Hypoxia-inducible Factor 1α Is Regulated by the Mammalian Target of Rapamycin (mTOR) via an mTOR Signaling Motif*

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Tumors that form as a result of heightened mammalian target of rapamycin (mTOR) signaling are highly vascularized. This process of angiogenesis is regulated through hypoxia-inducible factor (HIF)-mediated transcription of angiogenic factors. It is recognized that inhibition of mTOR with rapamycin can diminish the process of angiogenesis. Our work shows that activation of mTOR by Ras homologue enriched in brain (Rheb) overexpression potently enhances the activity of HIF1α and vascular endothelial growth factor (VEGF)-A secretion during hypoxia, which is reversed with rapamycin. Mutants of Rheb, which do not bind guanine nucleotide (D60K, D60V, N119I, and D122N) and are unable to activate mTOR, inhibit the activity of HIF when overexpressed. We show that regulatory associated protein of mTOR (Raptor) interacts with HIF1α and requires an mTOR signaling (TOS) motif located in the N terminus of HIF1α. Furthermore, a mutant of HIF1α lacking this TOS motif dominantly impaired HIF activity during hypoxia and was unable to bind to the co-activator CBP/p300. Rapamycin treatments do not affect the stability of HIF1α and modulate HIF activity via a Von Hippel-Lindau (VHL)-independent mechanism. We demonstrate that the high levels of HIF activity in cells devoid of TSC2 can be reversed by treatments with rapamycin or the readdition of TSC2. Our work explains why human cancers with aberrant mTOR signaling are prone to angiogenesis and suggests that inhibition of mTOR with rapamycin might be a suitable therapeutic strategy.

The supply of nutrients and oxygen is quickly depleted as solid tumors grow in size. Nutritional and oxygen homeostasis within solid tumors is reestablished by vascularization that consequently results in tumor expansion. Oxygen sensing is known to control the mechanism of vasculogenesis through hypoxia-inducible factor (HIF)2-mediated transcription of two key angiogenic factors; vascular endothelial growth factor (VEGF)-A (1) and angiopoietin-2 (Ang-2) (2). VEGF-A and Ang-2 encourage angiogenesis by stimulating the formation of blood vessels that migrate into the tumor.

As well as vascularization, HIF is known to control the expression of >70 genes involved in energy metabolism, apoptosis/survival, and metastasis (for reviews see Refs. 3, 4). Low oxygen enhances the activity of HIF by stabilizing the α-subunit of HIF. Oxygen-dependent prolyl hydroxylase domain (PHD) proteins become active in the presence of oxygen that sequentially leads to hydroxylation of two proline residues within the oxygen-dependent degradation domain (ODDD) of the HIF α-subunit. Proline hydroxylation results in ubiquitin-mediated degradation of the α-subunit that requires the Von Hippel-Lindau (VHL) tumor suppressor protein, which is a component of an E3 ubiquitin ligase complex (5). VHL syndrome, caused through loss of function of this tumor suppressor, is a dominantly inherited familial syndrome that predisposes the patient to renal cell carcinoma and cerebellar hemangioblastomas. In conditions of low oxygen tension, the HIF α protein is stable and binds to HIFβ (also known as aryl hydrocarbon receptor nuclear translocator-ARNT). The HIFα/HIFβ dimer is then rapidly translocated to the nucleus. As a dimer, they bind to RCGTG DNA sequences called hypoxia response elements. Transcription is sequentially activated when the C terminus transcriptional activation domain (C-TAD) of HIFα binds to the co-activator CBP/p300 (for review see Ref. 6).

The tuberous sclerosis complex (TSC), which is a hamartomas syndrome, occurs through loss of function of the tumor suppressor proteins TSC1 (hamartin) and TSC2 (tuberin). Tumors that occur in TS patients are highly vascularized, implying that HIF might be involved and is regulated downstream of TSC1/2. TSC is an autosomal genetic syndrome that occurs with an estimated prevalence of one in 6,000 newborns and is characterized by slow growing benign tumors that form in the heart, brain, kidneys, eyes, and skin (see Ref. 7 for review). These tumors can lead to both renal and neurological compi-

human embryonic kidney; MEFs, mouse embryonic fibroblasts; mTOR, mammalian target of rapamycin; TOS, mTOR signaling; NF1, neurofibromin 1; PI3K, phosphoinositide 3-kinase; PHD, prolyl hydroxylases domain; PTK, phosphatase and tensin homolog; Raptor, regulatory associated protein of mTOR; Redd1/2, regulated in development and DNA damage responses; Rheb, Ras homologue enriched in brain; Rictor, rapamycin-insensitive companion of mTOR; S6K1, ribosomal protein S6 kinase 1; STK11, serine/threonine kinase 11; TSC, tuberous sclerosis complex; VEGF, vascular endothelial growth factor; VHL, Von Hippel-Lindau; HA, hemagglutinin; FITC, fluorescein isothiocyanate.
cations. TSC1 and TSC2 function as a heterodimer that inhibits cell growth by impairing the Ser/Thr protein kinase called the mammalian target of rapamycin (mTOR) (see review in Ref. 8). TSC1/TSC2 heterodimers function to inhibit mTOR, by acting as a GTPase-activating protein (GAP) toward the small G-protein, Ras homologue enriched in brain (Rheb). Rheb has been shown to interact with mTOR, and promotes signal transduction when Rheb is in the active GTP-bound state (9). When in a complex with TSC1, TSC2 inhibits Rheb-induced mTOR signaling by reverting Rheb to its inactive GDP-bound state (10).

Therefore, when the normal function of TSC1/TSC2 heterodimers becomes compromised, Rheb becomes constitutively active, and mTOR signaling is significantly enhanced and mTOR-directed phosphorylation of downstream targets (9, 10). We show that mTOR directly enhances the transcriptional activity of HIF1α that does not involve increased HIF1α stabilization. Furthermore, mTOR activation of HIF1α requires the interaction of HIF1α with Raptor. Given that Raptor functions as a scaffold protein that recruits downstream mTOR substrates with a TOS motif to mTOR (15), this work reveals that HIF1α is a downstream target of mTOR.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Materials**—[3H]GTP, [3H]GDP, and [35S]methionine-radiolabeled reagents were purchased from American Biosciences (GE Healthcare UK Ltd.). Dimethylxalylglycine was purchased from Frontier Scientific Europe Ltd (Lancashire, UK). Deferoxamine mesylate, MG-132 (carbonyl-1-leucyl-1-leucyl-1-leucinal), rapamycin, and LY294002 were obtained from Merck Biosciences Ltd. (Nottingham, UK). CHAPS was ordered from Pierce. DSP (dithiobis(succinimidyl propionate)) was purchased from Apollo Scientific Ltd. (Stockport, UK). Rat anti-HA antibodies were purchased from Roche Applied Science. Mouse anti-HA antibodies were kindly provided by M. Chou (University of Pennsylvania, Philadelphia, PA). Cell Signaling Technology anti-S6K1 and anti-S6K1 phospho-Thr389 antibodies were purchased from New England Biolabs Ltd. (Hertfordshire, UK). Anti-HIF1α antibodies (clone: H1alpha67) were obtained from Novus Biologicals (Stratech Scientific Ltd.). Anti-Coactivator p300 (C-20) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Anti-FLAG antibodies (clone: M2), anti-Myc (clone: 9E10) and all other reagents (unless stated) were obtained from Sigma-Aldrich.

**Plasmids and Molecular Biology**—N-terminal FLAG-tagged pRRK7/Rheb was generated as described in Ref. 10. Human HAF was a kind gift from N. Sonenberg (McGill University, Montreal, Canada). HIF1α was cloned into a modified pRRK7 vector so it expressed a modified N-terminal HA tag using human HIF1α cDNA as a template (obtained from Novus Biologicals (Stratech Scientific Ltd.)). Myc-tagged Raptor/pRK5 was a kind gift from D. M. Sabatini (Whitehead Institute for Biomedical Research, Boston, MA). Human 4E-BP1 and human Rheb were subcloned into pGEX-2T/GST to generate GST-tagged recombinant protein. Point mutations were generated as described in the QuikChange® site-directed mutagenesis kit (Stratagene). The N-terminal FLAG-tagged pRRK7/TSC2 construct was generated as previously described (26). The pHVL-HA vector was kindly provided by P. Ratcliffe (Henry Wellcome Building of Molecular Physiology, Oxford University, UK) as described (27). The firefly luciferase reporter pGL2-TK-HRE plasmid was generated by subcloning three copies of the hypoxia response element (HRE) (5′-GTGACTACGTGCTGCCTAG-3′) from the inducible nitric-oxide synthase promoter into the promoter region of the pGL2-TK vector as previously described (28) and was kindly provided by G. Melillo (National Cancer Institute at Frederick, Maryland).

**Tissue Culture and Analysis of Cell Lysates**—Human embryonic kidney 293 (HEK293) cells were cultured (at 37 °C within 5% CO2) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. DMEM and fetal bovine serum were purchased from Invitro-
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gen Ltd. (Paisley, UK). TSC2−/− mouse embryonic fibroblasts (MEFS) were a kind gift from D. J. Kwiatkowski (Harvard Medical School, Boston, MA). The human renal carcinoma VHL−/− cells (786-O) were bought from ATCC. Cell transfections were carried out as described in the SuperFect® manufacturer’s protocol (purchased from Qiagen Ltd. (West Sussex, UK)). HEK293 cells were transfected with >95% efficiency, while transfection of TSC2−/− MEFS and human renal carcinoma VHL−/− cells were <5% efficient. Hypoxic treatments were carried out in a MACS VA500 Microaerophilic Work station (Don Whitley Scientific, Shipley, West Yorkshire) containing a humidified atmosphere equilibrated to 1% O2, 5% CO2, and 94% N2. To create cell lysates, cells were washed twice in phosphate-buffered saline, and then harvested with lysis buffer (10 mM KH2PO4, 1 mM EDTA, 10 mM MgCl2, 50 mM β-glycerophosphate, 5 mM EGTA, 0.5% Nonidet P-40, 0.1% Brij 35, 1 mM sodium orthovanadate, 40 mg/ml phenylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg pepstatin, pH 7.2). Cell lysates were sonicated to help break down the nucleus and then spun at 14,000 rpm for 8 min at 4°C to remove the cell debris. For Western blot analysis, these lysates were prepared as described for running NuPage® Novex gels (bought from Invitrogen Ltd.) Proteins resolved on Novex gels were transferred to Millipore Immobilon-P (purchased from Upstate Ltd. (Hampshire, UK)) according to the manufacturer’s protocol (purchased from GE Healthcare UK Ltd.) and blotted with the appropriate antibody followed by horseradish peroxidase-conjugated secondary antibodies. Enhanced chemiluminescence was carried out with Amersham Biosciences ECL™ Western blotting detection reagents (purchased from GE Healthcare UK Ltd.).

Immunoprecipitation—Proteins were immunoprecipitated using the relevant antibodies coupled to protein G-Sepharose (Amersham Biosciences). HIF1α immunoprecipitations were carried out overnight at 4°C, and the protein G-Sepharose was added for 1 h prior to being washed. Immunoprecipitates were washed twice each with both buffer A (50 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM MgCl2, 1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 1% Triton) and buffer B (50 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM MgCl2, 0.1% Triton) in the presence of protease inhibitors.

Luciferase Reporter Assay—The Dual-Luciferase® Reporter Assay System (purchased from Promega UK Ltd. (Southampton UK)) was carried out using cell lysis buffer as recommended by the manufacturer’s protocol with a Wallac Victor 2 1420 Multi-label counter. We ran luciferase assays using an empty pGLO3 vector (Promega) as a negative control.

VEGF Secretion—Secreted VEGF protein was detected using the human VEGF DuoSet® ELISA Development System (R&D Systems, Abingdon, UK) according to the manufacturer’s instructions. After treatment, the cell medium was recovered following gentle agitation to maximize the recovery of secreted proteins and immediately frozen in liquid N2. The cells were then washed in phosphate-buffered saline and lysed for later analysis of protein content using Bradford reagent. The quantity of VEGF secreted into the medium for each treatment was expressed as a fraction of the respective cellular protein content.

Immunocytochemistry—Cells transfected with either empty vector, HA-HIF1α, or HA-HIF1α (F99A) were cultured at 1% O2 for 18 h on glass coverslips. Cells were then fixed in methanol at −20°C, quenched in 0.1% Na+ borohydrate in Tris-buffered saline (TBS; 50 mM Tris-Cl (pH 7.4), 150 mM NaCl) and then blocked for 1 h in TBS containing 10% goat serum. Monoclonal Anti-HA (clone HA-7) FITC conjugate (Sigma) was then applied to the cells in TBS containing 0.1% bovine serum albumin overnight at 4°C and at 1:5000 dilution. After washing, coverslips were counterstained with DAPI antifade (Q-Bio) and mounted. The intracellular distribution of FITC and DAPI fluorescence was observed using a Zeiss LSM 510 confocal microscope.

Raptor Binding to mTOR Targets—To generate Raptor protein to be used in the overlay assays, Myc-tagged Raptor was overexpressed in HEK293 cells. These cells were lysed with 50 mM β-glycerophosphate, pH 7.4; 1 mM EDTA; 1 mM EGTA; 0.5 mM Na3VO4; 1 mM benzamidine hydrochloride; 1 mM dithiothreitol; 0.1 mM phenylmethane sulfonyl fluoride; 1% (w/v) Triton X-100, and 1 μg/ml each of pepstatin, antipain, and leupeptin. Proteins were transferred to Immobilon-P and blocked with 5% (w/v) milk in Tris-buffered saline. The blots were then incubated with anti-Myc antibodies for 2 h in 2% (w/v) bovine serum albumin Tris-buffered saline followed by anti-mouse horseradish peroxidase-conjugated secondary antibodies and ECL as described for Western blotting. Raptor association with mTOR substrates within cells was determined as previously described (15). Monitoring HIF1α Stability in Vivo—HEK293 cells transfected with HA-HIF1α (grown on 6-cm² plates) were incubated in methionine-free medium and labeled with 10 μCi of [35S]methionine for 4 h in the hypoxic chamber set at 1% O2. The cells were washed twice in fresh media and then incubated for 0, 0.5, 1, and 2 h in DMEM before cell lysates were generated. HA-HIF1α was immunoprecipitated for 1 h with anti-HA antibodies bound to protein G-Sepharose. Immunoprecipitated HIF1α was subjected to SDS-PAGE. The gel was stained, fixed, incubated with Amplify® (Amersham Biosciences), dried, and then exposed to x-ray film.

Rheb Nucleotide Binding—To examine [3H]GTP and [3H]GDP binding to Rheb in vitro, 2.5 μg of recombinant Rheb protein was incubated with either 1 μCi of [3H]GTP or [3H]GDP in 25 μl of loading buffer (50 mM HEPES, pH 7.5, 5 mM EDTA, and 5 mg/ml bovine serum albumin). After 30 min, 0.5 μl 1 mM MgCl2 and 25 μl of cold 50 mM HEPES, pH 7.4, were added. The [3H]GTP- or [3H]GDP-loaded Rheb was then applied to 0.2 μm cellulose nitrate membrane filter paper (Whatman) under suction in a vacuum manifold and washed three times with Rheb wash buffer (50 mM HEPES, pH 7.5, 0.5 mM NaCl, 0.1% Triton X-100, 5 mM MgCl2, 0.005% SDS plus protease inhibitors).

Statistics—One-way analysis of variance with posthoc significance assessed with Tukey’s honestly significant difference test using SigmaStat (version 2.0) was used on the data represented by bar charts. Values are given as means with ± S.D. A p value of <0.05 was considered to be statistically significant.
RESULTS

We wanted to examine whether the activity of HIF1α was enhanced by Rho overexpression during hypoxic conditions. Similar to the DMOG-treated cells (Fig. 1A), Rho enhanced the activity of HIF1α during hypoxia, which was significantly reduced with treatment with rapamycin (Fig. 2A).Increased activity of mTOR by Rho overexpression, as observed by increased Thr389 phosphorylation of S6K1, did not enhance the protein levels of endogenous HIF1α (Fig. 2B). We also observed a similar pattern of HIF1α expression within hypoxic cells (Fig. 2B), when compared with DMOG-treated cells (Fig. 1B). Given that Rho induced HIF1α transcription without increasing its protein levels, we propose that mTOR targets the transcriptional activity of HIF1α rather than its stability. To confirm that Rho overexpression was increasing the activity of HIF1α within these cells, we analyzed the secreted protein levels of VEGF-A, which is a well-characterized downstream gene target of HIF involved in angiogenesis (Fig. 2C). We observed that the levels of VEGF-A secretion paralleled the activity of HIF during...

**FIGURE 1.** mTOR activation enhances HIF1α transcription during treatment with DMOG. A, HEK293 cells transfected with a HIF-inducible luciferase reporter and empty pRK7 or pRK7/FLAG-Rho were serum-starved and where indicated were treated with 1 mM DMOG and/or 50 nM rapamycin for 18 h. Lysates prepared were analyzed for luciferase fluorescence. The HIF1α transcriptional activity from the DMOG transfected without Rho was standardized to 100%. B, protein levels of HIF1α were analyzed after being immunoprecipitated with anti-HIF1α antibodies. C, levels of VEGF-A secretion within the cell media was measured as pg/mg cell protein. 

**FIGURE 2.** mTOR activation enhances HIF1α transcription during hypoxia. A, serum-starved HEK293 cells transfected with a HIF-inducible luciferase reporter and empty pRK7 or pRK7/FLAG-Rho were transferred to low oxygen (1%) or maintained at 21% O2, where indicated, for 18 h. B, protein levels of HIF1α were analyzed after being immunoprecipitated with anti-HIF1α antibodies. C, levels of VEGF-A secretion within the cell media was measured as pg/mg cell protein.
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A

FIGURE 3. Guanine nucleotide binding mutants of Rheb inhibit the transcription activity of HIF1α. A, binding of both [3H]GTP and [3H]GDP to recombinant GST-Rheb proteins (wild-type, D60V, D60K, N119I, and D122N) or GST control were analyzed as described under “Experimental Procedures.” B, serum-starved HEK293 cells transfected with a HIF-inducible luciferase reporter and either empty pRK7 or pRK7 vector with FLAG-Rheb (wild-type, D60V, D60K, N119I, or D121N mutants) were transferred to a hypoxic chamber set at 1% O2 for 18 h. Lysates prepared in the hypoxic chamber were subjected to SDS-PAGE and a Raptor overlay assay. The band that corresponds to the Raptor was detected with anti-FLAG antibodies as shown. Co-purified Myc-Raptor was analyzed.

B

FIGURE 4. HIF1α interacts with the Raptor and requires an mTOR signaling motif. A, equal levels of recombinant GST-4E-BP1 and GST-4E-BP1(F115A) were subjected to SDS-PAGE and a Raptor overlay assay (lower panel). Equal levels of protein are shown using anti-GST antibodies (upper panel). B, HEK293 cells were treated with 100 μM DFX for 24 h, where indicated. Cells were treated with the MG-132 (50 μM) for 30 min prior to prevent degradation of HIF1α. Total lysates and purified HIF1α samples after immunoprecipitation with anti-HIF1α antibodies were resolved on SDS-PAGE and subjected to a Raptor overlay assay. The band that corresponds to HIF1α is marked. C, HEK293 cells transfected with HA-HIF1α and Myc-Raptor, where indicated, were lysed in the presence of reversible cross-linker DSP and non-ionic detergent (CHAPS). HA-HIF1α was immunoprecipitated using anti-HA antibodies and associated Myc-Raptor was determined. D, HEK293 cells were co-transfected with Myc-Raptor and either empty pRK7 vector, HA-4E-BP1, HA-HIF1α, or HA-HIF1α(F99A), where indicated. Cells were lysed in the presence of reversible cross-linker DSP and non-ionic detergent (CHAPS). Exogenous 4E-BP1 and HIF1α was immunoprecipitated using anti-HA antibodies as shown. Co-purified Myc-Raptor was analyzed.

hypoxia. Interestingly, Rheb overexpression enhanced the levels of VEGF-A secretion during conditions of normoxia that was sensitive to rapamycin.

Small G-protein mutants that do not bind guanine nucleotide typically form inactive complexes with their upstream regulators, i.e. the putative guanine exchange factor (GEF), which impedes small G-protein-mediated signal transduction. Studies in yeast identified dominant-negative mutants of Rheb (D60V and D60K), which were unable to efficiently bind to guanine nucleotides and impaired TOR signaling (31). We also generated two additional Rheb(N119I) and Rheb(D122N) mutants where these two mutated residues lie within the NKXD nucleotide binding motif of Rheb. Guanine nucleotide binding assays reveal that these D60V, D60K, N119I, and D122N mutants of Rheb are unable to bind to guanine nucleotides in vitro (Fig. 3A). These guanine binding-deficient mutants of Rheb are unable to efficiently impair acute activation of S6K1 by insulin treatment (data not shown, and described previously (32), but do possess some ability to impair mTOR (33). We wanted to investigate whether these Rheb mutants, which are deficient at binding guanine nucleotide, could alter the levels of HIF1α transcriptional activity during hypoxia. Interestingly, these Rheb mutants dominantly inhibited the transcriptional activity of HIF1α (Fig. 3B), when expressed to the same level as wild-type (Fig. 3C). This shows that these Rheb mutants can sufficiently impair the basal mTOR-mediated activation of HIF1α within HEK293 cells. Indeed, these mutants of Rheb were unable to induce Thr389 phosphorylation of S6K1 (Fig. 3C).

Well-characterized downstream targets of mTOR, eIF4E-binding protein 1 (4E-BP1) and S6K1, are recruited to mTOR through their association with Raptor. To examine Raptor interaction, we utilized a Raptor overlay assay to detect Raptor-interacting proteins resolved on SDS-PAGE. This Raptor overlay assay specifically detects proteins containing a TOS motif. For instance, the Raptor-overlay assay only detected wild-type 4E-BP1 and not a mutant of 4E-BP1 where the phenylalanine within the TOS motif at position 115 was mutated to an alanine (Fig. 4A). To determine whether Raptor interacted with HIF1α, we carried out a Raptor overlay assay on immunoprecipitated HIF1α. HEK293 cells were treated with 100 μM deferoxamine mesylate (DFX) for 24 h and then incubated with the proteo-
The HIF1α(F99A) TOS motif mutant dominantly inhibits HIF1α transcription during hypoxia. A, serum-starved HEK293 cells transfected with a HIF-inducible luciferase reporter with either empty prK7, HA-HIF1α, or HA-HIF1α(F99A) in the presence of absence of FLAG-Rheb were treated with 50 nM rapamycin, where indicated, and transferred to a hypoxic chamber set at 1% O2, for 18 h. Lysates prepared in the hypoxic chamber were analyzed for luciferase fluorescence. The HIF1α transcripitional activity of the cells transfected with empty prK7 vector and FLAG-Rheb was standardized to 100%. n = 6. *, p < 0.05 relative to activity of empty prK7 vector control group. **, p < 0.01 relative to activity of wild-type HA-HIF1α vector control group. B, protein levels of HA-HIF1α, FLAG-Rheb, S6K1, and Thr389 phosphorylation of S6K1 were analyzed.

FIGURE 5. Regulation of HIF1α by mTOR

mTOR does not modulate the stability of HIF1α. HEK293 cells co-expressing FLAG-Rheb with either wild-type HA-HIF1α or HA-HIF1α(F99A) were grown at 1% O2 in media containing [35S]methionine for 4 h, which was then replaced with unlabeled media in the presence or absence of rapamycin, where indicated. These cells were lysed after 0, 0.5, 1, and 2 h in the 1% O2 hypoxic chamber. Exogenous HA-HIF1α was immunoprecipitated with anti-HA antibodies and the relative level of [35S]methionine incorporation into HA-HIF1α was determined as described under “Experimental Procedures.” Protein levels of HA-HIF1α, FLAG-Rheb, S6K1, and Thr389 phosphorylation of S6K1 were also analyzed. The level of [35S]methionine-labeled HIF1α wild-type and HIF1α(F99A) at the zero time point was standardized at 100%. n = 3. There was no significant difference of [35S]methionine incorporation when comparing wild-type HIF1α with the F99A mutant or when comparing cells treated with or without rapamycin at similar time point.
either the wild-type or the F99A mutant of HIF1α. This experiment supports our hypothesis that mTOR does not directly modulate the stability of HIF1α.

It is known that VHL is required for the ubiquitin-mediated degradation of the α-subunit of HIF (35). To verify that signaling through mTOR does not modulate the stability of HIFα through a VHL-dependent mechanism, we analyzed HIF transcription within human renal carcinoma VHL−/− cells. In normoxic conditions these VHL-null cells possess heightened transcriptional activity of HIF (Fig. 7) showing that HIF is stabilized and active in these cells during high oxygen tensions. The transcriptional activity of HIF in these cells was due to the loss of VHL as the levels of HIF activity was completely ablated when VHL was transfected back. We observed that rapamycin potently reduced the levels of HIF transcription in the absence of VHL. This experiment shows that rapamycin inhibits HIF transcription independently of VHL and again supports the notion that TOR does not influence the stability of HIF1α.

Given that mTOR does not appear to affect the stability of HIF1α, the increased transcriptional activity of HIF1α by Rheb must be either caused by enhanced nuclear translocation of HIF1α or increased binding of HIF1α to other components of the transcription activation complex. First of all we examined whether the cellular distribution of the HIF1α(F99A) mutant was different to that of wild-type HIF1α. Confocal immunofluorescence studies showed that both wild-type and HIF1α(F99A) mutant was mainly nuclear (Fig. 8), as observed by co-localization with the nuclear stain, DAPI. We next examined if the HIF1α(F99A) mutant was deficient at forming transcriptional complexes. To examine this possibility, we measured the interaction of p300 with both the HIF1α(F99A) mutant and wild-type HIF1α (Fig. 9). We observed a significant loss of p300 binding to the HIF1α(F99A) mutant, which suggests that mTOR enhances HIF1α transcription through assembly of the HIF1α transcriptional machinery.

We wanted to examine the levels of HIF transcription within cells lacking TSC2. It is known that the loss of function of TSC2 potently enhances cell signaling through mTOR (8). We transfected TSC2−/− MEFs with the HIF reporter construct to measure the relative levels of HIF transcriptional activity within these cells during normoxic and hypoxic conditions (Fig. 10). We observed that rapamycin robustly inhibited the hypoxia-induced activation of HIF in cells without TSC2 by 70%. This rapamycin-sensitive level of HIF transcriptional activity during hypoxia was caused by the loss of TSC2 because transient expression of TSC2 also potently blocked the activity of HIF by 70%. This result demonstrates that the loss of function of TSC2 potently drives HIF-mediated transcription during conditions of low oxygen and is sensitive to treatments with rapamycin.

It is known that HIF-mediated gene expression is regulated through PI3K- and mTOR-dependent mechanisms (35–37). To investigate PI3K and mTOR induced activation of HIF in more detail, we treated HEK293 cells with insulin for 18 h in the presence and absence of LY294002 (to inhibit PI3K) and rapamycin (to inhibit mTOR) during hypoxia. We investigated the activity of HIF (Fig. 11A) and the amount of VEGF-A protein that was secreted by these cells (Fig. 11B). Insulin increased the...
phosphorylation of S6K1 (as observed by a mobility shift of the p70 and p85 isoforms of S6K1 to the higher phosphorylated bands) (Fig. 11C), showing that treatment with 100 nM insulin is sufficient to activate PI3K/mTOR signaling after 18 h of treatment. Treatments with either LY294002 or rapamycin were sufficient to block insulin-induced phosphorylation of S6K1 showing that we were inhibiting PI3K and mTOR-mediated signaling in these cells. It is important to note that LY294002 also inhibits mTOR. Therefore, the difference of inhibition we observe with rapamycin and LY294002 represents the level of HIF transcriptional activity (Fig. 11A) and VEGF-A secretion (Fig. 11B) that is dependent on PI3K. Upon insulin stimulation, we observed a marked increased in the HIF transcriptional activity within cells during hypoxia that was completely blocked by treatments with LY294002 and significantly impaired by rapamycin (Fig. 11A). Similarly, LY294002 blocked insulin-induced VEGF-A secretion during hypoxia while VEGF-A secretion was significantly repressed by rapamycin (Fig. 11B). These experiment shows that mTOR is necessary for the maximal activation of HIF-mediated transcription upon insulin stimulation.

**DISCUSSION**

We show that mTOR positively enhances the level of HIF-mediated transcription. Rheb-specific activation of mTOR enhanced the transcriptional activity of HIF during conditions that favored HIF1α stabilization, i.e. during hypoxia (Fig. 2A) and treatments with DMOG (Fig. 1A). This enhanced HIF activity was blocked by treatments with rapamycin showing that Rheb-induced HIF activity was caused by the heightened activity of mTOR. A potential function of mTOR would be to regulate the expression levels of HIF1α. Dogma has it that stability is the rate-limiting factor that determines the protein levels of HIF1α. However, it is important to appreciate that the regulation of HIF1α expression is multifaceted with additional inputs that function at the level of transcription and translation (30, 35–37). Indeed, it is known that the translation of HIF1α can be modulated by mTOR through cap-dependent mechanisms that is driven by eIF4E and repressed by the translation repressor, 4E-BP1 (30). The reduction of endogenous HIF1α that we observe after rapamycin treatment in the presence of DMOG or hypoxia conditions (Figs. 1B and 2B, respectively), could be accountable by the reduced rates of HIF1α protein synthesis. For added complexity, the translation of HIF1α can also be maintained through an internal ribosomal entry site (IRES). IRES-mediated translation does not require eIF4E and so confers a rapamycin-insensitive mechanism to promote HIF1α translation during times when mTOR signaling is switched off (38). Given that rapamycin did not reduce the levels of HIF1α protein in cells over-expressing Rheb (Figs. 1B and 2B), the reduction of Rheb-induced HIF transcriptional activity by rapamycin was not caused by a loss of the HIF1α protein. This rules out the possibility that mTOR modulates the stability of HIF1α and is supported by the observation that rapamycin treatment did not alter the rates of protein breakdown of HIF1α (Fig. 6). Furthermore, rapamycin potently impaired the activity of HIF in VHL-null cells (Fig. 7). VHL is required for the ubiquitin-mediated degradation of the α-subunit of HIF. Therefore, our data suggests that mTOR promotes
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The transcriptional activity of HIF1α and does not involve VHL-mediated degradation of HIF1α.

The mechanism by how mTOR modulates HIF within cells has remained elusive to date. In this manuscript, we have identified a potential FVMVL TOS motif within a previously undefined region of HIF1α. This motif is similar to well known TOS motifs found in both 4E-BP1 and S6K1, which are FEMDI and FD(L/I)DL, respectively. Raptor is thought to bind to the TOS motif within mTOR substrates. This interaction recruits these substrates to mTOR for their optimal phosphorylation. For instance, mutation of the TOS motif by alanine substitution of the phenylalanine within the TOS motif is mutated to an alanine (Fig. 4D). We observed a lower level of raptor interaction with HIF1α when compared with 4E-BP1 (Fig. 4D) and this might be caused by the differences in the cellular distribution of both HIF1α and 4E-BP1, i.e. 4E-BP1 is cytoplasmic while HIF1α is mainly nuclear. We show that mutation of the TOS motif renders HIF1α transcriptionally inactive and this TOS mutant is unresponsive to enhanced mTOR signaling when Rb is overexpressed (Fig. 5A). The HIF1α TOS mutant was predominantly nuclear (Fig. 8). These data suggest that raptor interaction with HIF1α does not influence the nuclear translocation of HIFα, a process that requires the interaction of HIFβ with HIF1α. Interestingly, mutation of the TOS motif within HIF1α, transforms HIF1α into a dominant negative mutant that inhibits endogenous HIF activity (Fig. 5A). It is possible that the HIF1α(F99A) mutant sequesters the HIFβ subunit to form inactive heterodimers, and thus competes with the interaction of HIFβ with endogenous HIF1α. Mutation of the TOS motif significantly impaired the interaction of HIF1α with the co-activator CBP/p300 (Fig. 9) and supports our view that the HIF1α(F99A) mutant is unable to form functional transcription complexes.

HIF1α is a reported phosphoprotein. It is possible that mTOR directly phosphorylates HIF1α, which is required to promote HIF1α ability to function as a transcription factor. We are currently pursuing this line of investigation to see whether there are any mTOR-dependent and rapamycin-sensitive phosphorylation sites within HIF1α. It would be interesting to examine whether a functional mTOR/raptor/LST8 complex could form within the nucleus. It has been reported that mTOR shuttles between the cytoplasm and the nucleus and that this cytoplasmic-nuclear interchange of mTOR is necessary for the mTOR-dependent phosphorylation of 4E-BP1 and S6K1 (39).

Several hamartomas syndromes that are caused through the loss of tumor suppressors have highly vascularized tumors. These include TSC, Cowden’s disease, Peutz-Jeghers syndrome and Neurofibromatosis that are caused through mutations that impair the normal tumor suppressor function of TSC1 or TSC2, PTEN, LKB1, and NF1, respectively. It is known that VEGF secretion is enhanced when there is a loss of function of either one of the TSC1, TSC2 (16), PTEN (20), LKB1 (21), or NF1 (22) tumor suppressors. This suggests that the normal function of these tumor suppressors is to repress VEGF expression and this process likely involves HIF. Lesions associated with each of these hamartomas syndromes are known to have high levels of mTOR activation (see review in Ref. 8). Interestingly, we observed high levels of HIF activity in cells lacking TSC2, which was reversed when we added back TSC2 or inhibited mTOR with rapamycin (Fig. 9). Our work suggests that the high degree of vascularization observed in tumors arising from these syndromes could be the direct consequence of high levels of mTOR activity. For instance, aberrant signaling through mTOR would enhance the activity of HIF and encourage the process of angiogenesis during hypoxia.

It is known that a negative feedback loop, which is activated by HIF, inhibits the mTOR pathway and HIF function. This feedback loop is regulated by Redd1/2 (Regulated in Development and DNA damage responses), which are also referred to as RPT801/801L and are transcriptionally up-regulated by HIF (40). The inhibition of mTOR by either Redd1 or Redd2 requires TSC2 (41) and suggests that Redd1/2 activates TSC1/2. The mechanism by how Redd1/2 signals through TSC2 is currently undefined. This negative feedback loop makes physiological sense, as it would be unfavorable for cellular HIF responses to be maintained for long periods of time. In our experiments, we override this Redd1/2 negative feedback loop by overexpressing Rheb, which is sufficient to potently enhance mTOR signaling during long term conditions of hypoxia (Fig. 2B). Cells lacking functional TSC1/TSC2 would also lack this hypoxic induced negative feedback loop through Redd1/2. The inability of Redd1/2 to activate the TSC1/TSC2 heterodimer in TSC2-null cells might be the reason why we observe high levels of HIF activity that is potently suppressed by treatments with rapamycin (Fig. 9). This study suggests that inhibition of mTOR might be a suitable strategy to treat hamartomas syndromes to repress mTOR-mediated angiogenesis through HIF.

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