

Tid-1 Interacts with the von Hippel-Lindau Protein and Modulates Angiogenesis by Destabilization of HIF-1 α

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Abstract

The von Hippel-Lindau protein (pVHL) is a major tumor suppressor protein and also associated with the inhibition of angiogenesis via HIF-1 α ubiquitination and proteasomal degradation. To further elucidate the biological activity of pVHL in angiogenesis, pVHL-interacting proteins were screened using the yeast two-hybrid system. We found that a mouse homologue of the long form of *Drosophila* tumor suppressor *l(2)tid*, Tid-1_L, directly interacts with pVHL *in vitro* and *in vivo*. Furthermore, Tid-1_L protein; enhanced the interaction between HIF-1 α and pVHL, leading to the destabilization of HIF-1 α protein; therefore, Tid-1_L protein decreased vascular endothelial growth factor expression and inhibited angiogenesis *in vivo* and *in vitro*. These findings propose that Tid-1_L may play a critical role in pVHL-mediated tumor suppression by modulating the pVHL-dependent HIF-1 α stability. (Cancer Res 2005; 65(7): 2520-5)

Introduction

von Hippel-Lindau (VHL) disease is a hereditary cancer syndrome leading to the development of a variety of vascular tumors, including renal clear cell carcinomas (1). These tumors are highly associated with mutation or transcriptional silencing of the *VHL* gene and subsequent loss or inactivation of the remaining *VHL* allele (1). The pVHL protein product has no significant sequence similarity to other known proteins thus giving no clues about its function. According to many reports, insights into the multiple functions of pVHL have come predominantly from the identification of proteins that bind to pVHL (1-3). Recent studies highlighted the action of pVHL on the hypoxia inducible factor-1 (HIF-1), consisting of HIF-1 α and HIF-1 β . Under normoxic conditions, oxygen-dependent degradation (ODD) domain within HIF-1 α interacts with the pVHL which functions as ubiquitin E3 ligase for HIF-1 α polyubiquitination, and polyubiquitinated HIF-1 α is rapidly degraded (4). Hypoxia or pVHL deficiency stabilizes HIF-1 α , resulting in activation of genes encoding erythropoietin and VEGF and of genes involved in glucose transport and metabolism (5). In the present study, we have conducted the yeast two-hybrid assay to identify novel proteins that interact with pVHL and to further elucidate the biological functions of pVHL. Thus, we identified a pVHL-

interacting clone, Tid-1_L (a mouse homologue of the long form of *Drosophila* tumor suppressor *l(2)tid*, tumorous imaginal disc) which is the first member of a DnaJ chaperone family to be classified as a tumor suppressor (6). The Tid-1 protein has two forms, Tid-1_L and Tid-1_S, by alternative splicing (7) apoptosis (7). Tid-1_L protein increases apoptosis, whereas expression of Tid-1_S dominantly suppresses apoptosis (7). However, their functions on tumor suppressive mechanisms in mammalian cells has not been fully characterized. Herein, we show that Tid-1_L down-regulates HIF-1 α protein levels by enhancing interaction of HIF-1 α with pVHL. Furthermore, we demonstrate that Tid-1_L transfectants display reduced new blood vessel formation by destabilizing HIF-1 α and decreasing VEGF expression.

Materials and Methods

Reagents and antibodies. Cobalt chloride and 2,2'-dipyridyl were purchased from Sigma (St. Louis, MO). Mouse monoclonal antibody against human Tid-1_{L/S} antibody, goat monoclonal antibody against human Tid-1_L, and vascular endothelial growth factor (VEGF) antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-VHL antibody was purchased from PharMingen (San Diego, CA).

Plasmids. For the yeast two-hybrid screening, human full-length VHL was subcloned into pGBT9 (Clontech, Palo Alto, CA). A series of deletion mutants of *pVHL* gene and full-length VHL were inserted into pGEX-4T (Amersham Pharmacia Biotech, Piscataway, NJ) and into pCMV-Tag (Stratagene, Cedar Creek, TX). To construct pET-Tid-1_L and glutathione *S*-transferase/ODD, the human Tid-1_L cDNA was inserted into pET28 (Novagen, Madison, WI) and ODD cDNA was inserted into pGEX-4T. pcDNA-Tid-1_L vector was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA). To construct antisense Tid-1_L vector, human Tid-1_L cDNA fragment (387-550) was inserted into pcDNA3.1 in reverse orientation.

Two-hybrid library screening and evaluation of protein-protein interactions. Yeast strains SFY526 and HF7c obtained from Clontech were used to assay protein-protein interactions and for library screening, respectively. Two-hybrid assays using the GAL4 system were done according to the instructions of the manufacturer (Clontech).

Cell culture. HEK293 cells and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Life Technologies, Inc., Gaithersburg, MD) and 1% antibiotics. Primary human umbilical vascular endothelial cells (HUVEC, passages 5-8) were grown on 0.3 % gelatin-coated plates in M199 supplemented with 20 % FBS, 3 ng/mL basic fibroblast growth factor, and 5 units/mL heparin. For hypoxic condition, cells were incubated at 5% CO₂ level with 1% O₂ balanced with N₂ in hypoxic chamber (Forma, Marietta, OH).

Glutathione *S*-transferase pull-down assay. GST pull-down assay was done as described (8) using GST or GST- full length VHL or VHL exons 1 to 3. For the binding assay with ODD and pVHL, GST-ODD fusion proteins and *in vitro* translated VHL or Tid-1_L were also prepared as above. GST-ODD fusion proteins were preincubated with HeLa cell extracts for 2 hours at 30°C and then binding assay was done as previously described (8).

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Coimmunoprecipitation. After 24 hours of transfection into HEK293 cells, cells were harvested and protein extracts were prepared. Transient transfection was done using LipofectAMINE reagent (Invitrogen). Immunoprecipitation and Western blot were done as described previously (8).

Conditioned media preparations. For preparation of conditioned medium (CM), the medium of Tid-1_L transfected cells was changed with 1% FBS-containing M199 and further incubated for 24 hours. The CM was filtered through 0.22- μ m pore membrane (Millipore, Bedford, MA) and directly applied onto HUVECs. To conduct the chorioallantoic membrane (CAM) assay, the CM was concentrated using an ultrafiltration kit (10-kDa cutoff, Millipore). Completion of transient transfection was confirmed by performing Western blotting and β -galactosidase assay.

Quantification of vascular endothelial growth factor in conditioned medium. Secreted VEGF was determined by using the ELISA development kit (R&D Systems, Minneapolis, MN) and done according to the manufacturer's instructions.

Chemoinvasion and wounding migration assay. Twenty-four-well transwell chambers of polycarbonate filter inserts were used, and cell invasion was determined by counting whole cell numbers at single filter (9). For migration assay, cells at 90% confluence were wounded with a razor blade 2 mm in width and marked at the injury line and then incubated in transfectant's CM and 1 mmol/L thymidine, followed by fixing and staining with Giemsa. Migration was quantitated with counting the number of cells that moved beyond the reference line (9).

Tube formation assay and chorioallantoic membrane assay. Matrigel (10 mg/mL) was polymerized for 30 minutes at 37°C. HUVECs were seeded on the Matrigel and grown in the transfectant's CM. After 12 to 16 hours, the cultures were observed at $\times 100$ magnification. CAM assay was conducted by using 4.5-day-old chick embryos as described (9). In CAM assay, at least 20 eggs were used for each concentrated CM and data on the number of positive CAM were analyzed by Student's *t* test with *P* < 0.05 as the level of significance.

Results

Identification of Tid-1_L as a von Hippel-Lindau protein-interacting protein. To isolate pVHL binding cellular proteins, we screened a mouse embryonic cDNA library and T cell library using a bait with full-length VHL cDNA as bait. From 6×10^6 transformants, strong His⁺/Lac⁺ double-positive clones were isolated. The 52 clones were further selected by β -galactosidase assays in another yeast strain, SFY 526, containing a GALIUS-LacZ reporter gene. Among these clones, some clones are known to be binding partners of pVHL, including elongin C and tat binding protein. DNA sequence and data base searches revealed that five clones encoded mouse homologue of *Drosophila* tumor suppressor *l(2)tid* long form (i.e., Tid-1_L). As previously reported, Tid-1 protein in HEK293 cells revealed two forms of Tid-1_L (~43 kDa) and Tid-1_S (~40 kDa), two splice variants (7). Direct interaction of pVHL and Tid-1_L *in vitro* was confirmed using GST pull-down assays. The results shown in Fig. 1A indicated that the [³⁵S]-labeled Tid-1_L was pulled down by interaction with the GST-fused full-length VHL protein *in vitro*. In the further GST pull-down assay using the deletion constructs of pVHL, *in vitro* translated Tid-1_L binds to exons 1 and 2, acidic and β -domains, but not exon 3, α -domain (Fig. 1B). The interaction between Tid-1_L and pVHL *in vivo* was verified by coimmunoprecipitation assays. Tid-1_L was coimmunoprecipitated when HEK293 cell extracts were treated with anti-VHL antibody (Fig. 1C).

Tid-1_L down-regulates HIF-1 α protein levels. It was reported that pVHL is the critical regulator of HIF-1 α stability depending on oxygen concentration (2, 4). We therefore checked the mRNA

and protein levels of Tid-1_L under hypoxic and normoxic conditions. As shown in Fig. 2A, Tid-1_L mRNA and protein levels were decreased by hypoxia and hypoxia-mimicking agents, cobalt chloride and 2,2'-dipyridyl. Because the pVHL is a negative regulator of HIF-1 α , we investigated whether Tid-1_L protein also influences HIF-1 α protein stability. We used HEK293 cells transfected with Tid-1_L expression vector. As shown in Fig. 2B, the overexpressed Tid-1_L down-regulated the protein level of HIF-1 α induced by hypoxia; however, it did not affect mRNA level of HIF-1 α , suggesting that the reduction of the HIF-1 α protein level by Tid-1_L was not due to the transcriptional reduction of the *HIF-1 α* gene. In Fig. 2C, we constructed the antisense Tid-1_L vector to test the ability of Tid-1_L to regulate HIF-1 α protein expression. From these results, HIF-1 α protein level was decreased by Tid-1_L, whereas the antisense Tid-1_L

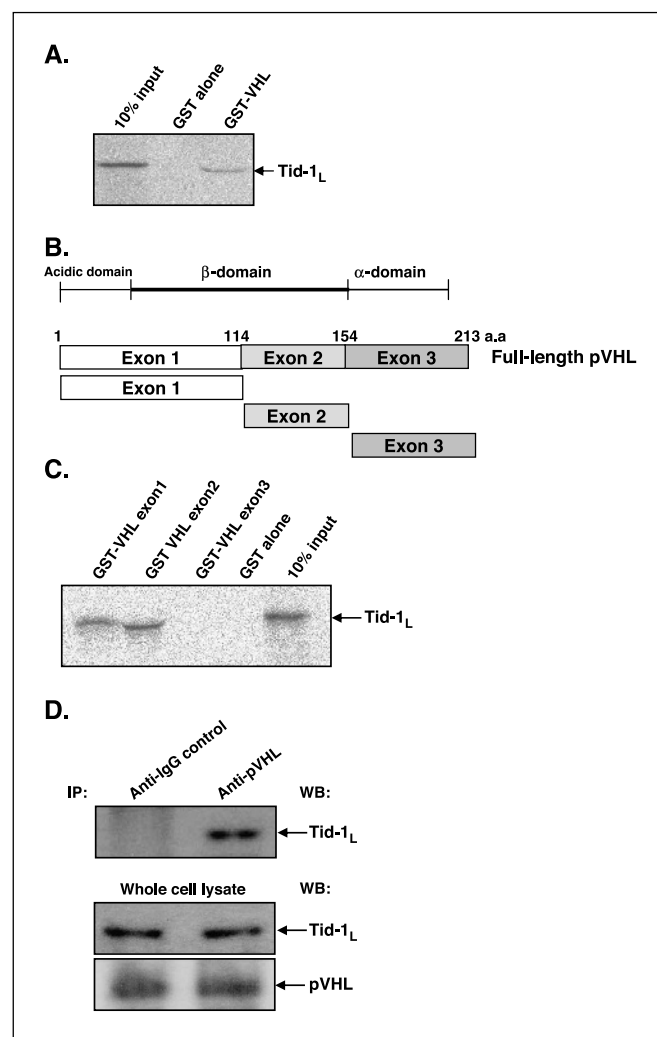


Figure 1. Tid-1_L interacts with pVHL *in vitro* and *in vivo*. **A.** ³⁵S-labeled Tid-1_L was mixed with GST or GST full-length VHL-bound beads. Ten percent of the material used in this assay (lane 1). **B.** Schematic representation of pVHL; pVHL has three exons containing an acidic domain, β -domain, and α -domain. ³⁵S-labeled Tid-1_L was mixed with GST-bound VHL exon 1, VHL exon 2, exon 3, and GST-bound beads. **C.** Endogenous pVHL was immunoprecipitated with HEK293 cell extracts and coprecipitated Tid-1_L was detected by Tid-1_L antibody. Anti-mouse IgG was used as a negative control. Expression levels of Tid-1_L and pVHL in input extracts were visualized by Western blot analysis. Immunoprecipitation (IP); Western blot (WB).

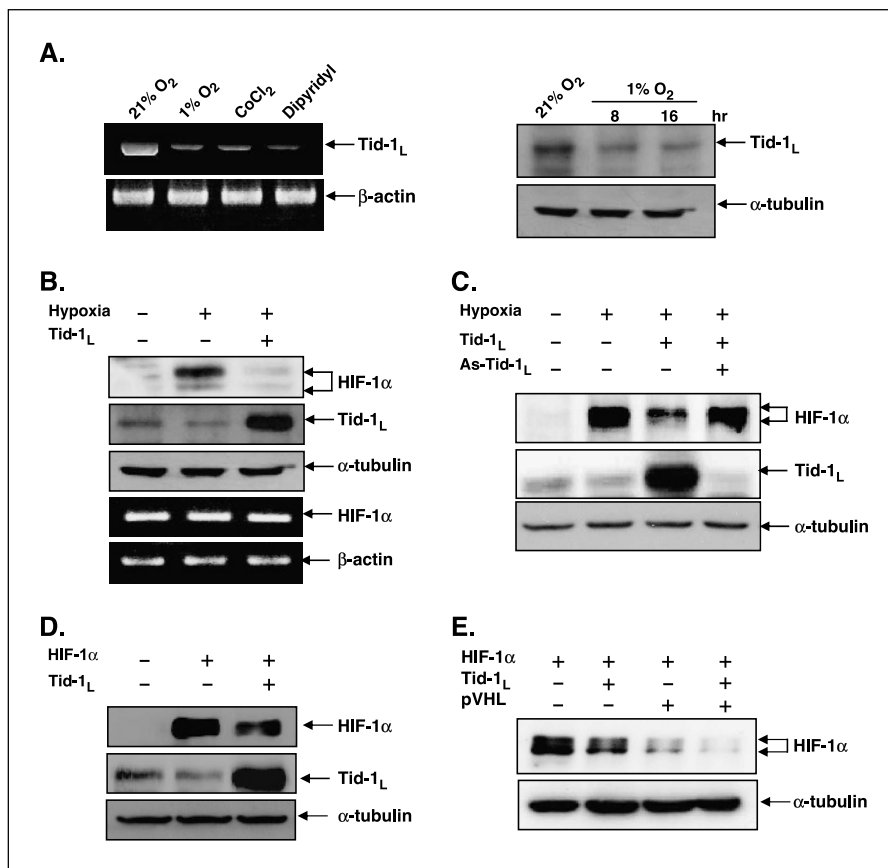


Figure 2. Tid-1_L decreases HIF-1α protein levels. **A**, HeLa cells were incubated for 4 hours under 1% O₂ or treated with 100 μmol/L cobalt chloride or 100 μmol/L 2, 2'-dipyridyl. Reverse transcription-PCR (RT-PCR) analysis was performed using specific primers for Tid-1_L (left). Tid-1_L protein levels were examined by anti-Tid-1_L Western blotting (right). β-Actin and α-tubulin served as loading controls. **B**, HEK293 cells were transfected with Tid-1_L expression vector and left untreated or exposed to 1% O₂ for 6 hours. Anti-HIF-1α or Tid-1_L Western blotting from protein extracts was performed (top two). RT-PCR analysis done using specific primer for HIF-1α and β-actin (bottom two). **C**, HEK293 cells were transfected with antisense Tid-1_L expression vector and exposed to 1% O₂ for 16 hours. HIF-1α and Tid-1_L protein levels were examined by Western blotting. **D** and **E**, HEK293 cells were transfected with the indicated expression vectors under normoxia. Immunoblot analysis done with anti-HIF-1α antibody. Representative results of at least three independent experiments.

increased HIF-1α protein level in hypoxic condition. The down-regulation of the HIF-1α protein level by Tid-1_L under normoxia was undetectable because endogenous HIF-1α is not present in this condition. According to recent report, at higher concentration of HIF-1α expression vector, HIF-1α protein expression levels were detected at normoxia (10). Therefore, we examined the effect of Tid-1_L on HIF-1α protein stability under the high level of HIF-1α at normoxia. Transient expression of Tid-1_L resulted in the reduction of HIF-1α protein level under normoxia (Fig. 2D). Moreover, HIF-1α protein level in coexpression of Tid-1_L and pVHL was much lower than that in Tid-1_L alone or pVHL alone (Fig. 2E).

Effect of Tid-1_L on the interaction between HIF-1α and pVHL. To investigate whether Tid-1_L participates in the association of HIF-1α and pVHL, the recombinant Tid-1_L or *in vitro* translated Tid-1_L was added to the reaction mixture of ³⁵S-labeled VHL and ODD domain of HIF-1α immobilized on beads. The recombinant Tid-1_L protein or *in vitro* translated Tid-1_L accelerated the binding of pVHL to HIF-1α (Fig. 3A). Moreover, the interaction between HIF-1α and pVHL was stimulated with increasing amounts of recombinant Tid-1_L protein (Fig. 3B). We further found that the overexpressed Tid-1_L increased the interaction of HIF-1α with pVHL in coimmunoprecipitation assay (Fig. 3C). These results suggest that Tid-1_L could function as a negative regulator of HIF-1α stability by accelerating the recruitment of pVHL to HIF-1α.

Tid-1_L inhibits vascular endothelial growth factor expression and angiogenesis *in vivo* and *in vitro*. We checked whether Tid-1_L modulates the expression of VEGF, a major angiogenic

factor regulated by HIF-1α. The mRNA and protein levels of VEGF were dramatically down-regulated by Tid-1_L compared with mock-transfected cells under both normoxic and hypoxic conditions (Fig. 4A). To investigate whether Tid-1_L shows antiangiogenic activities, we did *in vitro* and *in vivo* angiogenesis assays using conditioned media from control or Tid-1_L-expressing cells. In tube formation assay, both the control medium and mock-CM enhanced the formation of elongated and complex networks of HUVECs. However, the CM from Tid-1_L-transfected cells, which showed no cytotoxic side effects on HUVEC, remarkably reduced the capillary network formation of HUVECs on Matrigel beds (Fig. 4B). Accordingly, the tube structures were not elongated and the cells were made a rump and were not developed into tube network. Furthermore, Tid-1_L CM markedly decreased the migration (Fig. 4C) and invasion (Fig. 4D) of HUVECs compared with control CM or mock CM. Moreover, we confirmed the antiangiogenic effect of Tid-1_L *in vivo* with chick embryo CAM assay. The concentrated (100×) Tid-1_L-CM remarkably reduced angiogenesis on the CAM (Fig. 4E). The antiangiogenic activity of Tid-1_L-CM was 65%, whereas the inhibitory activity of mock CM was only 14%, similar to that of the empty coverslip on chick CAM. These results strongly suggest that Tid-1_L potently represses angiogenesis by inhibiting migration, invasion, and tube formation of endothelial cells.

Discussion

Hypervascularization is a typical feature of tumors in VHL disease, due to in part from high expression of VEGF (1). Under

normal physiological conditions, the functional pVHL physically binds to HIF-1 α and mediates HIF-1 α protein degradation via ubiquitin-proteasome pathway, leads to low levels of VEGF. pVHL is also known to interact with other signaling proteins, but the binding partners of pVHL and their physiologic roles in the suppression of hypervascular tumors have not been fully characterized.

In this study, we used the yeast two-hybrid screening to identify novel pVHL-interacting proteins. One of the interacting proteins, Tid-1_L, has a high homology to Tid56, the protein encoded by the tumor suppressor gene *l(2)tid* in *Drosophila melanogaster* in which recessive mutations lead to malignant transformation of the imaginal discs of the larva (11). Schilling et al. (12) reported that Tid-1_L interacts with the human papilloma virus type 16 E7 oncoprotein. Moreover, Tid-1_L interacts with a number of proteins involved in cell signaling molecules, such as Ras GTPase-activating protein (13), HTLV-1 tax, Hsp70 (14), and c-jun-NH₂-kinase (15).

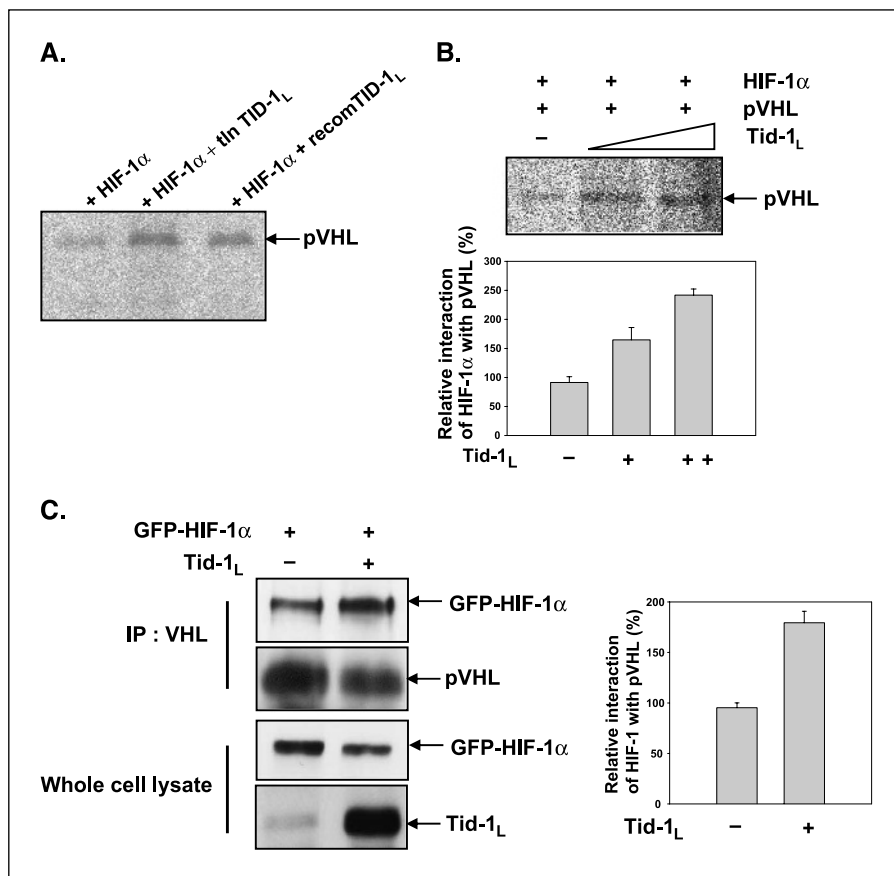
Tid-1_L is a member of DnaJ chaperone protein family which contains J domain, a highly conserved domain that binds to Hsp70 chaperones. The DnaJ/Hsp70 systems are involved in protein folding (16), protein degradation, and assembly and disassembly of multiprotein complex (17). We observed in this report that Tid-1_L protein increased the binding of pVHL to HIF-1 α protein *in vitro* and *in vivo*. Based on our observations, we propose that Tid-1_L may enhance the assembly of pVHL and HIF-1 α to promote HIF-1 α protein degradation. Moreover, Tid-1L could not reduce HIF-1 α protein level and VEGF expression in VHL-defective cells in contrast to VHL-positive cells (data not shown).

From these results, we strongly suggest that destabilization of HIF-1 α protein accelerated by Tid-1_L is pVHL-HIF1 α dependent. Of course, further investigations would be necessary to clarify the chaperone activity of Tid-1_L in pVHL conformational change and assembly of multiple complex structures including pVHL and HIF-1 α .

Although Tid-1 has not previously been recognized as a tumor suppressor in humans, it is tempting to find out its tumor suppressor function in mammalian cells. In addition, recent report showed *in vitro* transformation-suppressive activity of Tid-1_L in human cancer cells (14). According to many recent reports, a number of tumor suppressor proteins, such as p53, pVHL, PTEN, and thrombospondin, suppressed angiogenesis by modulating VEGF which is a key mediator of tumor angiogenesis (18–20). Importantly, the data shown in “Results” pointed to a potential role for Tid-1 protein in HIF-1-induced VEGF signaling. In this capacity, Tid-1_L tumor suppressor protein may promote HIF-1 α protein degradation through enhancement of pVHL-HIF-1 α interaction and suppress angiogenesis. Additional investigations of the biochemical function of Tid-1_L and pVHL complex may improve our understanding of how Tid-1_L protein may exert its effects on tumor suppression via inhibition of hypoxia-induced angiogenesis.

Taken together, we suggest that Tid-1_L interacts with pVHL and this interaction accelerates binding to HIF-1 α , leads to reduced HIF-1 α protein levels. Moreover, our data show that Tid-1_L, as a negative regulator of HIF-1 α stability, modulates neovascularization of endothelial cells *in vitro* and *in vivo* by decreasing the VEGF expression. Further characterizations of detailed physiological and

Figure 3. Tid-1_L regulates the association between HIF-1 α and pVHL. **A**, GST-ODD mixed with ³⁵S-labeled VHL under treatment of *in vitro* translated Tid-1_L or recombinant Tid-1_L. **B**, GST-ODD bound to bead was incubated with constant amounts of ³⁵S-labeled VHL in the absence or presence of increasing amounts (0.5-, 1-, 2-fold molar excess) of recombinant Tid-1_L. Interaction signals from three independent experiments were quantified (*bottom*). **C**, HEK293 cells were cotransfected with expression vectors encoding GFP-HIF-1 α and Tid-1_L. Anti-VHL immunoprecipitates (IP) and whole cell lysates were analyzed by immunoblot with antibodies against HIF-1 α , pVHL, and Tid-1_L. Relative interaction from their five independent experiments was quantified by densitometry. Interaction of HIF-1 α with pVHL was set to 100% (*right*).



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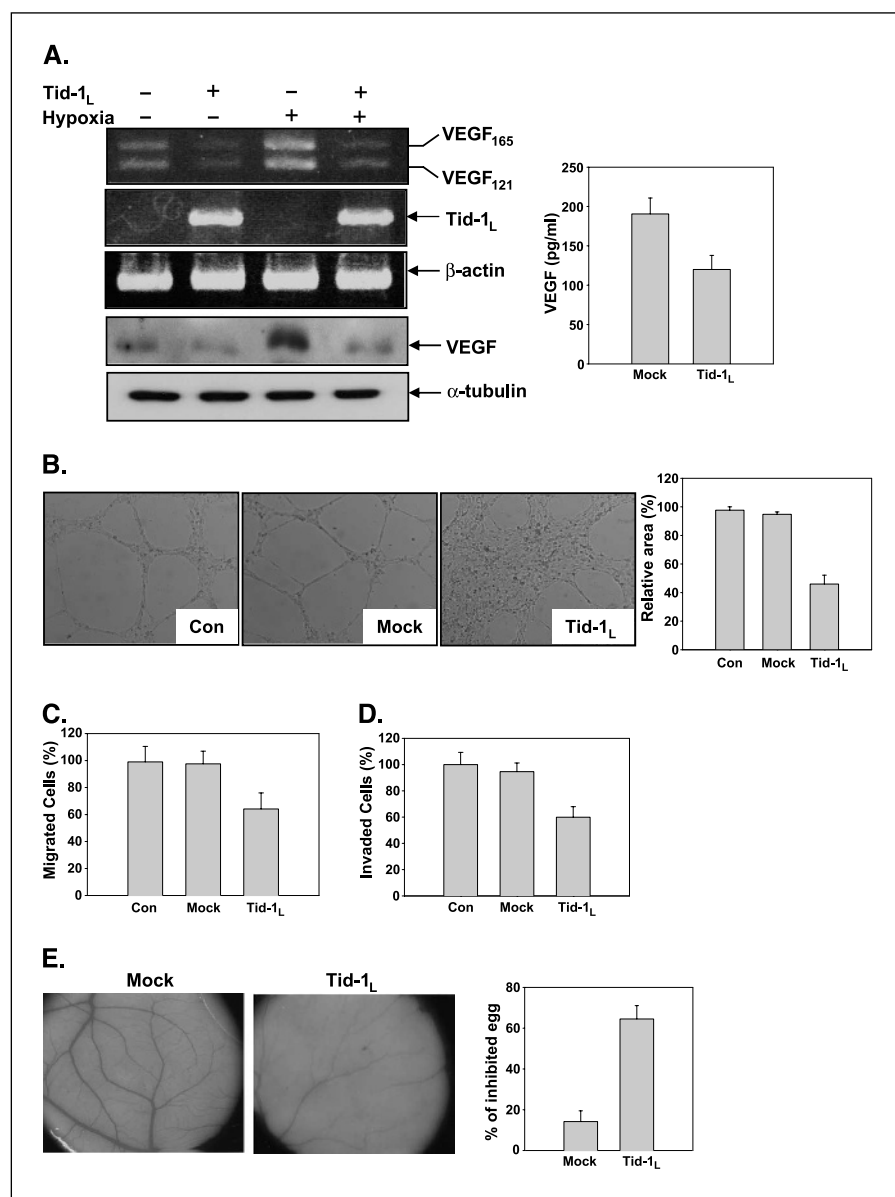


Figure 4. Tid-1_L modulates VEGF expression and angiogenesis. *A*, HEK293 cells were transfected with Tid-1_L expression vector and left untreated or exposed to 1% O₂ for 16 hours. RT-PCR analysis was performed using specific primer for VEGF. Anti-VEGF Western blotting (α-VEGF) from protein extracts was performed (*bottom two*). Secreted VEGF proteins in mock CM and Tid-1_L CM under hypoxia determined by ELISA (*right*). *B*, HUVECs were incubated on the Matrigel with CMs from nontransfected (*con*), mock-transfected (*mock*), and Tid-1_L transfected (Tid-1_L) cells for 8 hours. Area covered by the tube network quantitated using Image-Pro Plus software. *Columns*, % from three different experiments with duplicate; *bars*, ±SE (*right*). *C* and *D*, migration of HUVECs for 16 hours (*C*) and invasion of HUVECs for 16 hours (*D*) observed in Tid-1_L-CM compared with control or Mock-CM. Independent experiments were repeated four times with triplicate. *Columns*, mean from triplicate assays; *bars*, ±SD. *E*, CAM surfaces chick embryos were treated with the concentrated Mock CM or Tid-1_L CM, respectively, and observed after 3days.

biochemical functions of the Tid-1_L as pVHL-interacting molecule may provide us with better understanding of the precise mechanism underlying in tumor angiogenesis and tumorigenesis in VHL-related tumors.

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