Mutual Regulation of Hypoxia-Inducible Factor and Mammalian Target of Rapamycin as a Function of Oxygen Availability

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Abstract

The mammalian target of rapamycin (mTOR) regulates cellular growth and proliferation, mainly by controlling cellular translation. Most tumors show constitutive activation of the mTOR pathway. In hypoxia, mTOR is inactivated, which is believed to be part of the program of the cell to maintain energy homeostasis. However, certain proteins are believed to be preferentially translated during hypoxia via 5′ terminal oligopyrimidine tract mechanisms with controversial discussion about the involvement of the mTOR-dependent ribosomal protein S6 (rpS6). The hypoxia-inducible transcription factor (HIF) is the master regulator of hypoxic adaptation and itself strongly implicated in tumor growth. HIF is translationally regulated by mTOR. The regulatory features and the involvement of molecular oxygen itself in this regulation of HIF by mTOR are poorly understood. mTOR inhibition leads to profound attenuation of HIFα protein in the majority of primary and cancer cells studied. Under severe hypoxia, no influence of mTOR inhibitors was observed; thus, stimulation of HIFα by mTOR may only be relevant under mild hypoxia or even normoxia. HIF expression and phosphorylated rpS6 negatively correlate in experimental tumors. In cell culture, prolonged hypoxia abolishes rpS6 phosphorylation, which seems to be partly independent of the upstream p70S6 kinase. We show that hypoxic repression of rpS6 is largely dependent on HIF, implicating a negative feedback loop, which may influence cellular translational rates and metabolic homeostasis. These data implicate that the hypoxic microenvironment renders tumor cells resistant to mTOR inhibition, at least concerning hypoxic gene activation, which would add to the difficulties of other established therapeutic strategies in hypoxic cancer tissues. (Mol Cancer Res 2009;7(1):88–98)

Introduction

The transcription factor hypoxia-inducible factor (HIF) is widely recognized as the master regulator of adaptive transcriptional responses to reduced availability of oxygen. Important processes such as erythropoiesis, angiogenesis, vascular tone, glucose transport, and glycolysis are regulated in large by HIF. Therefore, HIF is implicated to be of relevance in several clinically important settings, such as ischemia and tumor growth (1). HIF is a heterodimer, consisting of a constitutive β-subunit and an oxygen regulated α-subunit. At least two oxygen-dependent HIFα subunits have been identified, HIF-1α and HIF-2α, with incomplete understanding of the individual function of these two isoforms. Regulation of the HIFα subunits occurs predominantly through oxygen-dependent hydroxylation of specific prolyl and asparagyl residues. The hydroxylated prolyl residues are recognized by an E3 ubiquitin ligase, containing the von Hippel-Lindau protein, and subsequently polyubiquitinated, leading to destruction by the proteasome. Hydroxylation of the asparagyl residue leads to inability to recruit the necessary transcriptional cofactor p300/CBP. Thus, HIFα regulation by molecular oxygen includes protein stability and transcriptional activity (2, 3).

Apart from the described oxygen-dependent regulation of HIFα subunits, there have been numerous reports of regulation by growth factors, mitogens, and oncogenic alterations. Among many others, insulin, angiotensin II, and epidermal growth factor have been shown to up-regulate HIFα in the presence of molecular oxygen (i.e., refs. 4, 5, respectively). The molecular mechanisms by which these stimuli exert their effects may be distinct; however, a predominant influence by the phosphatidylinositol 3-kinase/Akt pathway has been suggested (6). In recent years, a potentially unifying pathway for oxygen-independent HIFα activation has been identified, which could integrate many of these stimuli: the mammalian target of rapamycin (mTOR; refs. 5, 7, 8). These studies show that the

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use of highly specific inhibitors of mTOR leads to attenuation of HIF activation, yet the molecular mechanisms and the biological relevance remain unclear.

mTOR is a central controlling mechanism of cellular growth and proliferation, sensing nutritional status and mitogens and allowing progression of the cell cycle from G_1 to S phase. The molecular effects of mTOR are pleiotropic and complex and therefore incompletely understood. mTOR is a serine/threonine kinase with its classic targets ribosomal p70S6 kinase (p70S6K) and eIF4E-binding protein. Together, these downstream targets lead to direct and indirect enhancement of translation and transcription, which enables cell growth and cell cycle progression (see refs. 9, 10 for review). Pathways upstream of mTOR as well as mTOR itself are activated in cancer (11) and mTOR inhibition potently suppresses tumor growth, partly by inhibiting tumor vascularization (12).

Interestingly, HIF itself is a tumor-promoting system, in most settings (see ref. 13 for review). Thus, both systems could potentiate each other in terms of tumor aggressiveness and disease progression, which may be particularly true for renal cell carcinoma (8). These parallel effects add attraction toward blocking both pathways in tumors, which may be achieved by mTOR inhibition.

Hypoxia is a state of cellular energy deprivation. Therefore, energy-consuming processes, such as translation, are reduced, which is partly achieved by inactivation of mTOR (see ref. 14 for review). Translation of a few proteins is privileged under hypoxia. The mRNA encoding these proteins is believed to bare 5′ terminal oligopyrimidine tract (TOP) sequences in their 5′ untranslated region (UTR), enabling Cap-independent translation. Many ribosomal proteins belong to this group of proteins, as does probably HIF-1α, with its 5′TOP accounting for more than 60% of the HIF translational rate (8, 15). Yet, there is ongoing controversial discussion about the involvement of ribosomal protein S6 (rpS6) in translation of mRNAs harboring such a 5′TOP (8, 16, 17). However, considering that the Cap-independent pathway is downstream of mTOR, it is unknown if and how this pathway remains active under hypoxia. Further to this, if mTOR is required for HIFα translation, how is HIF up-regulated in hypoxia when it is most needed?

Given the important implications of an interaction of these two powerful systems and the limited information that is available so far, we aimed to analyze the effect of pharmacologic and hypoxic inhibition of mTOR and its differential effects on downstream effectors.

Results

To study the role of mTOR in the regulation of HIF, we first analyzed the efficacy of everolimus, a specific mTOR inhibitor, in HeLa cells under 10% serum and HIF stimulation with cobalt chloride ($CoCl_2$). The latter leads to stabilization of HIFα in normoxia and has been thoroughly studied in the context of mTOR inhibition with rapamycin (7). Increasing levels of everolimus (10-100 nmol/L) led to a strong and dose-dependent attenuation of HIF-1α protein levels. Interestingly, although the capability of mTOR to phosphorylate its downstream target p70S6K, with subsequent phosphorylation of rpS6, is completely abrogated at 30 nmol/L, HIF-1α levels continue to decline with rising concentrations of everolimus (Fig. 1A). Because mTOR has been shown to influence the subcellular localization of the HIF-1α homologue in *Drosophila* (6), we next evaluated the effect of everolimus on HIF-1α by immunocytochemistry. Although the decrease of HIF-1α protein in cobalt-treated cells was reproducible, HIF-1α is strictly nuclear with or without mTOR inhibition (Fig. 1B). The alternate HIFα isoform, HIF-2α, is regulated very similarly to HIF-1α in tissue culture cells (18). Regulation of HIF-2α by mTOR is also cell type dependent, albeit not corresponding to the pattern observed for HIF-1α. Hep3B cells show a strong and HeLa cells a moderate response toward everolimus treatment, whereas HKC-8 and HepG2 cells remain unaffected concerning modulation of HIF-2α protein levels. Overall, the suppression of HIF-2α by mTOR inhibition seems to be less pronounced than that of HIF-1α (Figs. 1C and 2D). Furthermore, a related mTOR inhibitor, rapamycin, shows comparable effects to everolimus on HIF-1α (Fig. 1D). Of note, frequently, 1 μmol/L of mTOR inhibitor was less effective in HIF attenuation than 100 nmol/L, which was variable in nature and not specific for rapamycin (see also Fig. 2D).

Transient transfection of a HIF reporter confirmed the suppressive effect of mTOR inhibition on the level of HIF transactivation, where $CoCl_2$ is a weaker stimulus than hypoxia (1% O_2; Fig. 2A). Further analysis of the regulation by RNase protection revealed that the effect was not dependent on mRNA expression of either HIF-1α or HIF-2α, as reported previously (8). Interestingly, the stimulated levels of the HIF target gene *carbonic anhydrase-9* (*CA-9*) showed a pronounced decrease by mTOR inhibition, whereas the *glucose transporter-1* (*Glut-1*) showed a moderate response (Fig. 2B), indicating some degree of target selectivity.

Considering the broad effect of mTOR on cellular translation and transcription, we questioned the specificity of mTOR inhibition toward HIFα. We therefore analyzed other short-lived proteins, such as p53, c-myc, and β-catenin, in parallel to HIF-1α. Under the conditions applied (6 hours of treatment), we were only able to observe the inhibitory effect for HIF-1α (Fig. 2C). Because the effect of mTOR inhibition on HIF has been previously shown in a restricted number of cells and frequently under serum reduction, we set out to study this regulation in a series of cells of different origin (at 10% serum). The majority of human cancer cell lines displayed a strong response to everolimus, including a virally transformed renal tubular cell line (HKC-8; Fig. 2D). Malignant cells very frequently have an increased activity of mTOR (11). Thus, the mTOR-HIF regulation may be confined to or most pronounced in cancer cells. We therefore included two human primary cell cultures in our analyses. The inhibitory effect of everolimus in the primary cells was comparable with the cancer cells studied, which further underlines the broad character of this interaction.

However, within the cells studied, we did find two lines, CAKI-1 and PC3, which were insensitive to everolimus under the conditions applied (Fig. 2D, *bottom two panels*). This finding was particularly unexpected in PC3 because this line was extensively studied before (7). When we reproduced the exact conditions as reported (7), namely, serum reduction to 2% or 0.1%, PC3 could be sensitized to everolimus but not CAKI-1

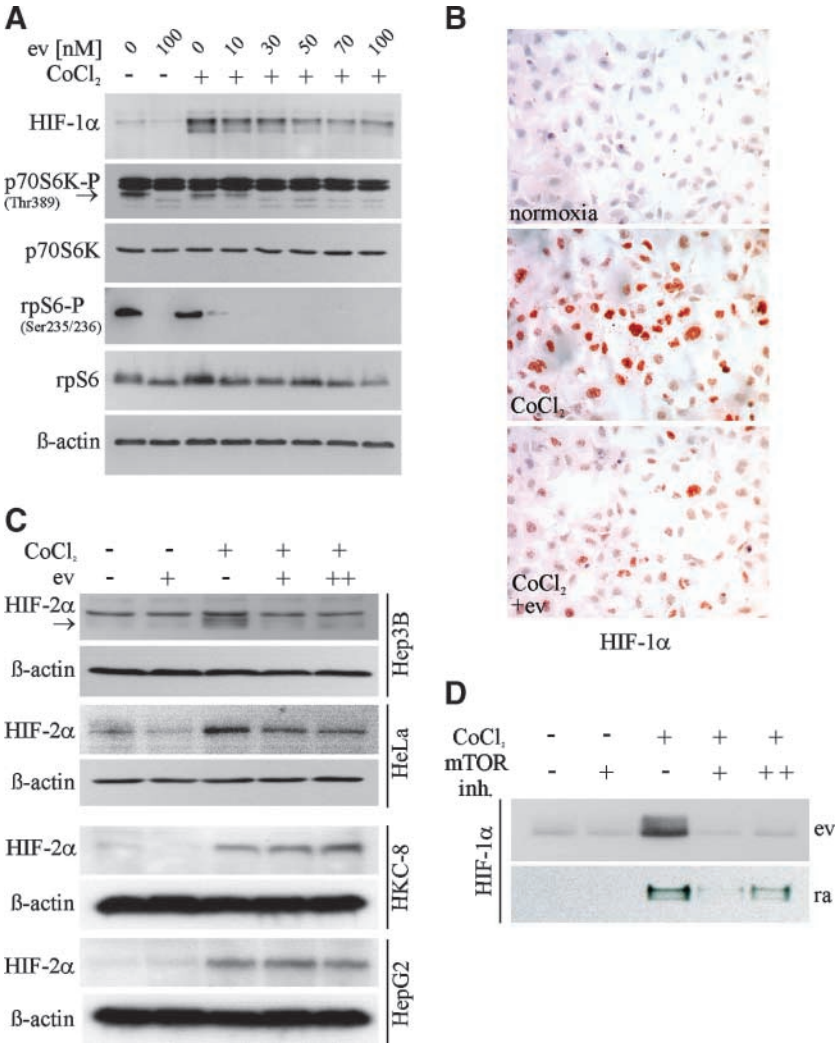

FIGURE 1. Effect of mTOR inhibition on HIFα protein expression. **A.** Immunoblots from whole-cell extracts of HeLa cells. Cells were pretreated for 45 min with everolimus (*ev*) at indicated concentrations and then exposed to the hypoxia mimetic cobalt chloride ($CoCl_2$, 100 μmol/L) for further 5 h. Attenuation of HIF-1α showed a dose-dependent response over the full range from 0 to 100 nmol/L everolimus. The activity of mTOR was assessed by the phosphorylation status of its downstream targets p70S6K (phosphorylation-specific antibody at Thr^{389}, *arrow*) and rpS6 (phosphorylation-specific antibody at $Ser^{235/236}$). Total amounts of these proteins were also measured, unselective for their phosphorylation status. β-Actin served as internal loading control. **B.** Immunocytochemistry for HIF-1α in HeLa cells, showing strictly nuclear localization of HIF-1α under stimulation with 100 μmol/L $CoCl_2$, with or without everolimus (100 nmol/L). **C.** Immunoblot analysis of HIF-2α showing a cell type–specific, differential response to mTOR inhibition. Hep3B cells show a strong and HeLa cells a moderate response to everolimus treatment, whereas HKC-8 and HepG2 cells remain unaffected by mTOR inhibition. Concentrations for everolimus were 100 nmol/L (+) or 1 μmol/L (++). **D.** Comparison of the efficacy of everolimus and rapamycin (*ra*) on HIF-1α regulation in HeLa cells.

cells (data not shown). Interestingly, in PC3, rpS6 is readily hypophosphorylated on exposure to everolimus at any serum concentration. In CAKI-1 cells, only small decreases can be observed for phosphorylated rpS6 at any serum concentration (data not shown).

Early on in our studies, we noticed that the effect of mTOR inhibition on HIF in hypoxia is more complex than under stimulation with $CoCl_2$. Cellular translation is a highly energy-consuming process. Thus, numerous checkpoints have been identified, where hypoxia leads to a decrease of translation, by mechanisms involving mTOR activity and other regulators (reviewed in ref. 14). Figure 3A compares the effect of everolimus on HIF-1α induced by iron chelation (desferrioxamine), $CoCl_2$, and severe hypoxia (0.3% O_2). Desferrioxamine and $CoCl_2$ led to a modest increase of HIF-1α levels, with a clear response to everolimus. On the other hand, hypoxia strongly up-regulates HIF-1α, with no obvious influence of mTOR inhibition. In each case, everolimus abrogated phosphorylation of the mTOR target p70S6K, which can also be seen at hypoxia. Intriguingly, in this setting, the phosphorylated rpS6 remains intact under hypoxia. To further characterize the effect of hypoxia, we compared severe (0.3%) with milder hypoxia of 1% and 3% O_2 (Fig. 3B). Under the latter conditions, less HIF-1α protein was stabilized, but now, everolimus did attenuate HIF-1α protein levels. Induction kinetics of HIFα have repeatedly shown to be strongest after a few hours, with decreasing levels at longer time points (i.e., refs. 18, 19). Recently, it has been suggested that the decrease of HIFα protein over time is part of a negative feedback loop, by up-regulation of prolyl hydoxylases (19). Increasing hypoxic exposure up to 48 hours, the induction rate of HIF-1α significantly and gradually decreased (Fig. 3C). Comparable with the situation in the former experiments, the phosphorylation of p70S6K was gone already at 6 hours of hypoxia and remained abolished at longer time points. Intriguingly, the phosphorylation of rpS6 showed a marked reduction only at 24 and 48 hours. It could therefore be speculated that the frequently observed reduction of HIFα at longer time points of hypoxia is at least partially dependent on the reduction of activated rpS6 at longer duration. These data could indicate that the influence of mTOR on HIF is greatest at mild hypoxia or even normoxia. However, it is not known whether HIF contributes to target gene expression also under normoxia. To address this question, we investigated the regulation of two

well-known HIF target genes under normoxic conditions. A HIF-1α and HIF-2α knockdown revealed HIF-1α–specific regulation of vascular endothelial growth factor (VEGF), but not Glut-1 (Fig. 3D), suggesting a role of HIF in normoxic signaling as well as target gene specificity in this context, possibly driven by mTOR.

Considering the strong repressive effects of hypoxia on mTOR activity and the phosphorylation status of its downstream targets, we were interested whether the hypoxic effects could be counterbalanced by mTOR stimulation or whether this would be a point of no return. Insulin is a strong inducer of mTOR and has frequently been shown to lead to HIF-1α accumulation in normoxia (thoroughly shown in ref. 4), which may be largely mediated by mTOR activation (6). Before studying the combined effects of hypoxia and insulin, we aimed to characterize the effect of insulin alone in our experimental setting. Because previous studies investigated the effect of insulin mostly under serum starvation (4), we reproduced the conditions in these experiments. In HeLa cells, which were studied most intensely by us, we did not have any indication of HIFα accumulation by insulin as judged by immunoblotting (data not shown; Fig. 4C). We therefore attempted to further maximize the stimulus by forced expression of p70S6K with additional stimulation by insulin (Fig. 4A). The latter led to increased abundance of the phosphorylated rpS6 and lower mobility of the transfected myc-tagged protein, which is most likely mediated through mTOR activation. However, we were not able to detect any effect on HIF-1α protein abundance. To increase the sensitivity of our observation, we next referred to a luciferase reporter assay. A very sensitive reporter assay for activation of endogenous HIFα is the "6xHRE-Luc" (20). Whereas $CoCl_2$ led to strong activation of the reporter, insulin did not (Fig. 4B, *lanes 1-4*). Furthermore, another reporter (HIF-1αProm+5′UTR-Luc), driven by the complete HIF-1α promoter with the 5′UTR and putative 5′TOP sequences included, was not induced by insulin or repressed by mTOR inhibition (Fig. 4B, *lanes 5-8*). In summary, the cellular and experimental system we were using did have a pronounced

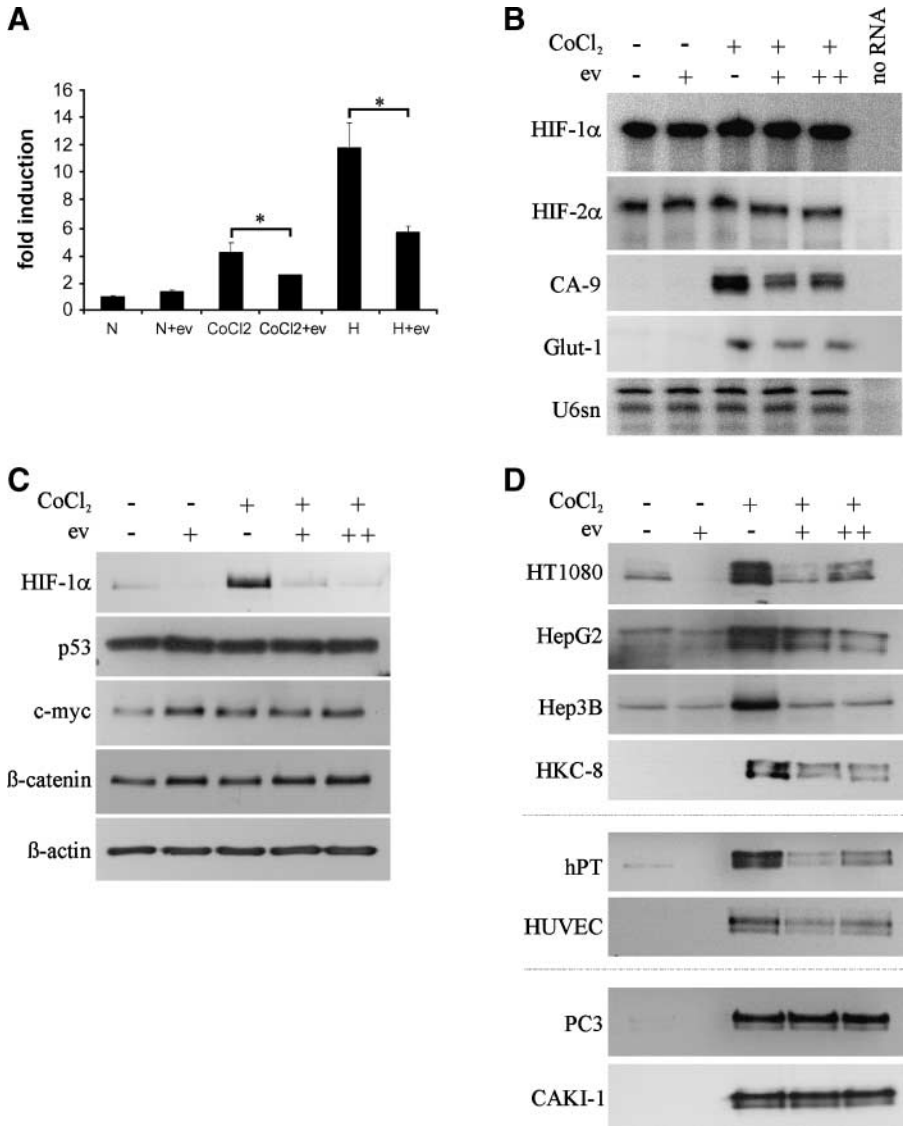

FIGURE 2. Characterization of regulatory features and specificity of mTOR inhibition. **A.** Decreased activity of the HIF reporter 6xHRE-Luc in a luciferase reporter assay after mTOR inhibition with everolimus and stimulation with $CoCl_2$ (100 μmol/L) or hypoxia (*H*; 1% O_2) for 18 h. The fold induction versus untreated, normoxic (*N*) cells is shown. **B.** RNase protection assays of HIF-1α and HIF-2α showed no modulation after mTOR inhibition, whereas the mRNA levels of the HIF targets Glut-1 and CA-9 declined at different magnitudes when treated with everolimus. Concentrations for everolimus were 100 nmol/L (+) or 1 μmol/L (++). U6sn was used as internal control. The lane "no RNA" controls for complete RNA digestion, where equal amount of radiolabeled probe was added. **C.** Immunoblot analysis of various proteins (p53, c-myc, β-catenin, and β-actin) not affected in their expression levels by mTOR inhibition, whereas HIF-1α was decreased in the same protein extracts. **D.** The cancer cell lines HT1080 (fibrosarcoma), HepG2 (liver), and Hep3B (liver) and the transformed renal tubular line HKC-8 all showed similar responses of HIF regulation to mTOR inhibition. Primary human tubular (*hPT*) cells as well as primary human umbilical vein endothelial cells (*HUVEC*) also revealed reduced HIF-1α levels after everolimus treatment. In contrast, human prostate cancer (PC3) and human renal clear cell cancer (CAKI-1) cell lines did not show a HIF response after mTOR inhibition.

effect on HIF, yet insulin signaling is neither required for nor effective in HIFα regulation. When combining hypoxia with insulin, again no effect was seen for HIF-1α protein levels. However, both the phosphorylated p70S6K and rpS6 increased on treatment with insulin, showing that the effect of hypoxia can be counterbalanced (Fig. 4C). Of note, serum starvation alone leads to a similar decrease of phosphorylation than hypoxia, explaining the moderate further effect of hypoxia in these experiments. Interestingly, not only insulin can restore the phosphorylation of downstream targets of mTOR but also reoxygenation, which indicates a dynamic cellular response to complex microenvironmental changes.

Considering the slow response of rpS6 inactivation in hypoxia, we hypothesized that this could be an active process requiring transcriptional activity. We therefore treated cells with actinomycin D simultaneously to hypoxia and investigated for the effects on the phosphorylation of rpS6. Figure 5A shows that indeed actinomycin D is able to rescue phosphorylation of rpS6 under hypoxia, which is evident at 24 hours of 1% O_2. Interestingly, this effect could also be observed for the phosphorylated p70S6K at 12 hours of hypoxia but not at 24 hours. The slight but consistent mobility changes for total rpS6 when applying actinomycin D at any experimental conditions are suggestive for a constitutive transcriptional activity to keep rpS6 in its hypophosphorylated form. Seeing that hypoxic inactivation of rpS6 requires transcriptional activity, we asked whether this could be dependent on HIF. Xenograft tumor tissues analyzed for expression of HIF-1α and the phosphorylated rpS6 in parallel further supported this notion. Consecutive sections from CAKI-1 xenografts in nude mice revealed regional staining patterns for both proteins, where microenvironmental hypoxia would be presumed to be the major stimulus for regional stabilization of HIF-1α. Of note, the areas of strong HIF-1α expression were consistently negative for phosphorylated rpS6 (Fig. 5B). In summary, these data are strongly suggestive that HIF could be involved in hypoxic rpS6 inactivation.

For this reason, we continued to investigate whether HIF had a direct role in phosphorylation of rpS6. ARNT-competent (C1C7) and ARNT-deficient (C4) Hepa-1 cells were exposed to 0, 1, 4, 6, and 24 hours of hypoxia (1% O_2; Fig. 6A). HIF-1α accumulated gradually in both cell lines up to 24 hours in a comparable fashion and was able to transactivate BNIP3, a hypoxia-inducible member of the Bcl-2 family of apoptotic regulators, in Hepa-1 C1C7, yet not in Hepa-1 C4 cells. p70S6K phosphorylation was completely abolished at 1 hour of hypoxia and remained shut off throughout the entire time course, whereas rpS6 phosphorylation analyzed showed a differential expression pattern in the two cell lines. Hypoxic exposure of Hepa-1 C4 cells resulted in hypophosphorylation of rpS6 to a much lesser degree than in wild-type cells, indicating a possible involvement of HIF in this regulation.

To confirm the involvement of HIF in this regulation, we did an ARNT knockdown in HeLa cells under both normoxic (24 hours, 21% O_2) and hypoxic (24 hours, 1% O_2) conditions. The knockdown led to a strong rescue of phosphorylation of

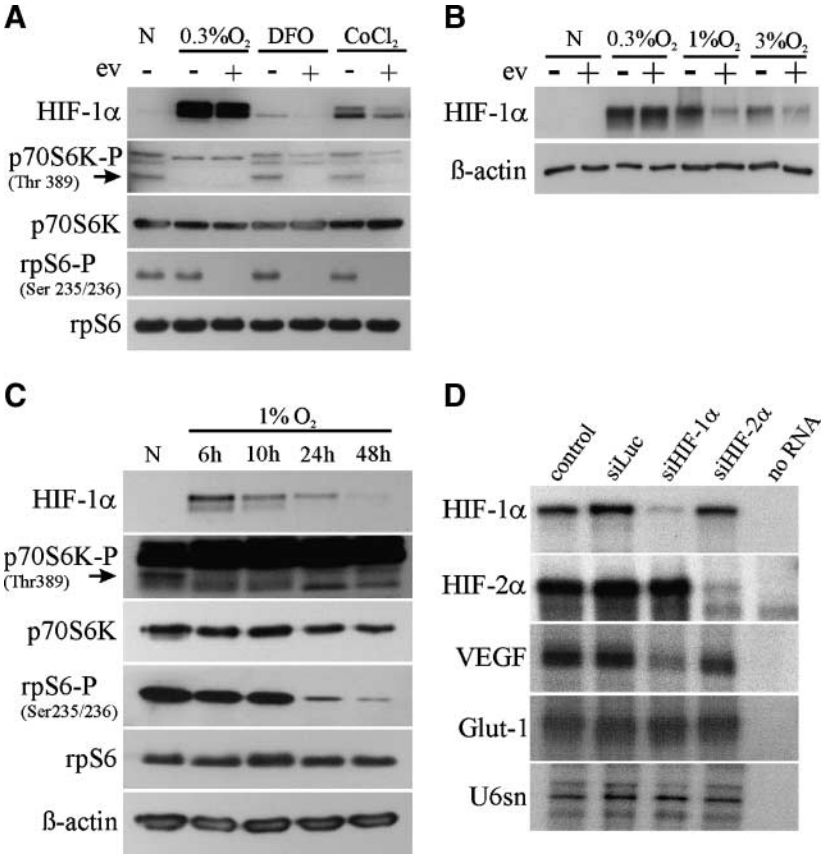

FIGURE 3. HIF regulation by mTOR is dependent on oxygen concentration. **A.** Immunoblot analysis of HIF-1α, phosphorylated p70S6K (*p70S6K-P*; at Thr^{389}), total p70S6K, phosphorylated rpS6 (*rpS6-P*; at $Ser^{235/236}$), total rpS6, and β-actin from HeLa whole-cell extracts. Cells were preincubated for 45 min with 100 nmol/L everolimus and then exposed to 0.3% O_2, the iron chelator desferrioxamine (*DFO*; 100 μmol/L), or $CoCl_2$ (100 μmol/L) for 5 h. Whereas a clear effect of HIF-1α attenuation was seen under desferrioxamine and $CoCl_2$ induction, no obvious response was visible under severe hypoxia. Effective mTOR blockage is shown by inhibition of p70S6K and rpS6 phosphorylation. Interestingly, p70S6K phosphorylation was completely abolished under hypoxia, whereas phosphorylated rpS6 remained intact. **B.** Immunoblot analysis of everolimus sensitivity after stabilization of HIF with increasing levels of O_2 (0.3%, 1%, and 3%). A response can only be seen in HeLa cells treated with 1% or 3% O_2 compared with HIF-1α induction under 0.3% O_2. **C.** Time courses of hypoxia (1%) up to 48 h reveal that HIF-1α reaches peak expression after 6 h and steadily declines up to 48 h. p70S6K phosphorylation disappears after 6 h of hypoxia, whereas rpS6 phosphorylation decreases at a comparable rate to HIF-1α. β-Actin serves as a loading control. **D.** RNase protection assays of samples treated with small interfering RNA for HIF-1α and HIF-2α under normoxic conditions in HeLa cells. Successful and specific knockdown of the two HIFα isoforms is shown by the respective mRNA levels. VEGF mRNA level is clearly decreased according to HIF-1α knockdown, indicating some degree of HIF-1α activity in normoxia. However, Glut-1 shows no regulation.

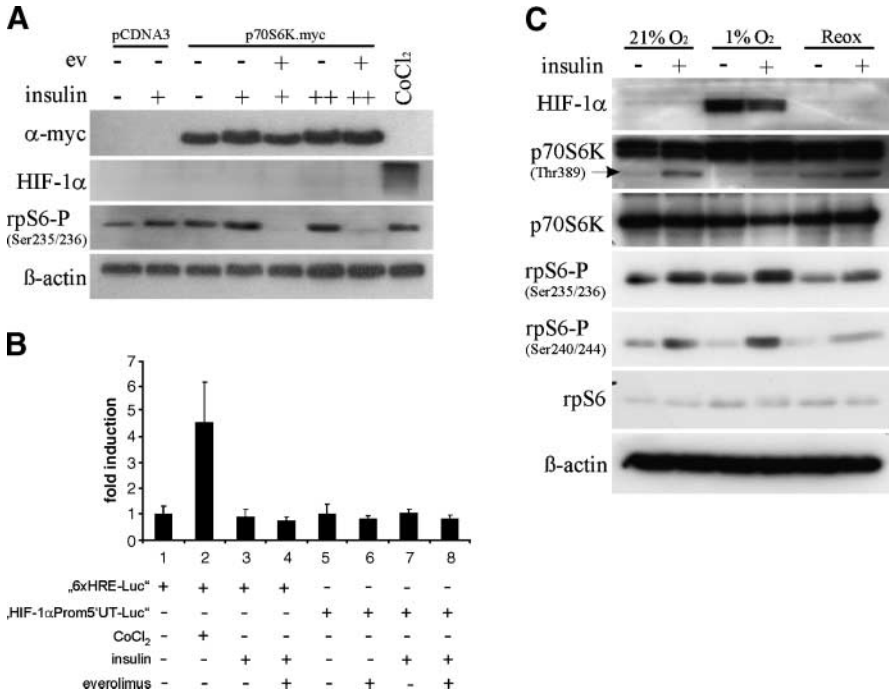

FIGURE 4. Influence of insulin and oxygen on HIF and phosphorylated rpS6. HeLa cells were serum starved at 1% FCS for 18 h to restrict the basal influence of mTOR. **A.** Overexpression of the myc-tagged p70S6K (p70S6K.myc) in HeLa cells failed to accumulate HIF-1α protein even when induced via insulin at two different concentrations (where "+" indicates 100 nmol/L and "++" indicates 500 nmol/L). Stabilization of endogenous HIF-1α is shown by addition of $CoCl_2$ (100 μmol/L). Principal insulin sensitivity of the mTOR/S6K/rpS6 pathway is implicated by compatible changes in migration of p70S6K.myc, which most likely displays the changes in phosphorylation. The mobility of the exogenous p70S6K.myc is slightly retarded when insulin is added (and lost again when mTOR is inhibited), indicating phosphorylation and activation of the transgene. Phosphorylation of rpS6 is abolished by mTOR inhibition. β-Actin serves as an internal loading control. **B.** Insulin (100 nmol/L) failed to induce 6xHRE-Luc and a reporter containing the complete HIF-1α promoter, including the 5′UTR and putative 5′TOP sequences linked to luciferase (HIF-1αProm+5′UTR-Luc), in HeLa cells. **C.** Hypoxic inhibition of rpS6 phosphorylation can be reversed by activating phosphatidylinositol 3-kinase/mTOR signaling via addition of insulin (100 nmol/L). Cells were subjected to insulin and/or hypoxia (1% O_2) for 2 h. Reoxygenation to ambient air was for 1 further hour.

rpS6 under hypoxic conditions, which was true for both independent small interfering RNAs (Fig. 6B). We also noticed a (re)phosphorylation of p70S6K after the ARNT knockdown under hypoxia, which could be, at least in part, due to inhibition of HIF activity upstream of mTOR. HIF-1α, total p70S6K, and total rpS6 levels remained unaffected.

Because ARNT is also involved in other pathways than HIF, we wished to confirm that the observed effects on the phosphorylation of rpS6 were indeed mediated by HIF. For this purpose, HeLa cells were subjected to a HIF-1α, HIF-2α, and ARNT knockdown and subsequently exposed to hypoxia for 6 and 24 hours. Immunoblotting was done and showed that p70S6K phosphorylation was completely abolished at 6 and 24 hours of hypoxia, with no apparent influence of either HIF-1α or HIF-2α knockdown, whereas ARNT knockdown again rescued p70S6K from hypophosphorylation (Fig. 6C). rpS6 phosphorylation declined when exposed to hypoxia, yet was rescued, at least in part, by HIF-1α and HIF-2α knockdown at 6 hours of hypoxia and to a lesser degree at 24 hours of hypoxic exposure, which led to an almost complete inhibition of rpS6 phosphorylation per se. Importantly, the ARNT knockdown was more effective in rescuing phosphorylation of rpS6 and p70S6K. This could indicate that the ARNT knockdown more potently takes effect on upstream pathways of mTOR (i.e., REDD1 and BNIP3) than the HIFα knockdown.

In the past, there have been reports that the activity of rpS6 is not strictly a function of upstream phosphorylation processes mediated dominantly by p70S6K, yet can also be controlled by active dephosphorylation downstream of mTOR. Two serine/threonine phosphatases (PP2A and PP1) are being discussed as possible candidates in this setting (21). By generally blocking serine/threonine phosphatases through application of NaF, we can assess their role in the regulation of rpS6. Because NaF exhibits toxic effects on vital cells after as little as 2 hours of exposure, we switched to a cell-free system, being able to analyze enzymatic reactions and simultaneously excluding HIF activity. NaF application and exposure of the lysates to 6 or 24 hours of hypoxia clearly enhanced rpS6 phosphorylation, showing the ability of this group of phosphatases to control rpS6 activity and implicating a constitutive influence (Fig. 6D). Interestingly, hypoxia did not alter the level of phosphorylated rpS6, neither with nor without NaF. Because HIF is not activated in this cell-free system despite of hypoxic exposure (data not shown), this may be seen as further correlative evidence of its involvement in rpS6 (de)activation.

Discussion

The findings pointing toward regulation of HIF by mTOR aroused our interest for several reasons. First, it offered a

plausible solution to several rather controversial data, showing that HIFα could be up-regulated in numerous ways besides hypoxia, as discussed above. Second, highly specific inhibitors of mTOR are available and already in clinical use. Thus, the possibility of experimentally (perhaps also clinically) exploiting this interaction was given. Finally, considering the broad importance of both systems, it seemed likely that processes of biological relevance would be influenced by the interaction. Among these, tumor growth may account to the most important ones. Putatively being able to target both systems simultaneously seems attractive and has been suggested for renal cell and prostate carcinoma (8, 22). However, considering the broadness of effects of mTOR and HIF, the likelihood of unwanted side effects is high. Thus, we aimed to further characterize the regulation of HIF by mTOR.

Although the evidence for HIF in enhancing tumor growth is not fully consistent, the vast majority of studies have shown a tumor-promoting effect. Many of the target genes of HIF have been independently identified as important tumor genes, such as *VEGF, glycolytic enzymes*, and *CA-9*. Thereby, inhibition of HIF and subsequently these targets would be expected to have a beneficial effect, in most settings. Particularly, tumor angiogenesis is thought to be an important mechanism of tumor growth, which is strongly influenced by VEGF (13) and which is one of the more important pathways of growth restriction by mTOR inhibitors (12). Interestingly, it has been shown that VEGF is under the influence of upstream regulators, which is only partially mTOR dependent (23). Thus, simultaneously blocking mTOR and HIF could prove to be more effective in tumor control. However, considering some of the conflicting data, where genetic inactivation of HIF resulted in reduced tumor growth (24, 25), application of HIF inhibitors may indeed be deleterious. In the absence of further knowledge of the molecular determinants, mTOR inhibitors may be safer to use because they should have a dual effect on tumor cell proliferation and possibly HIF inhibition. We have shown that the effect of mTOR inhibitors on HIF can be dependent on several intrinsic and extrinsic determinants (Fig. 2). These circumstances will make the response of an individual tumor hard to predict. Furthermore, microenvironmental changes within any given tumor could cause a heterogeneous pattern of sensitive and insensitive cells.

The seemingly parallel pathways of mTOR and HIF are conflicted by an apparent paradox. HIF is regarded to be of relevance under reduced oxygen tensions, which is the classic condition inducing HIF. Under hypoxia, HIFα hydroxylation and subsequent polyubiquitination are disabled, leading to protein accumulation and increased transcriptional activity. However, mTOR activity and thereby cellular translation are reduced by hypoxia (26), which is considered to be one of the strategies of the cell to save energy. In our studies, we reveal distinct effects of hypoxia on downstream targets of mTOR, leading to hypophosphorylation of p70S6K and rpS6 (Fig. 3). Rather unexpectedly, we found this effect to be largely dependent on HIF itself, which would argue for a negative feedback loop in repression of cellular translation in hypoxia. Several upstream regulators of mTOR have been described to be influenced by hypoxia: REDD1 (27), LKB1 and AMPK (28), BNIP3 (29), and PML (30); some of which are directly dependent on HIF. As previously described by Liu et al. (28) and Arsham et al. (31), a certain extent of HIF-dependent and HIF-independent influences is to be expected in hypoxic regulation of mTOR. We were, however, surprised by the magnitude of HIF involvement on rpS6 phosphorylation in our hands, which seems to be influenced independently of mTOR signaling. The effect of upstream modulators of mTOR activity would be expected to take place on p70S6K as well as rpS6. However, we noticed a discrepancy between the responses of p70S6K and rpS6. Whereas pharmacologic inhibition eradicates phosphorylation

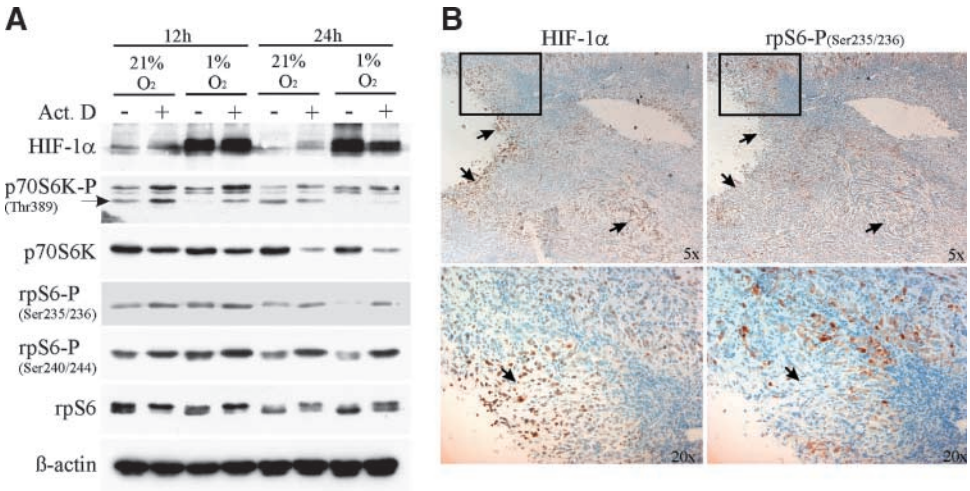

FIGURE 5. rpS6 regulation by hypoxia requires transcriptional activity and correlates to HIF-1α expression in a CAKI-1 xenograft tumor. **A.** Inhibition of transcription by actinomycin D (2 μg/mL) prevents hypophosphorylation of rpS6 by long-term (24 h) hypoxic exposure, at least partially independent of p70S6K. Immunoblots show lysates of HeLa cells that were subjected to actinomycin D for 45 min and then exposed to 21% or 1% O_2 for 12 or 24 h. **B.** Immunohistochemical analysis of HIF-1α and phosphorylated rpS6 ($Ser^{235/236}$) in CAKI-1 xenograft sections. Cells (5×10^6) were injected subcutaneously into nude BALB/c mice and grown for 6 wk. Arrows indicate regions where HIF-1α (nuclear staining) is expressed due to hypoxic microenvironment, yet no or little phosphorylated rpS6 ($Ser^{235/236}$) (cytoplasmic staining) expression is detectable. Magnifications, ×5 or ×20.

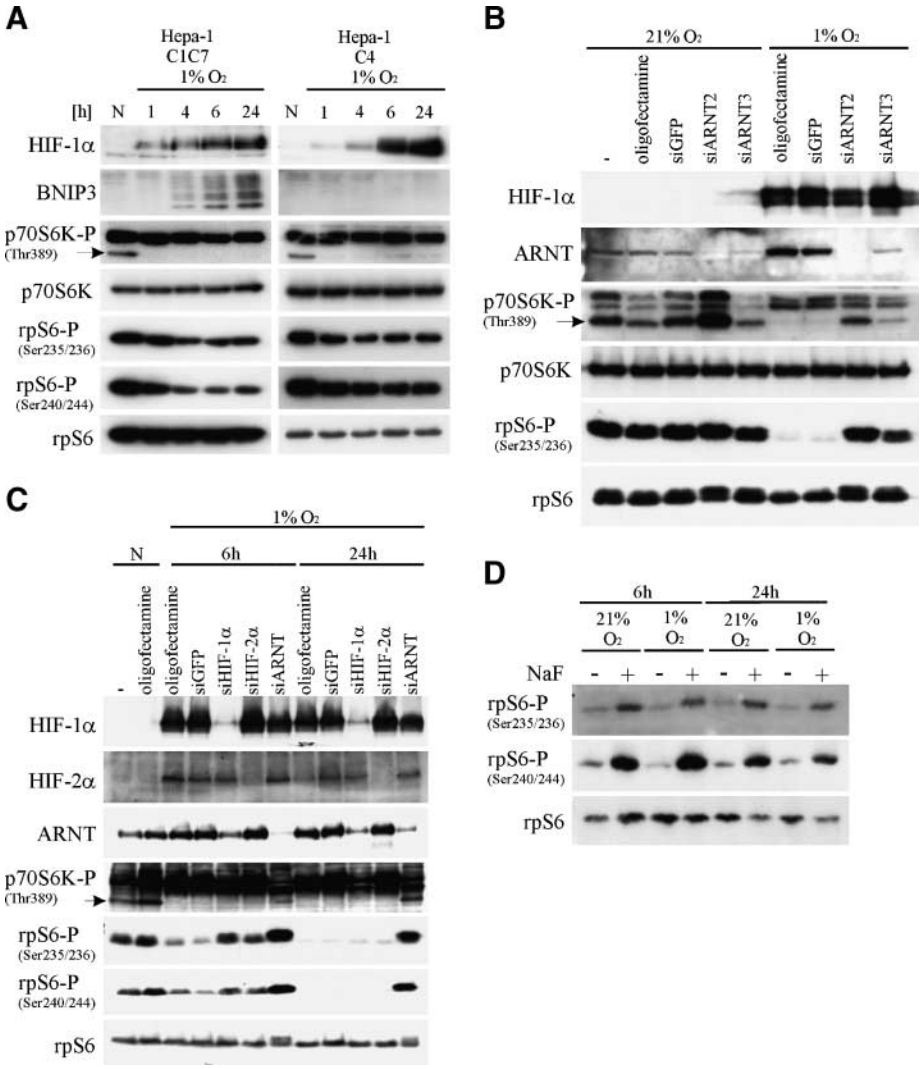

FIGURE 6. Involvement of HIF in regulation of rpS6. **A.** Hepa-1 cells (either C1C7, wild-type, or C4, ARNT deficient) were exposed to 0, 1, 4, 6, or 24 h of hypoxia (1% O_2) and then lysed. Whole-cell extracts were then subjected to immunoblot analysis. Phosphorylation of either $Ser^{235/236}$ or $Ser^{240/244}$ decreases quicker and to a stronger degree in Hepa-1 C1C7 cells, whereas p70S6K is immediately and sustainably hypophosphorylated by hypoxia in both cell lines. **B.** ARNT knockdown was done in HeLa cells and exposed either to 21% or 1% O_2. Immunoblotting was done and revealed a markedly decreased rpS6 phosphorylation on hypoxic exposure and a massive recovery on ARNT knockdown. p70S6K phosphorylation also recovered after ARNT knockdown. This was true for two individual small interfering RNAs. **C.** HIF-1α, HIF-2α, and ARNT knockdown was done in HeLa cells, which were then exposed to either 6 or 24 h of hypoxia (1% O_2). As expected, rpS6 phosphorylation ($Ser^{235/236}$ and $Ser^{240/244}$) levels were decreased by hypoxia. Knockdown of HIF-1α and HIF-2α causes a partial recovery, independent of p70S6K phosphorylation status. In contrast, ARNT knockdown leads to a recovery of rpS6 phosphorylation in a p70S6K-dependent fashion. **D.** HeLa cells were lysed and lysates were supplemented with or without NaF (50 mmol/L) and then subjected to normoxic (21% O_2) or hypoxic (1% O_2) conditions for 6 or 24 h. Inhibiting serine/threonine phosphatases by NaF leads to an accumulation of phosphorylated rpS6, independently of HIF and hypoxia.

of both proteins in a parallel fashion, hypoxia very quickly leads to dephosphorylation of p70S6K, yet rpS6 remains phosphorylated for over 10 hours (Fig. 3C). The delay in rpS6 hypophosphorylation as well as the dependency on transcriptional activity (Fig. 5A) already point toward an involvement of HIF. Consequently, hypoxic exposure, following knockdown of HIF-1α, HIF-2α, or ARNT, had distinct effects on the phosphorylation status of rpS6, with differential impact on p70S6K (Fig. 6A-C). These findings could implicate a direct effect of HIF on dephosphorylation activity, which is supported by the results of blocking serine/threonine phosphatase activity (Fig. 6D). It is tempting to speculate that a phosphatase is a transcriptional target of HIF, which will be explored in future studies.

Figure 7 attempts to illustrate our findings of complex regulatory pathways between HIF and mTOR, which are mutual and strongly dependent on the level of oxygen availability. Under normoxia or mild hypoxia, HIF would be synthesized to some extent under the influence of mTOR (*left*). In severe hypoxia, HIF protein is abundant, mainly by inactivation of its degradation apparatus (*right*). Hypoxic exposure leads to a general reduction of the cellular translation rate for most genes, serving as an energy-saving mechanism to the cell. Nevertheless, translation for certain genes is upheld even in hypoxia, which is also true for HIF. After continuous hypoxia, a negative feedback loop is enforced, which is to some extent a function of HIF itself. The effect of HIF on rpS6 dephosphorylation could clearly be mediated via the transactivation of genes such as *REDD1* or *BNIP3* and their inhibitory effect on mTOR; our data now also suggest that HIF may be involved in direct rpS6 regulatory processes downstream of mTOR and p70S6K.

Another putative escape mechanism from hypoxic translational repression next to 5′TOP-driven mechanisms is internal ribosome entry sites (IRES). It has been shown previously that HIF-1α mRNA contains a functional IRES (32). However, recent data indicate that this IRES contributes only to a very minor degree in HIF-1α translation (33).

The profound effects on p70S6K and rpS6 by hypoxia and HIF that we observed in our study could be of particular interest because recent data have pointed toward other functions than

cellular translation. Genetic deletion of both isoforms of p70S6K in mice leads to diminished embryonic and postnatal growth with perinatal lethality (16). Interestingly, single knockout of the p70S6K1 isoform was sufficient to suppress embryonic growth and lead to hypoinsulinemia and mildly impaired glucose intolerance (34, 35). Mutation of all known phosphorylatable serine residues of rpS6 in mice found little evidence for an involvement in 5′TOP translation. However, a functional role was defined for regulation of cellular size and proliferation (17) in derived primary fibroblasts. Of note, the same study showed that these mice develop hypoinsulinemia and impaired glucose tolerance. Because HIF is strongly involved in maintenance of glucose and energy metabolism in hypoxia (36, 37), these data could expand the role of HIF in cellular metabolic homeostasis.

Solid tumors frequently display severe hypoxia, which is of clinical relevance, because hypoxic tumors are believed to be more resistant to therapeutic attempts of chemotherapy and radiation (recently reviewed in ref. 38). We have shown that HIF is not influenced by mTOR in severe hypoxia. Therefore, if the effect on HIF contributes significantly to the tumor-suppressive effects of mTOR inhibitors, no benefit of these substances can be expected in these areas, which further complicates the treatment of hypoxic tumors.

In summary, we have shown that mTOR has the potential of stimulating HIF in a large panel of cells, of both malignant and primary origin. However, specific influences, such as cell type, amount of serum, and degree of hypoxia, considerably interfere with this response. Under severe hypoxia, HIF seems to be operating independently of mTOR. We observed clear hypophosphorylation of the downstream targets p70S6K and rpS6 under hypoxia, which was, strongly, dependent on HIF. Thus, the regulation of HIF and mTOR seems to go both ways. Our data suggest a direct influence of HIF on dephosphorylation of rpS6, which could involve specific phosphatases and should be addressed in further studies.

Materials and Methods

Cell Culture

HeLa, PC3, CAKI-1, HepG2, and Hep3B cells were cultured in DMEM (PAN) containing 1.0 g/L glucose, 10% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin; HT1080 cells were cultured in DMEM containing 4.0 g/L glucose; HKC-8 cells (kindly provided by Dr. L. Racusen, Johns Hopkins Medical Institutions, Baltimore, MD) were cultured in DMEM/Ham's F12 (1:1) complemented with 10% FCS, 2 mmol/L L-glutamine, 10 μg/mL insulin, 5 μg/mL transferrin, 100 units penicillin, and 100 μg streptomycin. Human primary tubular cells were cultured in DMEM/Ham's F12 (1:1), additionally complemented with 10 ng/mL EGF, 36 ng/mL hydrocortison, and 4 pg/mL T_3. Human primary tubular and human umbilical vein endothelial cells were generated as previously described (39, 40). The collection and use of the human primary

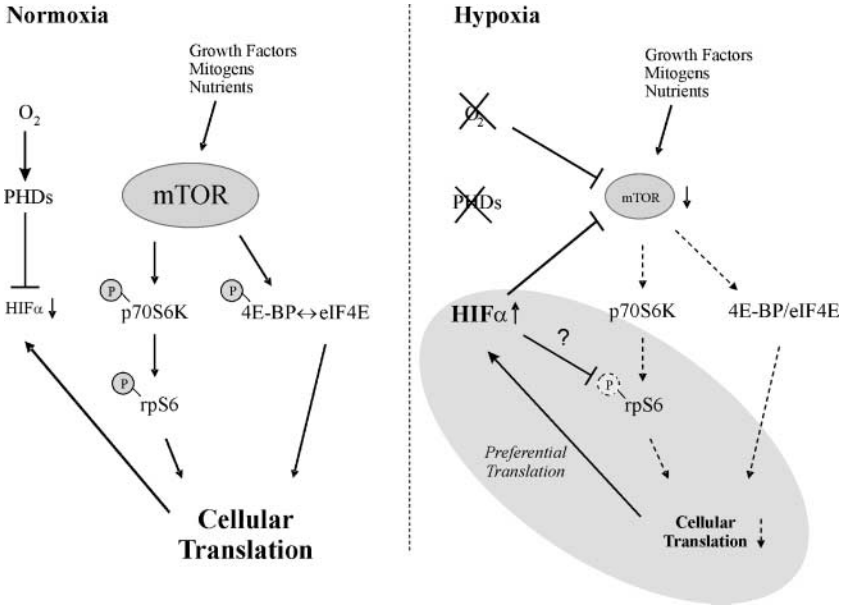

FIGURE 7. Model of the complex interactions between HIF and mTOR in dependency of oxygen availability. In the presence of molecular oxygen (normoxia, *left*), HIFα subunits are efficiently hydroxylated by prolyl hydroxylases (*PHDs*) and directed toward proteasomal degradation, with only marginal HIFα levels detectable. Growth factors, mitogens, and nutrients activate mTOR, which phosphorylates and thereby activates downstream effectors [p70S6K and 4E-binding protein (*4E-BP*)] involved in cellular translation. Phosphorylation of 4E-binding protein leads to dissociation and activation of eIF4E, which mediates classic Cap-dependent translation. Phosphorylation of p70S6K leads to activation of rpS6, which is controversially discussed to be involved in translation of 5′TOP-dependent mRNAs. Because HIF-1α is believed to belong to the 5′TOP mRNA family, low levels of HIFα protein in normoxia or hypoxia could be upheld by mTOR. In hypoxia (*right*), the prolyl hydroxylases remain inactive and consequently HIFα subunits accumulate. mTOR is potently inactivated, which leads to diminished cellular translation and rapid hypophosphorylation of p70S6K, which is in part a function of HIF itself. The rpS6 is hypophosphorylated in a much slower manner, which may enable a certain degree of preferential translation under hypoxia. However, prolonged hypoxia also inactivates rpS6, probably in part driven by HIF and representing a negative feedback loop. The gray oval highlights the assumed processes involved during hypoxic regulation of rpS6. Dotted arrows indicate a reduced activity in hypoxic compared with normoxic conditions.

cells was approved by the local ethic committee and written informed consent was obtained from each patient. Cells were incubated at 37°C at 5% CO_2 in humidified air. Hypoxic exposure took place in an "invivo400" hypoxic workbench (Ruskinn Technology Ltd.) with 1% oxygen, 5% CO_2, and balance N_2.

Protein Extraction and Immunoblotting

For protein extraction, cells were seeded 24 h before experiments and then pretreated for 45 min with mTOR inhibitors [everolimus (Novartis Institutes for Biomedical Research) and rapamycin (Sigma-Aldrich)] and subsequently subjected to 5 h of stimulation (conditions essentially as established in ref. 7). Unless indicated, all experiments were done with 10% serum. Cells were washed twice with PBS and homogenized into extraction buffer [8 mol/L urea, 10% glycerol, 1% SDS, 10 mmol/L Tris-HCl (pH 6.8), protease inhibitor Complete (Roche), 1 mmol/L sodium vanadate, 1 mmol/L AEBSF]. Equal amounts of protein were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and stained with antibodies as detailed in Supplementary Table S1. Signals were visualized using enhanced chemiluminescence systems from GE Healthcare. For the cell-free system, cells were lysed in 150 mmol/L NaCl, 5 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 7.4), and 1% Triton X-100. NaF was used at 50 mmol/L and actinomycin D at 2 μg/mL. Unless otherwise stated, chemicals and reagents were purchased from Sigma-Aldrich.

RNase Protection Assays

Total RNA was extracted from cell culture using RNAzol B (Biogenesis) according to the manufacturer's instructions. ^{32}P-labeled riboprobes were synthesized using SP6 or T7 RNA polymerase (Roche). RNase protection assays were done essentially as described previously (18). RNA was hybridized to radiolabeled riboprobes, which are then protected from RNase digestion. Details of the different riboprobes used, as well as amounts of total RNA, are shown in Supplementary Table S2. U6sn served as an internal control. Samples were separated on a polyacrylamide gel and signals were quantified using a PhosphoImager (Fujix BAS 2000, Fuji).

Immunohistochemistry and Cytochemistry

Immunohistochemical staining was done using rabbit polyclonal HIF-1α (Cayman Chemical) antibody as described earlier (41, 42). For phosphorylated rpS6 staining, rabbit polyclonal Ser$^{235/236}$ antibody from Cell Signaling was used. Sections were counterstained with aqueous hematoxylin. For immunocytochemical analysis, HeLa cells were cultivated on glass slides and fixed in 3% paraformaldehyde for 5 min.

Transient Transfections and Reporter and Knockdown Assays

Plasmids pGL3PGK6TKp (6xHRE-Luc; ref. 20), HIF-1αProm+5′UTR-Luc reporter (AF050115, nucleotides 4-931), S6K.myc, and pCMV-β-galactosidase (Stratagene) were transfected using TransFectin (Bio-Rad). pCMV-β-galactosidase was used to normalize reporter assays. Luciferase activity of cell lysates was determined using the Luciferase Assay Reagent (Promega). Unless indicated, data given are mean of three independent experiments with four replicates per sample ± SD. For small interfering RNA experiments, cells were seeded 24 h before transfection and knockdown was done as reported previously (43). The following sequences were used: HIF-1α, 5′-GCCACUUCGAAGUAGUGCUdTdT (sense); HIF-2α, 5′-GCGACAGCUGGAGUAUGAAdTdT (sense); siARNT2 (Qiagen); and siARNT3 (Qiagen).

Animal Experiments

The study was approved by the institutional review board for the care of animal subjects and was done in accordance with NIH guidelines. CAKI-1 cells (5×10^6) were injected subcutaneously into BALB/c nude mice. Xenograft tumors were immersion fixed in 3% paraformaldehyde for immunohistochemical analysis.

Disclosure of Potential Conflicts of Interest

The authors were supported by a small research grant from Novartis Pharma GmbH, Germany.

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References

1. Wenger RH. Cellular adaptation to hypoxia: O_2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O_2-regulated gene expression. FASEB J 2002;16:1151–62.

2. Schofield CJ, Ratcliffe PJ. Oxygen sensing by HIF hydroxylases. Nat Rev Mol Cell Biol 2004;5:343–54.

3. Kaelin WG, Jr. The von Hippel-Lindau tumor suppressor protein and clear cell renal carcinoma. Clin Cancer Res 2007;13:680–4s.

4. Treins C, Giorgetti-Peraldi S, Murdaca J, Semenza GL, Van Obberghen E. Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. J Biol Chem 2002;277:27975–81.

5. Zhong H, Chiles K, Feldser D, et al. Modulation of hypoxia-inducible factor 1α expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res 2000;60:1541–5.

6. Dekanty A, Lavista-Llanos S, Irisarri M, Oldham S, Wappner P. The insulin-PI3K/TOR pathway induces a HIF-dependent transcriptional response in *Drosophila* by promoting nuclear localization of HIF-α/Sima. J Cell Sci 2005;118:5431–41.

7. Hudson CC, Liu M, Chiang GG, et al. Regulation of hypoxia-inducible factor 1α expression and function by the mammalian target of rapamycin. Mol Cell Biol 2002;22:7004–14.

8. Thomas GV, Tran C, Mellinghoff IK, et al. Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer. Nat Med 2006;12:122–7.

9. Fingar DC, Blenis J. Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene 2004;23:3151–71.

10. Proud CG. The multifaceted role of mTOR in cellular stress responses. DNA Repair (Amst) 2004;3:927–34.

11. Bjornsti MA, Houghton PJ. The TOR pathway: a target for cancer therapy. Nat Rev Cancer 2004;4:335–48.

12. Guba M, von Breitenbuch P, Steinbauer M, et al. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat Med 2002;8:128–35.

13. Pouyssegur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature 2006;441:437–43.

14. Koritzinsky M, Wouters BG. Hypoxia and regulation of messenger RNA translation. Methods Enzymol 2007;435:247–73.

15. Thomas JD, Johannes GJ. Identification of mRNAs that continue to associate with polysomes during hypoxia. RNA 2007;13:1116–31.

16. Pende M, Um SH, Mieulet V, et al. S6K1(−/−)/S6K2(−/−) mice exhibit perinatal lethality and rapamycin-sensitive 5′-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. Mol Cell Biol 2004;24:3112–24.

17. Ruvinsky I, Sharon N, Lerer T, et al. Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. Genes Dev 2005;19:2199–211.

18. Wiesener MS, Turley H, Allen WE, et al. Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1α. Blood 1998;92:2260–8.

19. Stiehl DP, Wirthner R, Koditz J, Spielmann P, Camenisch G, Wenger RH. Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system. J Biol Chem 2006; 281:23482–91.

20. Vaux EC, Wood SM, Cockman ME, et al. Selection of mutant CHO cells with constitutive activation of the HIF system and inactivation of the von Hippel-Lindau tumor suppressor. J Biol Chem 2001;276:44323–30.

21. Williams AJ, Werner-Fraczek J, Chang IF, Bailey-Serres J. Regulated phosphorylation of 40S ribosomal protein S6 in root tips of maize. Plant Physiol 2003;132:2086–97.

22. Majumder PK, Febbo PG, Bikoff R, et al. mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. Nat Med 2004;10:594–601.

23. Brugarolas JB, Vazquez F, Reddy A, Sellers WR, Kaelin WG, Jr. TSC2 regulates VEGF through mTOR-dependent and -independent pathways. Cancer Cell 2003;4:147–58.

24. Blouw B, Song H, Tihan T, et al. The hypoxic response of tumors is dependent on their microenvironment. Cancer Cell 2003;4:133–46.

25. Carmeliet P, Dor Y, Herbert JM, et al. Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 1998;394:485–90.

26. van den Beucken T, Koritzinsky M, Wouters BG. Translational control of gene expression during hypoxia. Cancer Biol Ther 2006;5:749–55.

27. Brugarolas J, Lei K, Hurley RL, et al. Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. Genes Dev 2004;18:2893–904.

28. Liu L, Cash TP, Jones RG, Keith B, Thompson CB, Simon MC. Hypoxia-induced energy stress regulates mRNA translation and cell growth. Mol Cell 2006;21:521–31.

29. Li Y, Wang Y, Kim E, et al. Bnip3 mediates the hypoxia-induced inhibition on mammalian target of rapamycin by interacting with Rheb. J Biol Chem 2007; 282:35803–13.

30. Bernardi R, Guernah I, Jin D, et al. PML inhibits HIF-1α translation and neoangiogenesis through repression of mTOR. Nature 2006;442:779–85.

31. Arsham AM, Howell JJ, Simon MC. A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. J Biol Chem 2003;278:29655–60.

32. Lang KJ, Kappel A, Goodall GJ. Hypoxia-inducible factor-1α mRNA contains an internal ribosome entry site that allows efficient translation during normoxia and hypoxia. Mol Biol Cell 2002;13:1792–801.

33. Young RM, Wang SJ, Gordan JD, Ji X, Liebhaber SA, Simon MC. Hypoxia-mediated selective mRNA translation by an internal ribosome entry site-independent mechanism. J Biol Chem 2008;283:16309–19.

34. Shima H, Pende M, Chen Y, Fumagalli S, Thomas G, Kozma SC. Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. EMBO J 1998;17:6649–59.

35. Pende M, Kozma SC, Jaquet M, et al. Hypoinsulinaemia, glucose intolerance and diminished β-cell size in S6K1-deficient mice. Nature 2000;408:994–7.

36. Seagroves TN, Ryan HE, Lu H, et al. Transcription factor HIF-1 is a necessary mediator of the Pasteur effect in mammalian cells. Mol Cell Biol 2001; 21:3436–44.

37. Lum JJ, Bui T, Gruber M, et al. The transcription factor HIF-1α plays a critical role in the growth factor-dependent regulation of both aerobic and anaerobic glycolysis. Genes Dev 2007;21:1037–49.

38. Brahimi-Horn MC, Chiche J, Pouyssegur J. Hypoxia and cancer. J Mol Med 2007;85:1301–7.

39. Lechler P, Wu X, Bernhardt W, et al. The tumor gene survivin is highly expressed in adult renal tubular cells: implications for a pathophysiological role in the kidney. Am J Pathol 2007;171:1483–98.

40. Grafe M, Steinheider G, Desaga U, et al. Characterization of two distinct mechanisms for induction of apoptosis in human vascular endothelial cells. Clin Chem Lab Med 1999;37:505–10.

41. Rosenberger C, Mandriota S, Jurgensen JS, et al. Expression of hypoxia-inducible factor-1α and -2α in hypoxic and ischemic rat kidneys. J Am Soc Nephrol 2002;13:1721–32.

42. Bernhardt WM, Wiesener MS, Weidemann A, et al. Involvement of hypoxia-inducible transcription factors in polycystic kidney disease. Am J Pathol 2007; 170:830–42.

43. Warnecke C, Zaborowska Z, Kurreck J, et al. Differentiating the functional role of hypoxia-inducible factor (HIF)-1α and HIF-2α (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2α target gene in Hep3B and Kelly cells. FASEB J 2004;18:1462–4.