Mutations in the PI3K/PTEN/TSC2 Pathway Contribute to Mammalian Target of Rapamycin Activity and Increased Translation under Hypoxic Conditions

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Abstract
Decreased oxygen causes a rapid inhibition of mRNA translation. An important regulatory mechanism of translational repression under hypoxic conditions involves inhibition of the mammalian target of rapamycin (mTOR). mTOR is a target of the phosphatase and tensin homologue detected on chromosome 10 (PTEN)/phosphatidylinositol 3-kinase/AKT/TSC2 pathway, a pathway that is frequently mutated in human cancers. Although hypoxia has been shown to inhibit mTOR activity, we show here that the hypoxia-induced inhibition of mTOR activity is attenuated in cells lacking TSC2 or PTEN, resulting in a higher translation rate even under hypoxic conditions. Comparison of mTOR inhibition by hypoxia alone or in combination with rapamycin showed that prolonged exposure to hypoxia was required to fully inhibit mTOR activity even in wild-type cells. Increased mTOR activity and protein synthesis did not translate into enhanced cell proliferation rates. However, lack of TSC2 resulted in a survival advantage when cells were exposed to hypoxia. Protection against hypoxia-induced cell death due to TSC2 deficiency is rapamycin-resistant, suggesting that TSC2 affects an apoptotic pathway. Tumors derived from TSC2 wild-type cells exhibited a growth delay compared with TSC2-deficient tumors, indicating that enhanced mTOR activity is advantageous in the initial phase of tumor growth. Therefore, failure to inhibit mTOR under oxygen-limiting conditions can be affected by upstream activating mutations and increases the survival and growth of hypoxic tumor cells. (Cancer Res 2006; 66(3): 1561-9)

Introduction
Hypoxia, resulting from an inadequate supply of oxygen, is a common finding in solid human tumors. Not only does hypoxia render tumor cells more resistant to anticancer therapy, it also affects the malignant potential of a tumor resulting in a more aggressive phenotype (1). Hypoxia induces the selective transcription and translation of genes that are involved in angiogenesis, glycolysis, cell survival, and invasion (2); whereas on a global level, it causes a rapid and significant inhibition of protein synthesis (3).

Protein translation is a highly regulated process. One mechanism of regulating translation is through the formation of the ternary complex comprised of the translation initiation factor eIF2, GTP, and methionyl-tRNA. The ternary complex joins the 40S ribosomal subunit and is subsequently recruited to the mRNA that is primed for translation through binding of several initiation factors. Hypoxia has been shown to activate the endoplasmic reticulum kinase PERK, resulting in a rapid but transient phosphorylation of the eIF2α subunit (4). When eIF2α is phosphorylated, the GTP nucleotide exchange, as mediated by eIF2B is inhibited, thus halting translation initiation of the primed mRNA. Another mechanism of translational control is through the formation of the cap-binding initiation complex eIF4F. Proteins that make up the eIF4F complex include the eukaryotic initiation factors eIF4A, 4E, and 4G. In response to conditions that repress translation, eIF4E is sequestered away by the 4E-binding protein 4E-BP1. Under permissive conditions for translation, 4E-BP1 is hyperphosphorylated by the mammalian target of rapamycin (mTOR), resulting in release of eIF4E and thereby freeing it to join the eIF4F complex.

Cap-dependent binding to the mRNA follows as a first step in translation initiation (5). In response to hypoxia, the association of 4E-BP1 with eIF4E in rat hepatocytes is increased (6), and mTOR and its downstream target 4E-BP1 are hypophosphorylated (7). These findings are indicative of repression of mTOR activity under hypoxic conditions, contributing to the down-regulation of mRNA translation.

The mTOR protein is a central controller of protein synthesis, cell growth, cell proliferation, and cell viability (8). It was first isolated in a screen for resistance to the bacterial macrolide rapamycin in yeast (9). The mammalian gene was subsequently cloned based on homology to the yeast proteins (10). Aside from its effects on 4E-BP1, mTOR also affects 5'-cap-dependent translation through ribosome biogenesis via p70S6K and the phosphorylation of the helicase eIF4A, the scaffolding protein eIF4G, and potentially the elongation factor eEF2 (5, 8). Furthermore, mTOR has been implicated in the function of the hypoxia-inducible factor-1 (HIF-1), a key regulator of the transcriptional response to oxygen deprivation (11, 12). Activity of mTOR is affected by nutrients, growth factors, energy metabolism, and oxygen levels (7, 8). The effects of growth factors on mTOR are relayed through the phosphatidylinositol 3-kinase (PI3K)/phosphatase and tensin homologue detected on chromosome 10 (PTEN)/AKT pathway (refs. 8, 13–15; Fig. 1A). Activated AKT phosphorylates the tuberin (TSC2) subunit of the tuberous sclerosis TSC1/TSC2 complex, thereby preventing the formation of this complex (16). TSC1/TSC2 is a suppressor of mTOR signaling. The complex has inhibitory GTPase-activating protein activity towards the small GTPase Ras homologue-enriched in brain (Rheb; refs. 17, 18). Rheb, when active, stimulates the phosphorylation of mTOR (19). Recent studies have suggested that inhibition of mTOR by hypoxia requires the TSC1/TSC2 complex and the hypoxia-inducible gene REDD1/RTP801 (20, 21), suggesting a feedback pathway in which hypoxia-induced
RED1 acts upstream of TSC2. The inhibition of mTOR does not require AMP-activated protein kinase or LKB1, indicating that energy depletion is not the underlying mechanism.

Several components of the PI3K/PTEN/AKT pathway are dysregulated in a wide spectrum of human cancers, including mutation and silencing of the PTEN tumor suppressor gene, activation of PI3K, amplification or overexpression of AKT and inactivation of TSC2 by extracellular signal-regulated kinase (ERK; refs. 22, 23). Given the importance of this pathway in mTOR and hypoxic signaling (11, 12, 24, 25), we hypothesized that mutations in this pathway will attenuate the hypoxia-induced inhibition of mTOR. We therefore analyzed different cell lines spanning different components of this pathway for mTOR activity under hypoxic conditions. We also investigated the effects of retained mTOR activity on translation, proliferation, and survival under hypoxic conditions. Finally, the effect of rapamycin treatment on mTOR activity, translation, cell proliferation, and survival was investigated under hypoxic conditions in cell culture and in tumor xenografts. The results presented in this study lead us to conclude that failure to fully inhibit mTOR under oxygen-limiting conditions can be affected by upstream activating mutations and increases the survival of hypoxic tumor cells.

Materials and Methods

Cell culture and hypoxia treatment. The human prostate cancer cell line PC-3, stably transfected with empty pcDNA3 vector or wild-type mTOR cDNA were a gift from Dr. R.T. Abraham (the Burnham Institute, La Jolla, CA; ref. 12) and were kept in RPMI supplemented with 10% FCS (Hyclone, Logan, UT) and 500 μg/mL genetin (Invitrogen, Carlsbad, CA). The human glioblastoma cell line U251 with doxycyclin-inducible wild-type PTEN and catalytically inactive PTEN C124S, a gift from Dr. M.M. Georgescu (M.D. Anderson, Houston, TX; ref. 26), were grown in DMEM with Tet-compatible 10% FCS (BD Biosciences, San Jose, CA), 500 μg/mL genetin, and 5 μg/mL blasticidin (Invitrogen). For experiments, PTEN expression was induced with 10 μg/mL doxycyclin 1 hour prior to treatment. TSC2+/−/p53−/− and TSC2−/−/p53−/− mouse embryonic fibroblasts (MEF) from Dr. D.J. Kwiatkowski (Harvard, Boston, MA; ref. 27) were maintained in DMEM with 10% FCS. Wild-type TSC2 and TSC2-deficient MEFs were transformed with EIA and RAS by retroviral infection for xenograft experiments and maintained in 5 μg/mL puromycin (Sigma, St. Louis, MO) and 100 μg/mL hygromycin B (Invitrogen) supplemented DMEM (plasmids were a gift from Dr. Laura Attardi, Stanford University, Stanford, CA). All media were supplemented with antibiotics (penicillin and streptomycin). Rapamycin stock solution was dissolved in 100% ethanol at a concentration of 2 mmol/L. Cells were pretreated with either 100 nmol/L rapamycin (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute) or mock-treated with 100% ethanol 1 hour prior to normoxic or hypoxic treatment. To expose cells to hypoxia (<0.02% O2), cells were placed inside an anaerobic chamber (Sheldon Corp., Cornelius, OR) attached to a 5% carbon dioxide, 5% hydrogen, and 90% nitrogen gas mixture. The normoxic control samples were kept in a regular incubator.

Immunoblot analysis. Cells were plated on 100 mm culture dishes in medium containing 2% FCS. Cells were lysed inside the hypoxia chamber in 9 mol/L urea, 75 mmol/L Tris-HCl (pH 7.5), and 0.15 mol/L β-mercaptoethanol followed by sonication. Proteins were separated on 7.5% to 15% SDS polyacrylamide gels and detected by Western blotting.

Figure 1. Mutations that activate mTOR result in sustained mTOR activity under hypoxia. Overview of the PTEN/TSC2/mTOR pathway (A). U251 cells with doxycyclin-inducible wild-type PTEN or catalytically inactive PTEN C124S (B); TSC2−/− and TSC2−/− MEFs (C); and PC-3 cells stably overexpressing empty vector or wild-type mTOR (D) were exposed to normoxia or hypoxia (<0.02% oxygen) for 6 hours in the presence or absence of rapamycin. Phosphorylated p70S6k and 4E-BP1 proteins were detected by immunoblot analysis. Detection of α-tubulin was included to assess equal loading.
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Biosciences) with Hyperfilm enhanced chemiluminescence (Amersham Biosciences).

\[^{[35S]}\text{Methionine/cysteine labeling.}\] Cells were plated on 60 mm culture plates in medium containing 10% FCS. One hour before harvesting, the medium was replaced with methionine/cysteine and FCS-free medium (Invitrogen). After 30 minutes, \[^{[35S]}\text{methionine/cysteine Premix (Amersham Biosciences)}\] was added to a final concentration of 25 to 50 μCi/ml for an additional 30 minutes. Cells were lysed inside the hypoxia chamber in 25 mmol/L Tris-HCl (pH 8.0), 25 mmol/L EDTA, and 0.5% SDS. Equal amounts of solubilized protein were precipitated with trichloroacetic acid (TCA) and are means ± SE.

\[^{[3H]}\text{Thymidine incorporation.}\] Equal cell numbers on 60 mm tissue culture dishes were exposed to the indicated hours of hypoxia. One hour before harvest \[^{[3H]}\text{thymidine (Amersham Biosciences)}\] was added directly to the medium to a final concentration of 1 μCi/ml. Cells were lysed in 25 mmol/L Tris-HCl (pH 8.0), 25 mmol/L EDTA, and 0.5% SDS inside the hypoxia chamber. TCA precipitates were transferred to glass microfiber filters (Whatman GF/C) for measurement of \[^{[3H]}\text{-incorporation in a Beckman scintillation counter.}\] Counts per minute were normalized to the xenografts reached before harvest and are means ± SE. Bars represent values derived from triplicate samples and are means ± SE.

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**Cell proliferation assays.** Fifty thousand cells were plated in triplicate on 60 mm dishes in medium containing 10% FCS. Three days later, cells were counted on a hemacytometer and 50,000 cells were replated. This was repeated 4 times. Data points represent values derived from triplicate samples and are means ± SE.

**Apoptosis assays.** Cells were plated on two-well chamber slides (Fisher Scientific, Hampton, NH) in medium with 10% FCS. After treatment, 5 μg/ml Hoechst dye 33342 was added directly to the medium and apoptotic cells were scored for changes in nuclear morphology. The percentage of apoptotic cells relative to the total number of cells in at least three independent fields was calculated. Data points represent means ± SE of three experiments.

**Survival assays.** Cells were plated on 60 mm culture dishes in medium with 10% FCS. After treatment, cells were kept in an incubator for 1 to 2 weeks. Colonies were fixed and stained with crystal violet and counted. Percentage of survival was calculated relative to untreated control cells. Data points represent values derived from triplicate samples and are means ± SE.

**Northern blot analysis.** Cells were plated on 100 mm culture dishes in medium with 2% FCS. Total RNA was extracted from cells with Trizol (Invitrogen). After running 2 μg total RNA on agarose gels, RNA was transferred to Nitran membrane (Schleicher & Schuell, Keene, NH) and hybridized with \[^{32P}]\text{-DNA probes of human vascular endothelial growth factor (VEGF), phosphoglycerate kinase-1 (PGK1), and Aldolase A.}\] Equal loading of samples was visualized by staining the 18S rRNA with methylene blue. Blots were visualized on a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**VEGF ELISA.** Cells were plated on 24-well plates in medium with 2% FCS. Levels of secreted VEGF were measured in supernatants with a human VEGF ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Bars represent values derived from triplicate samples and are means ± SE.

**In vivo xenograft models.** Male nude mice (age 32 days, Charles River Laboratories, Inc.) were injected with 5 × 10^6^ cells on each flank. After the xenografts reached ~150 mm^3^ in size, as determined with volume = (length × width^2^)/2, selected mice were injected i.p. with 4 mg/kg bodyweight rapamycin diluted in 52% Tween 80 and 5:2% PEG-8000 from a 10 mg/ml stock in ethanol daily. An hour before the mice were sacrificed, 10 μg/l bodyweight EF-5 (2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3-penta-fluoropropyl)acetamide), a pentafluorinated derivative of etanidazole (a gift from Cameron Koch, University of Pennsylvania) from a 10 mmol/L stock in ethanol daily. One hour before the mice were sacrificed, 10 μg/l bodyweight EF-5 (2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3-penta-fluoropropyl)acetamide), a pentafluorinated derivative of etanidazole (a gift from Cameron Koch, University of Pennsylvania) from a 10 mmol/L stock was injected i.p. Tumors were flash-frozen in optimum cutting temperature Tissue-Tek embedding medium (Sakura Finetek) on dry ice and sectioned at 6 μm. Slides were dried overnight and then fixed in cold acetone for 1 minute. Immunohistochemistry was done using the following antibodies: rabbit polyclonal anti-phospho-4E-BP1 T37/46 (dilution 1:200) and anti-EF-5-Cy3-conjugated antibody (dilution 1:25, a gift from Cameron Koch). Biotinylated secondary antibodies (dilution 1:500, Jackson Immunologicals) were detected using fluorescent Alexa-Fluor 488-streptavidin conjugates (dilution 1:200, Molecular Probes, Eugene, OR).

**Results**

**Cells with mutations in the PTEN/PI3K/Akt pathway retain mTOR activity under hypoxic conditions.** Mutations of components of the PI3K/PTEN/AKT signaling network are frequent events in human cancers (28). Given the importance of the PI3K/PTEN/AKT pathway in mTOR activation and its contribution to proteomic changes in response to hypoxia, we hypothesized that mutations in this pathway permit cells to retain mTOR activity under hypoxic conditions. In contrast, in cells without these mutations, mTOR activity will be inhibited by hypoxia. To address this hypothesis, we assessed mTOR activity by Western blotting for the phosphorylation status of its downstream targets 4E-BP1 and p70S6K. We exposed the PTEN null glioblastoma cell line U251 stably transfected with doxycyclin-inducible wild-type PTEN (Fig. 1B, lanes 1–4) or phosphatase-inactive PTEN C124S (lanes 5–8) to 6 hours of normoxia or hypoxia in the presence or absence of rapamycin. Under hypoxic conditions, mTOR activity is inhibited significantly in U251 cells expressing wild-type PTEN (compare lanes 1 and 3). Exposure to hypoxia results in dephosphorylation of p70S6K T389 and a reduction in the intensity of hyperphosphorylated 4E-BP1 T37/46 bands (indicated by α and β) in favor of the downshifted, hyperphosphorylated band (indicated by γ) of 4E-BP1. These observations are all indicative of mTOR repression. Treatment with rapamycin alone or in combination with hypoxia (lanes 2 and 4) showed an even further inhibition of mTOR activity toward these targets, suggesting an incomplete repression of mTOR by hypoxia alone. In U251 cells expressing the mutant version of PTEN, phosphorylation of p70S6K T389, although attenuated, was still visible under hypoxic conditions as opposed to the disappearance in wild-type PTEN-expressing cells (compare lanes 5 and 7). Hyperphosphorylated 4E-BP1 T37/46 bands (indicated by α and γ) remained largely unaffected by hypoxia in these cells. Given the fact that rapamycin treatment in addition to hypoxia still resulted in the dephosphorylation of these mTOR targets (lane 8), it seems likely that the mTOR signaling pathway remained intact in these cells and that the absence of an effect of hypoxia on phosphorylation status of these proteins is not due to additional, activating mutations downstream of mTOR. Combined, these results indicate that mutant PTEN attenuates the hypoxia-induced inhibition of mTOR activity.

Next, we investigated the effects of hypoxia on TSC2^-/-^ and TSC2^-/-^ MEFs (Fig. 1C). As described above, cells were exposed to 6 hours of normoxia or hypoxia in the presence or absence of rapamycin. Hypoxia caused a significant reduction of p70S6K T389 phosphorylation (indicated by the arrow) in TSC2^-/-^ cells (compare lanes 1 and 3) but not in the TSC2^-/-^ cells (compare lanes 5 and 7). The effects on 4E-BP1 T37/46 phosphorylation were quite subtle. Although the hyperphosphorylation band of 4E-BP1 (indicated by α) was reduced in intensity in favor of the hypophosphorylated band of 4E-BP1 (indicated by β) in TSC2^-/-^ cells, it remained abundant. Whereas the hypophosphorylated band was already visible under normoxic conditions in TSC2^-/-^ cells, it was absent in untreated TSC2^-/-^ cells (compare lanes 1 and 3).
and 5). As was shown in the U251 cells, rapamycin treatment alone or in addition to hypoxia further decreased mTOR activity, indicative of incomplete repression of mTOR activity by hypoxia alone in both TSC2+/+ and TSC2−/− cells (lanes 4 and 8).

The consequence of PTEN or TSC2 loss is dysregulated mTOR activity. A direct approach to mimic these mutations is to overexpress mTOR. We investigated the effects of hypoxia on mTOR in the human prostate cancer cell line PC-3 that is either stably transfected with empty pcDNA3 vector or with a wild-type mTOR cDNA-overexpressing construct (Fig. 1D). In mTOR-overexpressing cells, phosphorylation of p70S6k remained largely unaffected by hypoxia (compare lanes 5 and 7). And whereas in the empty vector-transfected control cells 4E-BP1 dephosphorylation is unaffected by hypoxia (compare lanes 1 and 5). As was shown above with the U251 cells and the TSC2+/+ and TSC2−/− MEFs, rapamycin treatment is a more efficient inhibitor of mTOR activity as measured by mTOR target phosphorylation than hypoxia.

The combination of the results in these three cell lines indicates that whereas hypoxia reduces mTOR activity towards these targets, as has been shown in previous reports (7), mutations in the PI3K/PTEN/AKT pathway can result in elevated mTOR activity under hypoxic conditions. The finding that treatment with rapamycin reduces phosphorylation of mTOR and its targets further than hypoxia treatment alone indicates that repression of mTOR by lowered oxygen levels is not complete or is not as efficient as rapamycin.

**Cells with mutations in the PTEN/PI3K/Akt pathway exhibit attenuated inhibition of protein synthesis under conditions of lowered oxygen levels.** Because we have shown that cells with mutations in the PI3K/PTEN/AKT pathway still have active mTOR under conditions of reduced oxygen, we aimed to assess the effect of mTOR activity on global translation at decreased oxygen tensions. Cells were labeled with [35S]methionine/cysteine and incorporation of the [35S]-label into equal amounts of protein was measured. The basal protein synthesis rate under normoxic conditions was 1.7-fold higher in U251 cells that contain mutant PTEN than in cells containing wild-type PTEN (Fig. 2A). Hypoxia is a potent inhibitor of general translation (4). Exposure to hypoxia for 6 hours reduced [35S]-incorporation by 38% relative to normoxic levels in PTEN wild-type cells and by 50% in PTEN mutant cells. Because the basal translation rate is higher in cells with mutant PTEN, the protein synthesis rate under hypoxic conditions in cells with mutant PTEN is still 1.4-fold higher than in cells with wild-type PTEN. Rapamycin treatment inhibited translation by 21% in wild-type PTEN cells and by 11% in PTEN mutant cells under normoxic conditions. Additional rapamycin treatment under hypoxic conditions further decreased the already attenuated protein translation rates by 19% independent of PTEN status. These results suggest that activated mTOR, as caused by PTEN inactivation, translates into attenuated inhibition of protein synthesis by hypoxia. The observation that rapamycin treatment further decreases protein synthesis supports our previous finding that mTOR inhibition by hypoxia is not as stringent as mTOR inhibition caused by rapamycin.

Normoxic levels of [35S]-incorporation were 1.7-fold higher for untreated TSC2−/− cells than for untreated TSC2+/+ cells, indicative of a higher translation rate in the TSC2 null cells (Fig. 2B). The observation of a higher translation rate in the absence of TSC2 has been reported previously (29). Incorporation of label is reduced by 50% after 6 hours of hypoxia in the TSC2−/− cells and continues to decline to 27% of the normoxia control samples by 24 hours. Rapamycin treatment alone decreased translation with 39% in the TSC2+/+ cells. The combination of hypoxia with rapamycin treatment resulted in an even greater reduction in [35S]-incorporation, to 30% of the untreated controls after only 6 hours. The additive effect of rapamycin is indicative of the presence of active mTOR in cells that have been exposed to hypoxia. Complete inhibition of mTOR activity requires extended exposure of at least 24 hours to lowered levels of oxygen. In the absence of TSC2, [35S]-incorporation still decreased under hypoxic

![Figure 2](http://cancerres.aacrjournals.org/content/cancerres/66/3/1564.F2.large)

**Figure 2.** Mutations that activate mTOR result in enhanced global translation under hypoxic conditions. U251 cells with doxycyclin-inducible wild-type or mutant PTEN (A), TSC2−/− and TSC2−/− MEFs (B), and vector control and mTOR-overexpressing PC-3 cells (C) were exposed to hypoxia in the presence or absence of rapamycin for the indicated amounts of time and labeled with [35S]methionine/cysteine. [35S]-Incorporation was measured in equal amounts of protein. Counts per minute were normalized to wild-type, untreated normoxia controls (0 hours) resulting in relative [35S]methionine/cysteine incorporation values. Columns, mean of values derived from triplicate samples; bars, ± SE.
hypoxia. Contributing to maintained translation under conditions of pathway upstream of mTOR resulted in continued mTOR activity persisted. Consistent with mTOR targets analyzed by Western induced translational repression, we next addressed the question activated mTOR increases translation and attenuates hypoxia—under conditions of lowered oxygen levels. Because hyper—causes a further repression of protein synthesis. Furthermore, under hypoxic conditions, which manifested itself in attenuated mutations in the PI3K/AKT/TSC2 pathway retained mTOR activity conditions corresponded to inhibition of translation. Cells with conditions. The attenuation of protein synthesis as caused by hypoxia. Rapamycin treatment reduces translation withing cells than in the vector control PC-3 cells throughout exposure of mTOR overexpressing cells (16% and 19% reduction after 6 hours and approximately 1.3-fold higher than that of the vector control PC-3 cells, protein synthesis rates remain higher in mTOR-overexpressing cells, than in the vector control PC-3 cells throughout exposure to hypoxia. Rapamycin treatment reduces translation with approx. 29% in both cell lines both under normoxic and hypoxic conditions. Overexpression of mTOR in PC-3 cells increased [35S]-incorporation 1.3— to 1.6-fold under both normoxic and hypoxic conditions (Fig. 2C). The attenuation of protein synthesis as caused by hypoxia is similar for both empty vector control transfected and mTOR-overexpressing cells (16% and 19% reduction after 6 hours and 68% and 60% reduction after 24 hours of hypoxia, respectively). Because the basal translation rate of mTOR-overexpressing cells is approximately 1.3-fold higher than that of the vector control PC-3 cells, protein synthesis rates remain higher in mTOR-overexpressing cells than in the vector control PC-3 cells throughout exposure to hypoxia. Rapamycin treatment reduces translation with ~22% to 29% in both cell lines both under normoxic and hypoxic conditions.

Decreased phosphorylation of mTOR targets under hypoxic conditions corresponded to inhibition of translation. Cells with mutations in the PI3K/akt/TSC2 pathway retained mTOR activity under hypoxic conditions, which manifested itself in attenuated inhibition of protein synthesis as caused by hypoxia. Furthermore, inhibition of the mTOR contribution to global translation by hypoxia is not complete, as additional treatment with rapamycin causes a further repression of protein synthesis.

Mutations that activate mTOR do not affect proliferation under conditions of lowered oxygen levels. Because hyperactivated mTOR increases translation and attenuates hypoxia—induced translational repression, we next addressed the question of whether increased protein synthesis is translated into increased cell proliferation. Hypoxia very rapidly and stringently induces a growth arrest (30—32). Here, we show that within 6 hours of exposure to hypoxia, virtually all DNA synthesis as measured by [3H]thymidine incorporation ceases in the TSC2-deficient and wild-type MEFs (Fig. 3A). The response is the same for both TSC2+/— and TSC2+/+ MEFs, suggesting that TSC2 status has no effect on the DNA synthesis rate. Inhibition of mTOR with rapamycin, on the other hand, causes a 10% to 25% decrease in DNA synthesis at normoxia, consistent with observations that rapamycin causes a G1 arrest (33). The arrest caused by hypoxia is so severe that combining hypoxia with rapamycin treatment had no additive effect. Growth curves under normoxic conditions confirmed the growth arrest caused by rapamycin treatment (Fig. 3B). Whereas TSC2 status had no effect on the growth rate, a slight but reproducible resistance to rapamycin-induced growth arrest was observed in cells lacking TSC2.

Similar results were obtained with PC-3 cells. Hypoxia caused a significant decrease in DNA synthesis and whereas overexpression of mTOR had no effect on cell proliferation rates, rapamycin treatment inhibited [3H]thymidine incorporation (Fig. 3C). The inhibitory effects of hypoxia and rapamycin on cell proliferation seem to be independent from their effects on mTOR target phosphorylation and protein synthesis.

TSC2 deficiency protects against hypoxia-induced cell death. Mutations that activate mTOR affect translation but not necessarily proliferation. We therefore asked the question whether increased mTOR activity results in a survival advantage for the cell. When TSC2+/— and TSC2+/+ MEFs were exposed to hypoxia and scored for apoptotic cells, TSC2+/— MEFs started to exhibit a significant amount of apoptosis after exposures to hypoxia that exceed 24 hours (Fig. 4A). By 48 hours, approximately half the cell population had undergone apoptosis. Because the MEFs were derived from mice that are also p53 null, the apoptosis is p53-independent. TSC2+/— MEFs, on the other hand, were resistant to apoptosis, suggesting that lack of TSC2 gives an advantage to these cells when exposed to hypoxic conditions. If the effect is due to

Figure 3. Cell proliferation is not affected by mTOR-activating mutations. A, TSC2+/— and TSC2+/+ MEFs were exposed to hypoxia in the presence or absence of rapamycin for the indicated amounts of time and labeled with [3H]thymidine. [3H]-incorporation was measured in extracts derived from an equal number of cells. Counts per minute were normalized to wild-type, untreated normoxia controls (0 hours) resulting in relative [3H]-thymidine incorporation values. Columns, mean of values derived from triplicate samples; bars, ± SE. B, growth curves of TSC2+/— and TSC2+/+ MEFs grown at normoxia in the presence or absence of rapamycin. Dashed lines, rapamycin-treated cells; solid lines, mock-treated cells; squares with black lines, TSC2+/— cells; circles with grey lines, TSC2+/+ cells. Points, mean of values derived from triplicate samples; bars, ± SE. C, [3H]thymidine incorporation of empty vector control and mTOR-overexpressing PC-3 cells as described in (A).
Increased mTOR activity results in enhanced transcription and translation of hypoxia-inducible genes. Experiments with rapamycin and mTOR overexpression have shown that mTOR contributes to the activation of the hypoxia-inducible transcription factor HIF-1, a key regulator of the cellular response to hypoxia (11, 12). Activated HIF-1 enhances the expression of genes involved in glycolysis, glucose transport, angiogenesis, cell survival, and invasion (2). Increasing mTOR activity through activating upstream mutations would therefore be likely to contribute to an enhanced cellular response to hypoxic stress. Indeed, as Brugarolas et al. have shown previously, TSC2 deficiency results in increased activation of HIF-1 target genes (29). To confirm these findings in a different setting, we evaluated the contribution of mTOR overexpression in PC-3 cells to mRNA levels of several HIF-1 target genes (Fig. 5A). The transcripts analyzed included PGK, VEGF, and Aldolase A. Under normoxic conditions, levels of transcript were increased when mTOR was overexpressed (compare lanes 1 and 5). Treatment with rapamycin decreased transcript abundance, indicating that increased mTOR activity, inhibition of mTOR with rapamycin in TSC2−/− cells would be expected to return the apoptotic potential to levels similar to that of TSC2+/+ cells. Surprisingly, rapamycin had no effect on apoptosis in either the TSC2−/− or TSC2+/+ cells. To corroborate the results of the apoptosis assays, we carried out colony survival assays. Cells were exposed to hypoxia for the indicated amount of time followed by incubation in a regular incubator for 1 to 2 weeks. In general, rapamycin-treated cells resulted in smaller colonies (data not shown), indicative of proliferation inhibition and required an additional week to reach similar, countable sizes as those formed by cells that were not treated with rapamycin. The survival data matched the findings in the apoptosis assays. TSC2−/− cells exhibited a higher survival rate than the TSC2−/+ MEFs after hypoxia and rapamycin treatment did not affect survival rates (Fig. 4B). Protection against hypoxia-induced apoptosis due to a lack of TSC2 seems to be either an mTOR-independent consequence of TSC2 deficiency or an mTOR-dependent, but rapamycin-resistant, phenomenon.

Figure 4. TSC2 deficiency protects cells against hypoxia-induced apoptosis. A, TSC2−/− and TSC2+/+ MEFs were exposed to hypoxia in the presence or absence of rapamycin and apoptotic cells were scored for changes in nuclear morphology after the addition of Hoechst dye 33342. Points, means of three experiments; bars, ± SE. B, colony survival assays of TSC2−/− or TSC2+/+ MEFs that were exposed to hypoxia in the presence or absence of rapamycin. The percentage of survival was calculated relative to untreated control cells (0 hours). Points, means of values derived from triplicate samples; bars, ± SE. *, P = 0.009 TSC2−/− versus TSC2+/+; P = 0.62 TSC2−/− controls versus rapamycin; P = 0.12 TSC2+/+ controls versus rapamycin as determined with Student’s t test.

Figure 5. Overexpression of mTOR enhances expression of hypoxia-inducible genes. A, empty vector control and mTOR-overexpressing PC-3 cells were exposed to normoxia or hypoxia for 18 hours in the presence or absence of rapamycin. Five micrograms of total RNA was run out on agarose gels and transferred to Nytran membrane followed by hybridization with 32P-cDNA probes of human VEGF, PGK1, and Aldolase A. Equal loading of samples was visualized by staining the 18S rRNA with methylene blue. B, VEGF ELISA of empty vector control and mTOR-overexpressing PC-3 cells exposed to normoxia or hypoxia in the presence or absence of rapamycin for 24 hours. Levels of secreted VEGF were measured in supernatants with a human VEGF ELISA. Columns, mean of values derived from triplicate samples; bars, ± SE.
under normoxic conditions, HIF-1 activity is affected by mTOR (compare lanes 1 and 2, and lanes 5 and 6). Upon exposure to hypoxia, transcript levels increased in both vector control cells and mTOR-overexpressing cells (compare lanes 1 and 3, and lanes 5 and 7). Overall levels of HIF-1 target transcripts, however, were higher in cells that overexpress wild-type mTOR. Therefore, increasing mTOR activity can enhance expression of HIF-1 target genes both under normoxic and hypoxic conditions. Inhibition of mTOR with rapamycin decreased the abundance of HIF-1 target mRNAs.

When endogenous VEGF protein levels were assessed, mTOR overexpression was also found to stimulate the amount of VEGF protein produced both under normoxic and hypoxic conditions (Fig. 5B). In addition, rapamycin treatment repressed VEGF protein production. These results correlated well with VEGF transcript levels.

Cells with activated mTOR in hypoxic areas of tumors. To assess whether activated mTOR actually occurs in hypoxic regions in tumors, we turned to a xenograft model. We used the TSC2-deficient and wild-type MEFs that were transformed with E1A and RAS in order to be able to grow them as tumors. In vitro growth curves showed that the effect of TSC2 status had not been altered by the transformation process (data not shown). Although growth of TSC2 wild-type tumors was initially delayed compared with that of TSC2-deficient tumors, the growth rate after the initial delay was similar for both cell types (Fig. 6A). This suggests that increased translation and expression of hypoxia-induced genes and/or resistance to hypoxia-induced cell death contributes to enhanced tumor initiation in vivo and that increased mTOR activity is only advantageous in the initial phase of tumor growth in this particular model. Rapamycin delayed tumor growth of both tumor types. However, the delay was transient, and eventually, rapamycin was no longer able to prevent tumor growth. The resistance to rapamycin-induced growth inhibition was not due to a cellular resistance to rapamycin, as a cell line derived from a TSC2 wild-type tumor still experienced growth arrest in response to rapamycin in vitro (data not shown).

Immunohistochemistry of tumor sections with anti-EF-5, a pentafluorinated derivative of etanidazole that forms adducts with cellular macromolecules under hypoxic conditions (34), and anti-phospho-4E-BP1 T37/46 showed that most phospho-4E-BP1–positive cells localized to the nonhypoxic regions (Fig. 6B–D). However, occasional hyperphosphorylated 4E-BP1–positive cells (indicated with arrows) did occur in hypoxic regions, supporting our theory that mTOR can be active under hypoxic conditions.

Discussion

Cells respond to hypoxia with a rapid and substantial down-regulation of mRNA translation in order to preserve energy (3, 4). Hypoxia has recently been shown to decrease mTOR activity as assessed by the phosphorylation status of its downstream targets 4E-BP1 and p70s6k (7, 20). Because these targets play central roles in cap-dependent translation initiation, down-regulation of their activity results in repression of translation. Aside from protein synthesis, mTOR also plays a role in the function of the hypoxia-inducible transcription factor HIF-1 (12). Therefore, given the role for mTOR in a hypoxia response pathway and the fact that components of the PI3K/PTEN/AKT signaling network are dysregulated in a wide spectrum of human cancers (28), we hypothesized that tumor cells with these mutations retain mTOR activity under hypoxic conditions. As we have shown in this report, lack of functional PTEN or TSC2 or overexpression of wild-type mTOR all result in continued mTOR activity when exposed to hypoxia as measured by the phosphorylation status of mTOR targets. In addition, even though hypoxia decreases phosphorylation of p70S6K and 4E-BP1 in cells with functional PTEN and TSC2 or normal levels of mTOR, the inhibitory effect of rapamycin treatment is much stronger than that of hypoxia. These results suggest that the inhibition of mTOR activity by lowered oxygen levels is not as complete as with rapamycin treatment, leaving room for mTOR function in HIF-1 signaling under hypoxic conditions.

Because mTOR plays a central role in translational control, continued mTOR activity under hypoxic conditions in cells lacking functional PTEN or TSC2 and cells overexpressing wild-type mTOR attenuates the hypoxia-induced translational repression. Furthermore, even though exposure to hypoxia causes a rapid and significant down-regulation of translation in wild-type cells, additional treatment with rapamycin even further decreases protein synthesis. Only after extended hypoxic treatment does activity...
hypothesis of translation by hypoxia alone reach similar levels of translational repression as the combination of hypoxia with rapamycin treatment. Thus, repression of mTOR activity requires prolonged exposure to hypoxia for complete inhibition.

Hypoxia causes a decrease in protein synthesis through multiple mechanisms, one of which is through regulation of mTOR activity (7). However, because mTOR is not the only factor contributing to translational control, hypoxia will still cause a decrease in mRNA translation even in the presence of mTOR-activating mutations. Similarly, inhibition of mTOR with rapamycin will not completely abrogate protein synthesis. The results presented here give an indication of the relative contribution of mTOR to translational repression by hypoxia.

The increase in protein synthesis as caused by increased mTOR activity does not translate itself into increased proliferation under unstressed conditions. Rather, the resulting phenotype is the appearance of larger cells, indicative of increased cell growth as opposed to cell proliferation (data not shown). Sensitivity to growth arrest caused by rapamycin, on the other hand, is affected by TSC2 status. Although both TSC2-deficient and wild-type cells display a severe inhibition of cell proliferation in response to rapamycin, TSC2-deficient cells still exhibit low levels of proliferation. Expression of cyclin D1 is at least partly regulated by mTOR and can be inhibited by rapamycin (35–37). Because TSC2 deficiency resulted in a 1.7-fold higher global translation rate, a potential explanation could be that levels of cyclin D1 remain sufficiently high in these cells, even after rapamycin treatment, to confer a moderate resistance to rapamycin-induced proliferation inhibition.

Although TSC2 deficiency positively affects mTOR activity, translation, and survival, but has no effect on proliferation, rapamycin treatment negatively affects mTOR activity, translation, and proliferation, but has no effect on survival. The fact that mTOR activity and inhibition thereof with rapamycin do not always have opposing effects is noteworthy. This leaves room for the interpretation that some effects of rapamycin are not dependent on mTOR activity or that some mTOR functions are not susceptible to rapamycin inhibition.

Hypoxia in solid tumors is the result of two processes: chronic hypoxia, caused by the limited diffusion range of oxygen across tissue and the relatively large distance of certain tumor cells away from the blood vessels; and acute hypoxia, resulting from fluctuating blood flow in the often aberrantly formed vascular network of the tumor (38). Activity of mTOR seems to be completely inhibited only after an extended period of exposure to hypoxia. It therefore seems feasible that cells throughout a tumor, even after rapamycin treatment, could contribute to tumor progression.

In conclusion, whereas hypoxia inhibits mTOR activity, mutations in the PTEN/Pik3r/tsc2 pathway that activate mTOR allow for continued mTOR activity even under hypoxic conditions. This can result in increased translation and hypoxic gene expression, contributing to tumor progression.

Acknowledgments


Grant support: National Cancer Institute grants 5/766 and 8/2566. N. Dornhofer was supported in part by a grant of the Else Kröner-Fresenius-Foundation.

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We thank Sharon Clarke for her invaluable administrative assistance and Pauline Chu at the Department of Comparative Medicine, Stanford University for sectioning and subsequent immunohistochemistry of tumors.

References

Mutations in the PI3K/PTEN/TSC2 Pathway Contribute to Mammalian Target of Rapamycin Activity and Increased Translation under Hypoxic Conditions

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