, France) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively, and anti-pan-Ras was from Calbiochem (San Diego, CA). The monoclonal anti-CD31 rat antibody was from BD Biosciences (Heidelberg, Germany). Glutathione transferase (GST)/TTP fusion protein was used to produce a Rabbit polyclonal antibody according to standard procedures (Eurogentec, Liege, Belgium). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were purchased from Promega (Madison, WI).

Cell Culture, Transfection, and Luciferase Assays

The Raf1-ER chimera, the A431 and Ras/Val12 (clone 5C) cells were grown as described previously (Milanini-Mongiat *et al.*, 2002). Transfections were carried out using the calcium phosphate technique. S19R443 cells that express the Tet repressor were used for transfection with pCDNA4/TO/myc-HisA (pCDNA4/TO-TTP) (pTrex-A; Invitrogen, Cergy Pontoise, France). Zeocine-resistant clones were screened by immunofluorescence and Western blotting after tetracycline induction. Raf1-ER/TTP cells were cotransfected with a plasmid coding for an activated form of the Ha-Ras oncogene (Seuwen *et al.*, 1988) and a plasmid coding for a puromycin resistance gene.

Transfection for luciferase assays were performed using 293Raf1-ER cells. Eight hours after transfection, cells were serum starved for 14 h, followed by stimulation or not with 1 μ M tamoxifen for 5 h. Luciferase assays were performed as described previously (Milanini-Mongiat *et al.*, 2002). Results were expressed as the percentage of inhibition over control cells.

RNA Preparation, Real-Time Polymerase Chain Reaction (PCR), and Northern Blotting

Total RNA was extracted with TRIzol Reagent (Invitrogen). Two micrograms of total RNA were used for reverse transcription, using the Superscript First-Strand Synthesis System (QIAGEN, Hilden, Germany), with oligo(dT) to prime first-strand synthesis. For real-time PCR, primers and dual-labeled probes (5'-5-carboxyfluorescein, 3'-5-carboxytetramethylrhodamine) were from Applied Biosystems (7300 Software System; Courtaboeuf, France). To calculate the relative expression of VEGF mRNA in Raf1-ER/TTP cells the $2^{-\Delta\Delta C_T}$ method was used (Livak and Schmittgen, 2001). Northern Blots were performed as described previously (Onesto *et al.*, 2004).

5,6-Dichlorobenzimidazole Riboside (DRB) Pulse Chase Experiments

Raf1-ER cells expressing inducible myc-tagged TTP were stimulated or not with 1 μ g/ml tetracycline for 24 h before the addition of 25 μ g/ml DRB and RNAs were prepared from 0 to 4 h thereafter. Relative VEGF and 36B4 mRNA amounts were determined by quantitative reverse transcription-PCR, and VEGF mRNA was normalized to 36B4 mRNA. The relative amounts of VEGF mRNA at time 0 before DRB addition were set to 100%.

RNA Interference Experiments

TTP small interfering RNAs (siRNAs) (Eurogentec) were designed in different regions of mouse TTP cDNA (NM 011576). The respective sequences are as following: TTP (1): 5'-UCGCCACCCCAAGUACAAAtt-3' and TTP (2): 5'-CUCUGCCACAAGUUCUACctt-3'. The control siRNA (Dharmacon RNA Technologies, Lafayette, CO) was used as an irrelevant siRNA. Raf1-ER cells were transfected with siRNA as described previously (Onesto *et al.*, 2004). Forty-eight hours after the second transfection, cells were serum starved and stimulated at indicated times with or without tamoxifen. Then, cells were analyzed for the expression of TTP and VEGF mRNAs by real-time PCR.

Measurement of Secreted VEGF

VEGF protein release was measured using an enzyme-linked immunosorbent assay (ELISA) kit Quantikine Mouse VEGF Immunoassay (R&D Systems, Minneapolis, MN).

Immunoprecipitation of Ribonucleoprotein Complexes

RNP complexes were immunoprecipitated as described previously (Niranjanakumari *et al.*, 2002) by using protein G-Sepharose beads preincubated with anti-myc antibody (9E10) or with an irrelevant antibody (anti-Pan-Ras) (Calbiochem). RNA was isolated from supernatants and immunoprecipitates and reverse-transcribed with Superscript II (Invitrogen). The transcripts were amplified by PCR by using the following primers (5' to 3'): VEGF, 5'-ATGGCAGAAGGAGGGCAGCAT-3' and 5'-TTGGTGAGTTTGATCCGCATCAT-3'; and 36B4, 5'-GCCAACC GCG-AGAAGATGACCCAG-3' and 5'-CTCGAAGTCCAGGCGACGTAGC-3'. The PCR products were analyzed by 2% agarose gels.

Western Blot Analysis, Alkaline Phosphatase (Calf Intestine Phosphatase [CIP]) Treatment, and Cycloheximide Pulse Chase

Cell extracts were prepared in Laemmli buffer, resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA). The immunoreactive proteins were visualized by the enhanced chemiluminescence detection system (ECL; Pierce Chemical, Rockford, IL). For experiments with CIP (New England Biolabs, Ipswich, MA), Raf1-ER/TTP cells were first serum starved and stimulated with 1 μ g/ml tetracycline for 24 h before stimulation or not with 1 μ M tamoxifen for 3 h. Cells were then lysed in lysis buffer (1% Triton X-100, 50 mM Tris, pH 8.5, 100 mM NaCl, and 0.5 mM EDTA). CIP (35 U) was added to the lysates for 1 h at 37°C. The reaction was stopped by adding Laemmli sample buffer.

For cycloheximide pulse chase, cells were cultured in the same conditions. After stimulation with tamoxifen for 3 h, Raf1-ER/TTP cells were treated with 10 μ g/ml cycloheximide to block protein neosynthesis in the presence or absence of 10 μ M U0126.

RNA Electromobility Shift Assays (REMSAs)

RNA transcripts were synthesized as described previously (Levy *et al.*, 1996). For REMSA experiments, 30 pmol of biotinylated RNA using Biotin RNA Labeling Mix (Roche, Penzberg, Germany) or radiolabeled RNA transcripts (200,000 cpm/reaction) was combined with 50 nM GST fusion proteins or GST alone, in a previously described binding buffer (Levy *et al.*, 1996). The reaction mixture was incubated for 30 min at 30°C and treated for 15 min at room temperature with 100 U of ribonuclease T1 (Roche). When specific or nonspecific competitors were used, they were incubated for 15 min at 30°C with the proteins in binding buffer before the addition of the radiolabeled or biotinylated transcripts. The reaction mixtures were resolved on 5% native polyacrylamide gels in 0.5 $\times$ Tris borate-EDTA (TBE) buffer. Gels were dried and autoradiographed or transferred to nylon N⁺ membranes in 0.5 $\times$ TBE at 400 mA and 4°C for 1 h. The RNAs were cross-linked to the membranes and detected by the Lightshift electrophoretic mobility shift assay kit (Pierce Chemical) by using streptavidin-horseradish peroxidase binding and chemiluminescent detection.

Tumorigenicity Assays

Raf1-ER/TTP (10⁶) cells transformed by Ha-Ras were injected subcutaneously in the flank of 6-wk-old nude (nu/nu) mice (Harlan, Indianapolis, IN). Doxycycline (750 μ g/ml) was added to the drinking water starting from day 3 after the injection. Tumors were measured at the indicated times, and volumes were calculated as $v = L \times l^2 \times 0.52$ (Auerbach *et al.*, 1978), where L and l represent the largest and the smallest tumor diameter, respectively, measured weekly. The observations were ended after 5 wk from the day of cells injection. Animals were killed, and tumors were immediately frozen in dry ice or fixed in Formalin. Care of the animals was provided according to institutional guidelines: European Directive 86/609/EEC.

Immunofluorescence Experiments

Cells and tumor sections (6- μ m cryostat sections) were fixed in 4% paraformaldehyde for 20 min at room temperature or cold acetone for 2 min, permeabilized with 0.2% Triton X-100 for 5 min, and blocked in 2% bovine serum albumin/phosphate-buffered saline (PBS) for 2 h. Cells or tumor sections were then incubated with the appropriate antibody for 1 h at room temperature. After three washes in PBS, cells were incubated with the appropriate fluorescent-conjugated secondary antibody or streptavidin conjugates or with Cy3-conjugated mouse anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), washed, and counterstained with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Preparations were mounted in PBS:glycerol (1:9), analyzed with a Leica microscope (Leica, Wetzlar, Germany), and counted at a 20 $\times$ magnification in six random fields from the tumor.

RESULTS

Increased VEGF mRNA Stability in Tumor Cell Lines with High ERK1/2 Activity

Although numerous studies, including ours, have described the role of ERKs on VEGF gene transcription (Milanini *et al.*, 1998), nobody has reported the impact of ERKs activity on VEGF mRNA stability. For that purpose, we analyzed the ERK1/2 activity and VEGF mRNA half-life in normal Chinese hamster fibroblasts (control cells), as well as in Ras-Val12-transformed cells (clone 5C) containing high amounts of the transgene, a cellular system where ERKs activity is increased. As shown in Figure 1A, ERK1/2 activity measured with anti-phospho-specific antibodies, is increased by

Figure 1. Expression of VEGF mRNA in normal and tumor cell lines. (A) Twenty micrograms of total protein extracts from control or Ras Val 12 (clone 5C)-transformed cells were subjected in duplicate to Western blotting by using a monoclonal pERK antibody. The amounts of actin are shown as a loading control. Average ERK activity normalized to actin amounts obtained by quantification of the two independent samples is also shown. (B) Total RNA isolated from normal Chinese hamster fibroblasts and Ras Val 12 (clone 5C) cells were analyzed by Northern blotting for the expression of VEGF mRNA after DRB treatment (25 μ g/ml). 36B4 RNA is shown as a loading control. (C) The amount of VEGF mRNA was quantified with a PhosphorImaging system (Storm 840; Amersham Biosciences, Roosendaal, The Netherlands). VEGF mRNA half-lives, deduced from two independent experiments, are indicated ($p < 0.05$).

almost six times in Ras cells compared with control cells (Figure 1A). VEGF mRNA expression and stability were examined by Northern blotting on the different cell lines by using DRB chase experiments (Figure 1B). Quantification of all the VEGF mRNA isoforms (see arrows; Milanini *et al.*, 1998) detected in these experiments illustrates not only an increase in VEGF mRNA amounts at the basal level but also an increase in VEGF mRNA half-life in Ras ($t_{1/2} = 168 \text{ min} \pm 10 \text{ min}$; $p < 0.05$) compared with normal cells ($t_{1/2} = 27 \text{ min} \pm 5 \text{ min}$; $p < 0.05$) (Figure 1C), indicating that an increase in ERK1/2 activity is correlated to an increase in VEGF mRNA stability. Equivalent results were obtained with A431 cells that highly expressed EGF receptor and present with constitutive ERK activity.

Specific Stabilization of VEGF mRNA by ERK Pathway

To more carefully address the relationship between ERK activity and VEGF mRNA stability, we have used CCL39 Chinese hamster fibroblasts expressing the Raf1-ER chimera (Lenormand *et al.*, 1996). In these cells, tamoxifen stimulation only results in the stimulation of ERKs. As described previously (Milanini *et al.*, 1998), after an overnight serum starvation (null ERKs activity, Figure 2A), VEGF mRNAs

Figure 2. VEGF mRNA expression is regulated by the ERK pathway. (A) Raf1-ER cells were serum starved and treated with 1 μ M tamoxifen for 3 h. Then, cells were treated or not with 10 μ M UO126. Protein extracts (20 μ g) were analyzed by Western blotting by using a monoclonal anti-pERK antibody. Data are representative of two independent experiments. (B) Raf1-ER cells were serum starved and treated with 1 μ M tamoxifen for 3 h. Cells were treated with 25 μ g/ml DRB in the presence or absence of 10 μ M UO126. VEGF mRNA was detected by Northern blotting and normalized against 36B4 RNA. (C) The amount of VEGF mRNA was quantified with a PhosphorImaging system. VEGF mRNA half-lives, deduced from two independent experiments, are indicated ($p < 0.05$).

were undetectable in these cells. To verify that VEGF mRNA stabilization is specifically caused by ERKs activation, cells were serum starved overnight and stimulated with tamoxifen for 3 h to allow VEGF mRNA accumulation. This VEGF mRNA accumulation is due in part to transcriptional activation (Milanini *et al.*, 1998). We have tested whether it also could implicate mRNA stability mechanisms. Hence, the rate of VEGF mRNA decay was evaluated by DRB chase experiments in the presence or absence of the MEK inhibitor U0126 at indicated times. Northern blot analysis showed that when ERKs are permanently activated by tamoxifen treatment, the half-life of VEGF mRNA ($t_{1/2} = 225 \text{ min} \pm 10 \text{ min}$; $p < 0.05$; Figure 2, B and C) was comparable with the half-life observed in Ras-transformed cells ($t_{1/2} = 168 \text{ min} \pm 10 \text{ min}$; $p < 0.05$; Figure 1C). Inhibition of ERKs by U0126 resulted in a rapid decay of VEGF mRNA ($t_{1/2} = 68 \text{ min} \pm 5 \text{ min}$; $p < 0.05$), confirming the results of Figure 1 showing that ERKs activity is directly implicated in VEGF mRNA stabilization. Figure 2A shows that, in Raf1-ER cells, ERKs activity is stimulated by tamoxifen and that U0126 is able to repress tamoxifen-induced ERKs activation.

Induction of TTP and VEGF mRNAs Is Dependent on ERKs Activation

The previous results suggest that the ERK pathway may be able to induce or activate proteins implicated in VEGF mRNA stability or to repress or inactivate proteins implicated in VEGF mRNA degradation. We then focused our attention on negative regulators of VEGF mRNA stability and particularly on the TTP protein for two reasons. First, TTP is a destabilizing-mRNA binding protein acting through AREs and reported to be a substrate for multiple kinases, including ERKs, c-Jun NH₂-terminal kinase, p38, and MAPKAP kinase 2 (Brook *et al.*, 2006). Second, VEGF mRNA 3'-UTR contains putative binding sites for TTP, suggesting that TTP may be implicated in the regulation of VEGF mRNA stability. Thus, we tested the impact of activation of the ERK pathway on TTP expression in Raf1-ER cells stimulated by tamoxifen. VEGF expression has also been tested in parallel to establish a potential relationship between both proteins. Figure 3A showed that tamoxifen induced a strong and rapid accumulation of TTP mRNA and protein peaking, respectively, at 1 and 2 h after stimulation (Figure 3, A and B). Although the expression of TTP mRNA decreased after 1 h, the amount of VEGF mRNA increases exponentially. These experiments show that ERKs induce a concomitant expression of TTP and VEGF mRNA. This observation suggests that ERKs promote simultaneously the production of angiogenic factors as well as proteins putatively responsible for their down-regulation, thus initiating a negative feedback loop. The fact that both proteins are present simultaneously implicates a subtle regulatory mechanism.

GST-TTP Interacts with the VEGF mRNA 3'-UTR In Vitro

To test whether TTP could interact directly with the VEGF mRNA 3'-UTR, gel shift assays were performed using recombinant GST-TTP protein. Figure 4B shows that GST-TTP binds to the entire rat VEGF mRNA 3'-UTR (full-length probe). No shifted band was observed with GST alone, demonstrating the specificity of the interaction. To restrict the interacting region, the 3'-UTR was divided into several regions (NsiI, Δ NsiI [Δ NsiI part containing most of the AREs], Stu I, and HP probes) (Figure 4A and Supplemental Figure 3SDB). RNA electrophoretic mobility shift assays showed that GST-TTP protein interacts specifically with NsiI, Δ NsiI, and HP regions (Figure 4B and Supplemental

Figure 3. ERK activation induces the expression of TTP and VEGF mRNA. (A) Raf1-ER cells were serum deprived, treated with 1 μ M tamoxifen for the indicated time, and total RNAs were isolated. Quantitative PCR was performed using specific primers for VEGF and TTP. Fold induction of VEGF and TTP RNA is relative to time 0 after normalization to 36B4 in each sample. (B) Protein extracts (30 μ g) prepared in parallel, were analyzed by Western blotting by using a polyclonal anti-TTP, monoclonal anti-pERK, and ERK antibodies. Data are representative of three independent experiments.

Figure 3SDB) and that Stu I construct failed to bind to the fusion protein.

This interaction is specific, because it can be inhibited by competition with 100 $\times$ M excess of unlabeled specific probe (Figure 4C and Supplemental Figure 3SDB). The same molar excess of unlabeled nonspecific probe (3'-UTR-ERK) poorly affects TTP interaction demonstrating the specificity of TTP RNA binding.

TTP Interacts with Endogenous VEGF mRNA

To determine whether TTP/VEGF mRNA 3'-UTR interaction also occurs in a cellular context, Raf1-ER cells were stably transfected with an expression vector encoding a tetracycline inducible myc-tagged TTP. Figure 5A shows tetracycline-inducible overexpression of myc-tagged TTP in different clones of exponentially growing Raf1-ER/TTP cells, treated or not with tetracycline for various times. Moreover, we observed by immunofluorescence analysis (Figure 5B) that ectopically expressed TTP displays the same subcellular localization than the endogenous form of TTP (Cao, 2004). To confirm that TTP not only interacts with VEGF 3'-UTR transcript in vitro but also with the endogenous VEGF mRNA, we performed ribonucleoprotein complexes immunoprecipitation experiments with whole cell extracts from Raf1-ER/TTP cells. Figure 5C shows that immunoprecipitation of TTP results in the coprecipitation of

Figure 4. TTP interacts with the VEGF mRNA 3'-UTR in vitro. (A) Schematic map of the VEGF 3'-UTR illustrating the templates used for generation of riboprobes for REMSA. (B) REMSAs were performed by incubating the radiolabeled VEGF 3'-UTR transcripts (full length, NsiI, and ΔNsiI) with purified GST-TTP or GST. The arrows point to RNA-protein complexes. (C) Competition with specific competitor (100 M excess) corresponding to unlabeled NsiI and ΔNsiI transcripts, respectively, was also performed in the presence of GST-TTP. The arrows point to RNA-protein complexes.

VEGF mRNA (lane 4, top). This coprecipitation is specific, because immunoprecipitation with an irrelevant antibody or using cells in which TTP was not induced does not coprecipitate VEGF mRNA (lanes 8, 10, 11, and 13, top). This in vivo interaction was not influenced by the ERK pathway as immunoprecipitation with specific antibody after U0126 treatment does not modify the amount of immunoprecipitated VEGF mRNA level (lane 6, top). VEGF mRNA was also detected in total RNA prepared from Raf1-ER/TTP cell extracts used for immunoprecipitations (input, lanes 1&2). Immunoprecipitation of TTP does not lead to coprecipitation of a nonspecific transcript such as 36B4 RNA (lanes 4 and 6, bottom), which was used as a negative control. These results provide convincing evidence that VEGF mRNA is specifically associated with TTP in vivo but ERKs do not influence its binding capacity.

TTP Overexpression Decreases Endogenous VEGF mRNA Half-Life and VEGF Secretion: Influence of ERKs

TTP effect on endogenous VEGF mRNA stability was performed by measuring the VEGF mRNA half-life by using DRB chase experiments. The influence of ERKs was estimated by stimulating the cells by tamoxifen in the absence or presence of U0126. The amounts of VEGF mRNA were evaluated by real-time PCR. As we demonstrated in Figure

Figure 5. TTP interacts with VEGF mRNA in vivo. (A) Raf1-ER cells were stably transfected with an expression vector encoding a tetracycline inducible myc-tagged TTP. Cells were stimulated for various times with 1 μg/ml tetracycline. Inducible overexpression of myc-TTP was then verified either by Western blotting or (B) by immunostaining, by using a monoclonal anti-myc antibody. (C) Tamoxifen-treated Raf1-ER/TTP cell extracts were immunoprecipitated with no antibody, with the anti-myc antibody, or with an irrelevant antibody (anti-Ras antibody) in the presence or absence of MEK inhibitor 10 μM U0126. Equal aliquots of purified total RNA isolated from the immunoprecipitates (P), and from the supernatants (S), were assayed by reverse transcriptase-PCR to detect the VEGF (top) and 36B4 (bottom) transcripts.

2B, Figure 6A confirms that, in control cells, when ERKs were activated by tamoxifen treatment (-TET, -U0, maximal ERKs activity), the half-life of VEGF mRNA was 4 h ($t_{1/2} = 240 \text{ min} \pm 20 \text{ min}$; $p < 0.05$). Inhibition of ERKs by U0126 (-TET, +U0, very low ERKs activity) resulted in approximately fourfold decrease of VEGF mRNA half-life ($t_{1/2} = 60 \text{ min} \pm 7 \text{ min}$; $p < 0.05$). When TTP was overexpressed and ERKs were activated (+TET, -U0, maximal ERKs activity), VEGF mRNA half-life is almost the same than in absence of TTP and even superior ($t_{1/2} > 240 \text{ min}$; $p < 0.05$). However, when TTP is overexpressed and ERKs were inactivated by U0126 (+TET, +U0, very low ERK activity), the half-life of VEGF mRNA is reduced by over eightfold ($t_{1/2} = 30 \text{ min} \pm 5 \text{ min}$; $p < 0.05$). Supplemental Figure 1SD shows that it exists a gradation in ERKs activity in these different situations (i.e., growth-arrested cells < treatment by U0126 < exponentially growing cells < Ras-transformed cells < tamoxifen-stimulated cells). These results strongly suggested that ERKs exert an inhibitory effect on TTP. They also suggested a molecular link between ERKs and TTP. Figure 6B (left) shows that tamoxifen stimulation results in a shift in electrophoretic mobility of TTP. Phos-

Figure 6. TTP overexpression decreases VEGF mRNA half-life in vivo. (A) Raf1-ER/TTP cells were serum-starved and stimulated (+) or not (–) with 1 μ g/ml tetracycline for 24 h. After 3 h of stimulation with tamoxifen, cells were incubated in the absence or presence of 10 μ M U0126 for one supplemental hour, and then in the presence of 25 μ g/ml DRB for the indicated times. During the DRB chase, cells were maintained or not in the presence of U0126. The amounts of VEGF mRNA remaining were quantified by real-time PCR. The values are normalized to 36B4, and the values at time 0 were taken as 100%. VEGF mRNA half-lives were deduced from the curves ($n = 3$; $p < 0.05$). (B) Raf1-ER/TTP cells were serum-starved and stimulated with 1 μ g/ml tetracycline for 24 h before stimulation or not with tamoxifen for indicated time. Left, time course of tamoxifen stimulation. Arrow and bracket indicate the unshifted and the retarded bands, respectively. Right, cell extracts were treated or not with CIP. Protein extracts (30 μ g) were then analyzed by Western blotting by using anti-myc, pERK and ERK antibodies. This experiment is representative of two independent experiments. (C) Raf1-ER or Raf1-ER/TTP cells were serum starved, stimulated with 1 μ M tamoxifen and incubated in the absence or presence of 10 μ M U0126 for one supplemental hour. Protein extracts (30 μ g) were analyzed by Western blotting using anti-TTP, myc, and ERK antibodies. This experiment is representative of two independent experiments. (D) Exponentially growing Raf1-ER or Raf1-ER/TTP cells were stimulated with or without 1 μ g/ml tetracycline for 24 h. Secreted VEGF was measured by ELISA. VEGF levels were normalized to the cell number. -Fold inhibition of secreted VEGF are presented as a mean of three independent experiments performed in triplicate.

phatase treatment induced a disappearance of the retarded bands (Figure 6B, right), demonstrating that, in our experimental model, tamoxifen stimulated ERKs induced TTP phosphorylation, as already suggested (Brook *et al.*, 2006). It is important to note that upon tamoxifen treatment, TTP amounts are increased compared with growth factor-deprived cells (null ERKs activity). This induction is specific, because the MEK inhibitor U0126 blocks the accumulation of both the endogenous and exogenous proteins (Figure 6C, left and right, respectively). This also strongly suggested that phosphorylation of TTP by ERKs results in increased stability of the protein as it was previously described by Brook *et al.* (2006). We have shown by cycloheximide pulse chase experiments that it was effectively the case (Supplemental Figure 2SD). Altogether, our results suggested that even if TTP is constitutively bound to VEGF mRNA, its ERKs-dependent phosphorylation status is directly implicated in its "degradative" action.

To correlate VEGF mRNA stability with VEGF production, secreted VEGF was measured by ELISA in supernatants of exponentially growing cells (intermediate ERK activity) overexpressing or not TTP (Raf1-ER/TTP, Raf1-ER). Under these conditions, TTP is still able to reduce VEGF mRNA stability. Figure 6D shows reduction of VEGF secretion when TTP was overexpressed, whereas no inhibition was detected in control cells. These results suggest that the reduction in VEGF mRNA stability mediated by TTP correlates with a decrease in VEGF production.

Silencing of TTP by RNA Interference Increases the Level of Endogenous VEGF mRNA: Influence of ERKs

To strengthen the conclusions drawn from the contribution of TTP in the regulation of VEGF mRNA expression, we used the RNA interference methodology. Forty-eight hours after transfection with two independent siRNA duplexes targeting different regions of TTP mRNA, Raf1-ER cells were treated or not with tamoxifen for the indicated periods in the presence or absence of U0126. TTP and VEGF mRNA expression were evaluated by real-time PCR. Quantification of TTP and VEGF mRNA level revealed that TTP gene expression was knocked down by ~50%, whereas nonspecific control siRNA (Dharmacon RNA Technologies) was ineffective on TTP silencing (Figure 7A). In tamoxifen-treated cells, control siRNA had no effect, because we obtained an equivalent-fold stimulation as reported in Figure 3A (~3-fold compared with untreated cells). However, TTP knockdown with two independent siRNA resulted in an approximately sevenfold increase of VEGF mRNA expression compared with untreated cells (2.5-fold more than in the absence of TTP-directed siRNA, Figure 7B). U0126, as expected, reduced the effects of tamoxifen in the presence of control siRNA (1.5-fold induction). However, TTP directed siRNA diminished the effect of U0126 (4-fold induction remaining compared with control cells), suggesting that TTP is an important mediator of VEGF mRNA down-regulation when ERK activity is inhibited.

Figure 7. TTP knockdown increases VEGF mRNA level. (A) Cells were transfected with siRNAs against TTP or with a control siRNA (Dharmacon RNA Technologies). Forty-eight hours after the second transfection, cells were serum starved overnight and stimulated with tamoxifen for 3 h in the absence or presence of 10 μ M U0126. Total RNA was isolated and real-time PCR analysis was performed to determine TTP and (B) VEGF mRNA expression levels. The data shown represent the mean $\pm$ SE of three independent experiments.

TTP Induced an Inhibitory Effect on VEGF 3'-UTR Luciferase Reporter Gene: Influence of ERKs

To determine whether TTP exerts its effect via the VEGF 3'-UTR, we have cloned downstream of luciferase reporter gene different domains of VEGF mRNA 3'-UTR. These constructs were transfected in 293 Raf1-ER cells, an easier transfectable cell line (Cagnol *et al.*, 2006). TTP induced a dose-dependent inhibitory effect on luciferase activity on the full-length 3'-UTR, reaching 80% inhibition in serum deprived cells. Tamoxifen stimulation results in an increase of luciferase activity demonstrating that ERKs are implicated in VEGF mRNA stability through its 3'-UTR. In the presence of tamoxifen, TTP is still able to reduce luciferase activity but to a lesser extent (Figure 8A). Thus, ERKs partially prevents TTP-dependent degradation of VEGF 3'-UTR reporter mRNA. Experiments described in Figure 6B suggested that it acts through a phosphorylation-dependent mechanism. To evaluate through which domain(s) TTP exerts its action, the effect of TTP was tested on different constructs of the rat VEGF 3'-UTR (Figure 8B). Figure 8C indicated that, whereas TTP had no effect on luciferase activity mediated by a control vector, it principally inhibits the activity of the VEGF 3'-UTR throughout the NsiI region. The Luc-HP construct, which contains five AU consensus domains that form a stable hairpin structure important for the posttranscriptional regulation of VEGF (Pagès *et al.*, 2000), similarly responded to TTP, although slightly less than the full-length construct. HP region contained two consensuses ARE motifs that are both conserved in the human VEGF sequence. Mutations of these two ARE alone or together do not affect the TTP effect in contrast to TIS11b, the activity of which is dependent on these ARE sequences (Ciais *et al.*, 2004). Deletion of a large fragment of the VEGF 3'-UTR containing all ARE motifs (StuI) did not abrogated the inhibitory effect of TTP on reporter gene activity. This suggests the existence of an indirect mode of regulation that does not depend on TTP

Figure 8. TTP induced an inhibitory effect on luciferase activity. (A) 293 Raf1-ER cells were cotransfected with a VEGF mRNA 3'-UTR-luciferase reporter construct and different amounts of TTP expression plasmids in the presence or absence of 1 μ M tamoxifen. Luciferase assays were conducted 24 h after transfection. Relative luciferase activity was normalized to total protein amount. The luciferase counts obtained with the construct Luciferase/VEGF 3'-UTR in the absence of TTP was taken as the value of reference (100%). The percentages of inhibition exerted by TTP are also indicated on the figure. Results are reported as the mean $\pm$ SE of three independent experiments performed in triplicate. (B) Mapping of different sequences in VEGF mRNA 3'-UTR used for TTP inhibition of reporter gene activity. (C) Transfections were performed as described in A in the presence or absence of 100 ng of TTP. The luciferase values obtained for each construct in the absence of TTP were taken as the value of reference (100%). Plotted are the percentages of remaining luciferase activity in the presence of TTP. Results are reported as the mean $\pm$ SE of three independent experiments performed in triplicate.

binding. The data of Supplemental Figure 3SD further support this interpretation as GST-TTP does not bind to the StuI domain.

Overexpression of TTP in Ras Cells Decreases VEGF Secretion

Because TTP negatively controls VEGF mRNA expression, we hypothesized that, in tumor cells, TTP is down-regulated or that its activity is inhibited. To validate this hypothesis, we evaluated the role of TTP in neovascularization and tumor growth in vivo by tumorigenesis assays on nude mice. We first transfected Raf1-ER/TTP cells (having a low tumorigenic potential) with a constitutively active form of Ha-Ras and isolated stable clones expressing Ras Val 12. As a negative control, we used the same cells transfected with

an empty vector. As a positive control, we used Ras 5C cells stably expressing the same active form of Ras (Seuwen *et al.*, 1988). Both expression of Ras Val 12 and TTP were verified by Western blot. As shown in Figure 9A (left), all stable

Figure 9. Inducible expression of TTP inhibits tumor angiogenesis in vivo. (A) Left, Raf-ER/TTP cells were transfected with a plasmid coding for Ha-Ras. Cells from stable clones were stimulated for various times with 1 µg/ml tetracycline before lysis. Total cell extracts were used to detect both Ras and myc-TTP proteins by Western blot analysis by using anti-Pan Ras and Myc antibodies, respectively. Protein levels of total ERK are shown as loading control. Right, supernatants from stable clones cultured in the presence or absence of 1 µg/ml tetracycline for 24 h were collected and analyzed by mouse VEGF-specific ELISA. Results are reported as the mean ± SE of three independent experiments each run in duplicate. (B) Left, eight nude mice per condition were inoculated with cells from stable clone or negative and positive control. Animals were randomized into two groups. One group received weekly 750 µg/ml doxycycline. Tumor size was measured at the indicated times and tumor volume was calculated. The medians of the tumor size (cubic millimeters) are indicated. Right, tumors were excised from animals at day 35 after tumor cell inoculation when the largest tumor had reached a diameter of 4 cm. Representative examples for each experimental group are shown. (C) Tumor tissues were lysed and intratumoral hemoglobin and VEGF contents were measured by Drabkin reagent kit 525 (Sigma) and ELISA (R&D systems), respectively. The data were corrected to the protein concentration. (D) Immunofluorescence staining of frozen tumor sections with anti-CD31 antibody. Data are representative of two independent experiments.

clones selected expressed Ras and TTP after tetracycline induction. Tetracycline treatment had no significant effect on the expression of Ras Val 12. TTP expression remained inducible by tetracycline in Ras Val 12-transformed cells. The level of Ras Val 12 expression in these cells was estimated to be 10 times lower than the levels found in Ras 5C cells. VEGF secretion was then checked in exponentially growing Raf1-ER/TTP-Ras and TTP control cells. It is important to note that ERKs activity in exponentially growing Raf1-ER/TTP-Ras cells is increased compared with control cells but remains inferior than in tamoxifen-stimulated cells (Supplemental Figure 1). Hence, ERKs cannot exert a maximal inhibitory effect on TTP "degradative" activity. As shown in Figure 9A (right), VEGF amounts in supernatants derived from Raf1-ER/TTP-Ras cells was higher compared with control cells. After 24 h of tetracycline treatment, overexpression of TTP was sufficient to lower VEGF production both in control cells (Raf1-ER/TTP cells) and in Raf1-ER/TTP-Ras cells by 65 and 45%, respectively. This result clearly shows that TTP was still able to reduce VEGF production in Raf1-ER/TTP-Ras cells. It is important to note that the inhibitory effect of TTP is not equivalent in Raf1-ER/TTP-Ras and in control cells. This inhibitory effect is directly proportional to ERKs activity in both cell types (Supplemental Figure 1).

Overexpression of TTP Slows Down the Growth Rate of Tumor Xenografts

To verify whether TTP, by reducing VEGF production, may have an impact on tumor growth in vivo, we used Raf1-ER/TTP-Ras cells to induce tumors in nude mice. We subcutaneously injected 10⁶ cells into the flank of nude mice. As a positive control, 10⁵ cells from the Ras 5C cells were injected into another group of mice. Then, mice were divided into two experimental groups (n = 8/group). Animals were given doxycycline (Dox-treated group) or not (control group) in their drinking water, starting 3 d after tumor cells inoculation and for the whole duration of the experiment. After injection, tumors were allowed to grow for 5 wk. Subsequent tumor growth was monitored every week and tumor volume calculated as indicated in *Materials and Methods*. We observed that doxycycline treatment had no non-specific effect on the time required for emergence and growth of 5C cells (data not shown). As shown in Figure 9B (left), tumors resulting from the injection of inducible cells emerged at day 15 in the control group. In contrast, at this time, tumors were not yet visible in Dox-treated mice. Tumors in these mice emerged only 2 wk later, and, in some mice, tumors never developed. The growth rate of control tumors was ninefold greater than in the doxycycline treated mice. For ethical reasons, we decided to end the observations five weeks after injection. As shown in Figure 9B, tumors in the control group had a median volume of 2494 mm³, which was 10-fold larger than the treated group (261 mm³). Tumor growth time was statistically significant (p < 0.0001) from day 15 after the beginning of doxycycline treatment until the day the mice were killed. This correlates with tumor weight that was reduced approximately by 12-fold in Dox-treated mice. These data clearly show that inducible expression of TTP is able to slow down the growth of Ras-derived xenografts and that it has a lasting effect on tumor development, being effective for at least 35 d.

Overexpression of TTP Reduces Intratumoral Hemoglobin and VEGF

To determine whether the difference of growth between the xenografts resulted from impaired angiogenesis, we both monitored hemoglobin and VEGF contents in tumors. Fig-

ure 9C (left) shows a 10-fold reduction of intratumoral hemoglobin in Dox-treated xenografts compared with control. Figure 9C (right) shows that control xenografts present with higher VEGF levels compared with Dox-treated-tumors. This decrease in VEGF amount correlate with the diminution observed in individual clones.

Dox-treated Tumors Have a Reduced Microvessel Density

Marked differences were observed both by morphological analysis (Figure 9B, right) and by specific immunofluorescence staining (Figure 9D). Figure 9D shows that control tumors seemed to be much more vascularized. In contrast, treated tumors were almost white and did not show any major vascular structure. These notable macroscopical differences represent our first *in vivo* indication that TTP is able to impair tumor growth rate by inhibiting angiogenesis. Immunostaining for the endothelial cell marker CD31 showed that Dox-treated tumors exhibited low vascular density compared with tumors from untreated mice (Figure 9D). Vessels from these tumors were generally thinner and displayed multiple branched points. Equivalent results were obtained by staining the tumors with von Willebrand factor (data not shown).

DISCUSSION

VEGF production is one of the most important regulators of tumor angiogenesis. Its expression, especially by oncogenes was particularly investigated at the transcriptional level. However, the relationship between oncogenes and VEGF mRNA stability was poorly documented. In this report, we have established a new molecular link between the Ras–Raf–MEK–ERK pathway and VEGF mRNA stability. Thus, we have described that the regulation of basal VEGF mRNA amounts in normal cells is dependent on an active mechanism involving the protein TTP, an ARE binding factor. Several reports support the observations that activation of several signaling pathway serve to stabilize ARE-containing transcripts (Dean *et al.*, 2004). Our data suggest that in tumor cells, activation of the ERK pathway favors VEGF mRNA stability. Interestingly, we have demonstrated that the ERK pathway induces the expression of VEGF mRNA. It also induces the expression of TTP, a potent protein inducing the degradation of VEGF mRNA. We found that the 3' UTR of VEGF mRNA contains independently functional destabilizing elements, not AU rich, and that the rapid degradation of the VEGF mRNA is due to the combined action of these elements. Our data are concordant with a previous report (Marderosian *et al.*, 2006) which showed that removal of specific AUUUA motifs from the 3'-UTR of Cyclin D1 mRNA resulted in transcripts that were still destabilized by TTP. It may be expected that removal of these elements would result in a transcript with increased stability as previously reported (Ciais *et al.*, 2004) for TIS11b, the first identified protein destabilizing VEGF mRNA. TIS11b acts through a 75-base pair domain, containing two consensus AU-rich motifs. This domain binds TIS11b and mediates its destabilizing activity. In the present work, we show that TTP could not only influence VEGF mRNA stability through this domain but it also through other non-AUUUA regions (Figure 8C, Stu I region). We concluded that other sequences besides the ARE were involved in the TTP-mediated overall steady-state turnover of VEGF mRNA. It also demonstrated that even if TTP and TIS11b belong to the same family of proteins, their action on VEGF mRNA stability is not redundant. Our data indicate that TTP action is probably independent on RNA binding in mRNA devoid of ARE. Some VEGF

mRNA resulting from alternative utilization of polyadenylation sites and devoid of ARE sequences (Claffey *et al.*, 1998) could remain TTP sensitive. Nevertheless, TTP binds to various elements located within the 3' UTR of VEGF mRNA and facilitates the recruitment of the decay machinery, leading to message destabilization and reduced VEGF production. However, activation of ERKs decreases this effect. It is important to stress that TTP "degradative" effect is inversely proportional to ERKs activity; TTP 100% activity (null ERKs activity: serum deprived cells or +U0126) > TTP strong activity (basal ERKs activity: exponentially growing control cells) > TTP intermediate activity (intermediate ERKs activity: exponentially growing Raf1-ER/TTP-Ras cells) > TTP null activity (full ERKs activity: tamoxifen-stimulated Raf1-ER/TTP cells) (Supplemental Figure 1SD). Our data are in agreement with studies demonstrating that p38 activity is antagonistic to ARE-directed deadenylation and subsequent decay of the TNF mRNA (Dean *et al.*, 2003). More recently, Hitti *et al.* (2006) reported that mitogen-activated protein kinase-activated protein kinase 2 regulates TNF mRNA stability and translation by altering TTP expression, stability, and binding to ARE elements. One could imagine that ERKs activity is essential for stabilization of TTP mRNA and protein as well as for stabilization of VEGF mRNA. Indeed, we have observed that TTP is phosphorylated in response to tamoxifen. Moreover, phosphorylation of TTP by ERKs reduces its proteasome-dependent degradation (Brook *et al.*, 2006) and increases its stability (Supplemental Figure 2SD). Our results strongly suggested that ERKs-dependent phosphorylation events could also modulate the capacity of TTP to induce mRNA degradation. However, this mechanism does not depend on the ability of TTP to bind VEGF mRNA, because we have observed that TTP *in vivo* binding is equivalent upon tamoxifen stimulation in the presence or absence of the MEK inhibitor U0126. A possible explanation for these observations is that phosphorylation of TTP allows the recruitment of other stabilizing proteins such as HuR, an ARE-binding protein responsible for increased VEGF mRNA stability upon hypoxia (Levy *et al.*, 1998). We are currently investigating this point. More recently, Cherradi *et al.* (2006) reported that TIS11b and HuR exert antagonistic effects on VEGF 3'-UTR *in vitro*. HuR seems to transiently stabilize VEGF transcripts after ACTH stimulation of adrenocortical cells, and TIS11b seems to subsequently trigger their degradation. The impact of TTP on the development of Ras-transformed cells has already been evaluated (Stoecklin *et al.*, 2003). Our study proposes an interpretation of these previous results, demonstrating that angiogenesis and in particular VEGF are key events. It is important to stress that the antagonist role of ERKs on TTP activity is not highlighted in *in vivo* experiments. It is probably because the intermediate ERKs activity observed in Raf1-ER/TTP-Ras is not sufficient to totally antagonize TTP degradative action (Figures 8A and 9A). Even if VEGF remains a key player in this phenomenon, Ras has been implicated in the production of another potent angiogenic factor interleukin-8 (Sparmann and Bar-Sagi, 2004). Even if it was demonstrated that Ras control IL-8 transcription, IL-8 mRNA 3'-UTR contains numerous AREs, suggesting that TTP could exert its anti-angiogenic potential through VEGF as we demonstrated but also by modulating IL-8 mRNA decay and consequently production. To strengthen the relationship between VEGF and TTP in the tumor development, it would have been interested to demonstrate that exogenous VEGF reverses the effect of TTP. However, this strategy would probably fail as chronic exposition of animals to VEGF results in hepatic and heart problems (Dor *et al.*, 2002). Another potential of TTP

consists on its ability to alter cell growth, because it modulates Fos, Myc, and Cyclin D1 mRNA (Marderosian *et al.*, 2006; Hau *et al.*, 2007). It could also induce apoptosis in particular by modulation of the expression of TNF- α (Johnson *et al.*, 2000; Johnson and Blackwell, 2002). We have never observed that TTP affects the growth or apoptosis of the Raf1-ER/TTP-Ras cells (Supplemental Figure 4SD). However, in our system, we do not know whether the decrease in tumor size and vascularization involve apoptosis mechanisms responsible for the reduction in the number of blood vessels, because VEGF has been described as an antiapoptotic factor for endothelial cells (Grosjean *et al.*, 2006). Because many tumor cells were recently demonstrated to express VEGF receptors, down-regulation of VEGF production could also result in apoptosis of the tumor cells by suppressing an antiapoptotic autocrine loop. In conclusion, our study suggests that TTP disappearance could represent a pertinent diagnosis and prognosis marker for tumor aggressivity as well as a potential indicator for the success of therapies in tumors with increased ERKs activity.

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