Hypoxia inducible factor 1 (HIF-1), a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits, serves as a key regulator of metabolic adaptation to hypoxia. The amount of HIF-1α protein is regulated either by attenuating von Hippel-Lindau protein (pVHL)-dependent ubiquitination and subsequent 26 S proteasomal degradation or by enhancing cap-dependent mRNA translation, presumably involving a phosphatidylinositol 3-kinase (PI3K)/Akt-regulated pathway. In addition, it became apparent that Hsp90 protects HIF-1α from oxygen-independent degradation. Here we present evidence that PI3K/Akt is required for heat shock proteins to stabilize HIF-1α. In pVHL-deficient renal cell carcinoma cells, PI3K inhibition by LY294002 and wortmannin or transfection of either a dominant-negative HIF-1α or a kinase-dead Akt mutant substantially lowered constitutively expressed HIF-1α without altering HIF-1α mRNA. Inhibitors of mitogen-activated protein kinase kinase (MAPKK) such as PD98059 or the p38 MAPK inhibitor SB203580 showed no interference. Considering that PI3K inhibitors down-regulated heat shock protein 90 (Hsp90) as well as Hsp70 expression and moreover attenuated heat- or hypoxia-induced Hsp70 as well as hypoxia-induced Hsp90 up-regulation, we conclude that PI3K inhibition promoted degradation of HIF-1α indirectly by reducing steady state concentrations of Hsp90 and/or Hsp70. PI3K co-immunoprecipitated with Hsp90/Hsp70 and direct binding of Hsp70 to the oxygen-dependent degradation domain (ODD) of HIF-1α was proven by a pull-down assay and a peptide array. PI3K-mediated degradation of HIF-1α was confirmed in HEK 293 cells under hypoxia, suggesting that heat shock proteins constitute an integral component for HIF-1α accumulation. We conclude that PI3K/Akt contributes to HIF-1α stabilization by provoking expression of heat shock proteins.

Hypoxia inducible factor-1 (HIF-1) is a heterodimeric transcription factor that senses low oxygen availability and enhances activation of hypoxia-inducible genes involved in energy/iron metabolism, angiogenesis, erythropoiesis, cell proliferation, and cell survival decisions (1–3). HIF-1 is composed of the basic-helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) proteins HIF-1α and HIF-1β, known as the aryl hydrocarbon nuclear translocator (4, 5). Under normoxia HIF-1α protein is kept at a low or undetectable level because of continuous degradation via the 26 S proteasome pathway, whereas HIF-1β is constitutively present.

HIF-1α is shown to physically interact with heat shock protein 90 (Hsp90) (6, 7) with the notion that only dephosphorylated HIF-1α associates with Hsp90 (8, 9). Recent results suggest that Hsp90 protects HIF-1α from being oxygen-independent, i.e. pVHL-independent degraded by an unidentified proteasome pathway (10, 11), and affects HIF-1α-DNA binding (12). Interestingly, several studies reported hypoxia-evoked Hsp70 expression (13–15). Hsp70 is regulated by several protein kinases such as protein-tyrosine kinase (16), protein kinase A (17), or phosphatidylinositol 3-kinase (PI3K) (18, 19). In some analogy, Hsp90 synthesis is attenuated by protein kinase A inhibitors (17). Furthermore, Beak et al. (20) noticed that activation of the heat shock transcription factor, which is a key regulator of heat shock proteins, is protein kinase Cδ (PKCδ)-dependent, which itself is affected by the upstream located PI3K.

Stabilization of HIF-1α under hypoxic conditions revealed a crucial role of prolyl hydroxylases (HIF-prolyl hydroxylases) (21–23). Hydroxylation at proline 564 and/or proline 402 of HIF-1α is necessary and sufficient for binding of the von Hippel-Lindau protein (pVHL) to HIF-1α with concomitant ubiquitination and 26 S proteasomal degradation of HIF-1α. Notably, hypoxia transition metals such as CoCl2 or the iron chelator desferroxamine block HIF-prolyl hydroxylases and in turn provoke HIF-1α stabilization. In addition, several studies pointed to increased translation (24–26) and possibly transcription (25) as mechanisms to accumulate HIF-1α. A PI3K/Akt-dependent signaling pathway that culminates in phosphorylation of essential components required for the cap-dependent translational machinery such as 4E-BP1 or p70 S6 kinase mediates HIF-1α accumulation in response to a variety of growth factors, hormones, or cytokines. Consistently, inhibitors targeting PI3K/Akt or FK506 binding protein-rapamycin-associated protein (FRAP/mTOR) prevent among others epidermal growth factor-, insulin-, interleukin-1β-, or tumor necrosis factor α-evoked HIF-1α responses (27–30). However, the PI3K pathway appears cell-type specific and is questioned by guest on April 27, 2019 http://www.jbc.org/ Downloaded from

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to participate in the hypoxic response (31, 32). Furthermore, it emerges that stabilization and transcriptional activation of HIF-1α are two separated processes that are regulated by hypoxia/activin single pathways. Resistance of hydroxylation at distinct residues. Hydroxylation of proline 564/402 affects protein stability, whereas HIF-1 transcriptional activity is affected by hydroxylation of Asn-803. These modifications are regulated through partial overlapping and/or distinguishable pathways (33, 34).

Herein, we determined molecular details of a pVHL-independent, but P13K/Akt-dependent signaling pathway to degrade HIF-1α. Attenuating P13K/Akt decreased HIF-1α protein in pVHL-deficient renal cell carcinoma (RCC) cells without altering the steady state of HIF-1α mRNA. An active P13K/Akt pathway is required for HIF-1α stabilization and degradation in RCC4 cells. We conclude that inhibition of P13K/Akt not only attenuates translation of HIF-1α as previously reported but also contributes to Hsp synthesis. Under conditions of long-lasting P13K inhibition expression of the Hsp is impaired, which in turn destabilized HIF-1α. This implies a so far unappreciated role of the P13K/Akt pathway in affecting HIF-1α degradation via Hsp expression regulation.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were of the highest grade of purity and commercially available. Fetal calf serum was from Biochrom, Medium and supplements were purchased from PAA Laboratories (Pasching, Austria). LY294002, wortmannin, MG132, cycloheximide (CHX), imidazol, anti-actin, and anti-glutathione S-transferase (GST) antibodies were ordered from Sigma. A protein assay kit was bought from Bio-Rad. Protease inhibitor mixtures were from Roche Diagnostics. Nitrocellulose membranes, ECL detection system, and horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies were delivered by Amersham Biosciences. The peqGOLD RNAse kit was from Peqlab and primers were ordered from MWG-Biotech. Platinum SYBR Green qPCR Super Mix UDG came from Invitrogen. HIF-1α antibody, Advantage RT-for-PCR kit, AdvanTaq PCR kit, and GSH-agarose were purchased from BD Biosciences. SuperFectTM transfection reagent, Ni-NTA agarose and incubated at 4°C, 10 s, 72°C, 10 s. Immunoprecipitation—Cells were treated, scraped off the dishes and collected. Cell pellets were reconstituted with 300 µl of buffer B (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture, pH 7.5), followed by centrifugation (35 000 × g, 10 min). Proteins were loaded on 7.5% SDS-PAGE. Western analysis was performed using anti-Hsp70 or anti-GST antibodies. Culture—Human embryonic kidney (HEK 293) cells and renal cell carcinoma (RCC4 or RCC4/pVHL) cells were cultured in Dulbecco’s modified Eagle’s medium with 4.5 g/liter D-glucose. Medium contained 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. RCC4/pVHL cells were cultured in the same medium with the addition of 500 µg/ml G418 and cells were maintained in medium without G418 for 24 h prior to experiments. Cells were transfected two times a week and medium was changed prior to experiments. Cells were kept in a humidified atmosphere of 5% CO2 in air at 37°C.

Cell Transfection—4 × 105 RCC4 cells were seeded in 6-cm dishes 1 day before transfection. At a rate of 60% confluence, cells were transfected using SuperFectTM transfection reagent following the instructions provided by the manufacturer.

Western Blot Analysis—HIF-1α was quantified by Western analysis. Briefly, cells were incubated, scraped off, lysed in 150 µl of buffer A (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture, pH 7.5), sonicated, and followed by centrifugation (15 000 × g, 15 min). 80 µg of protein was added to the same volume of 2× SDS-PAGE sample buffer and boiled for 5 min. Proteins were resolved on 7.5% SDS-polyacrylamide gels. Gels were washed with blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 5 min, proteins were blotted onto nitrocellulose membranes by a semi-dry transfer cell (Bio-Rad), and unspecific binding sites were blocked with 5% milk/TTBS (50 mM Tris-HCl, 140 mM NaCl, 0.05% Tween 20, pH 7.2) for 1 h. The primary antibody was diluted in 1% milk/TTBS (1:1000 for anti-HIF-1α antibody, 1:500 for anti-actin antibody, 1:2000 for anti-Hsp90, anti-Hsp70, and anti-GST antibodies) and incubated with the membrane overnight at 4°C. After washing, nitrocellulose membranes were washed 3 times for 5 min each with TTBS. For protein detection, blots were incubated with a horseradish peroxidase-labeled goat anti-mouse secondary antibody (1:2000 in 1% milk/TTBS) or horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:2000 in 1% milk/TTBS) for 1 h, washed 3 times for 5 min each with TTBS, followed by ECL detection.

Peptide Array Assay—The peptide array containing HIF-1α peptide spots was generated following SPOT synthesis. Briefly, 272 overlapping
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RESULTS

PI3K Is Required to Maintain HIF-1α Expression in RCC4 Cells—Considering some controversy on the role of PI3K in affecting HIF-1α expression we wanted to better understand the involvement of PI3K in stability regulation of the HIF-1α protein. We started experiments in RCC4 cells because they should show constitutively expressed HIF-1α because of a pVHL deficiency. RCC4, but as expected not RCC4 cells with pVHL being reintroduced (RCC4/pVHL), revealed basal HIF-1α expression. Cells were treated with PI3K inhibitors such as 3 to 30 μM LY294002 or 10 to 100 nM wortmannin for 4 h (Fig. 1, A and B) and HIF-1α disappeared following dose-dependent inhibition of PI3K.

To confirm HIF-1α down-regulation by interfering with PI3K/Akt signaling and to exclude potential side effects of inhibitory drugs we transfected RCC4 cells with dominant-negative kinase mutants of either PI3K or Akt (Fig. 1, C and D). RCC4 cells were transfected with 1 μg of plasmids allowing expression of a dominant-negative PI3K (pSRα-Ap85) or a kinase-dead mutant of Akt (pCMV5-m/p-PAK179A). Compared with transfection with wild-type PI3K (pSRα-Wtp85) or an empty vector plasmid (pCMV5) dominant-negative kinases decreased HIF-1α expression substantially. In contrast to PI3K inhibitors, the MAPK kinase inhibitor PD98059 or the p38 MAPK inhibitor SB203580 left HIF-1α expression in RCC4 cells unaltered (Fig. 1E). These experiments imply that pharmacological intervention or a molecular approach to attenuate PI3K/Akt signaling decreased HIF-1α expression in RCC4 cells. It remains to be clarified how an inactive PI3K/Akt pathway contributes to HIF-1α down-regulation.

Inhibition of PI3K Promoted Degradation of Constitutively Expressed HIF-1α—HIF-1α accumulation either results from enhanced protein synthesis or attenuated protein degradation. As a first potential target of PI3K inhibitors we examined HIF-1α mRNA levels in RCC4 cells by semi-quantitative RT-PCR (Fig. 2A) and quantitative real-time PCR (Fig. 2B). A total overlap of traces from controls, LY294002-, and wortmannin-treated samples showing the dependence on the cycle number relative to the fluorescence increase during quantitative real-time PCR excluded variations in HIF-1α mRNA to account for alterations noticed at the HIF-1α protein level. Quantification showed that neither LY294002 (mRNA amount is 1 ± 0.03
Impact of PI3K inhibitors on HIF-1α protein expression. RCC4 cells were first treated with 30 μM LY294002 (LY) or 50 μM cycloheximide (CHX) for 8 h. Thereafter, cells additionally received 5 μM of the proteasome inhibitor MG132 and incubations were continued for 4 h. B, RCC4 cells were treated with 5 μM of the proteasome inhibitor MG132 for the time indicated. C, RCC4 cells were first treated with 50 μM cycloheximide for 2–8 h. Thereafter, cells additionally received 5 μM MG132 and incubations were continued for 4 h. Accumulation of HIF-1α was determined by Western analysis as described under “Experimental Procedures.” Figures are representative of three independent experiments.

 normalized to the control) nor wortmannin (mRNA amount is 1 ± 0.08 normalized to the control) caused mRNA alterations.

In a second step we approached the question of HIF-1α degradation. For these experiments LY294002 or wortmannin were used during an 8-h incubation period to decrease HIF-1α expression in RCC4 cells (Fig. 3A). Thereafter we supplied the proteasome inhibitor MG132 at a concentration of 5 μM for 4 h, which allowed full recovery of HIF-1α expression that otherwise was compromised by PI3K inhibition. As expected, decreasing HIF-1α expression by blocking protein translation with CHX did not allow regaining of the protein upon MG132 treatment, implying that LY294002 did not block basal translation of HIF-1α mRNA compared with CHX. The proteasome inhibitor MG132 blocked HIF-1α protein degradation but did not disturb HIF-1α protein synthesis. Choosing a low exposure time of the x-ray film allowed detection of increased HIF-1α protein after 2 h of MG132 (5 μM) treatment with a maximal HIF-1α response seen around 4 h with no further increase noticed at 8 h (Fig. 3B).

In contrast, the protein translation inhibitor CHX blocked HIF-1α protein synthesis. As seen in Fig. 3C, a 4-h CHX treatment lowered HIF-1α expression substantially compared with controls and HIF-1α disappeared when CHX was supplied for 8 h. Once HIF-1α had been depleted with CHX, the protein could not be recovered by subsequent addition of 5 μM MG132 for 4 h.

We conclude that a pVHL-independent but proteasome-dependent degradation process explains the HIF-1α disappearance by PI3K inhibitors. Considering that besides the HIF-prolyl hydroxylases/pVHL/proteasome degradation system Hsp90 may play a role in pVHL-independent HIF-1α degradation, we investigated whether heat shock proteins are involved in decreasing HIF-1α upon PI3K inhibition.

Expression of Hsp90 and Hsp70 Is Decreased by PI3K Inhibition—Hsp90 is known to participate in HIF-1α stability regulation (10, 11). Therefore, it was our intention to search for a connection between PI3K signaling and heat shock proteins. For these experiments RCC4 cells were treated with 1 μM geldanamycin, 30 μM LY294002, or 100 nM wortmannin for 8 h (Fig. 4A). As anticipated the Hsp90 inhibitor geldanamycin decreased HIF-1α expression without altering protein amounts of Hsp90 or Hsp70. In contrast, the PI3K inhibitors LY294002 and wortmannin not only decreased the amount of HIF-1α but also abrogated expression of Hsp90 as well as Hsp70. A time-dependent study revealed that LY294002 effects became apparent after 8 h and persisted at least up to 24 h (Fig. 4B). Interestingly, Western analysis showed that protein disappearance of HIF-1α, Hsp90, and Hsp70 followed a similar time...
FIG. 4. PI3K inhibitors but not geldanamycin attenuate Hsp90 and Hsp70 expression. RCC4 cells were incubated with PI3K inhibitors LY294002 (30 μM) (A and B), wortmannin (100 nM) (A), the MAPKK inhibitor PD58059 (50 μM), the p38 MAPK inhibitor SB203580 (20 μM) (C), or 1 μM geldanamycin (A and C). HIF-1α, Hsp90, and Hsp70 expression was determined by Western analysis as described under “Experimental Procedures.” Unless indicated otherwise, incubations were done for 8 h. Blots are representative of three independent experiments.

response. Decreased protein expression of all three proteins was seen at 8 h and stayed low up to 24 h.

In contrast we established that the MAPKK inhibitor PD58059 (50 μM) and the p38 MAPK inhibitor SB203580 (20 μM), both incubated for 8 h, left HIF-1α expression as well as protein amounts of Hsp90 and Hsp70 unaltered (Fig. 4C). For comparison, 1 μM geldanamycin decreased HIF-1α, left Hsp90 expression unaltered but increased the Hsp70 protein amount, whereas LY294002 (30 μM) reduced expression of all three proteins.

Interactions of HIF-1α with Hsp90 and Hsp70—To prove that HIF-1α interacts with Hsp90 and Hsp70 in vivo, immunoprecipitation studies were performed using the anti-HIF-1α antibody (Fig. 5). RCC4 cells were treated with 30 μM LY294002 or left untreated. Immunoprecipitation results confirmed a direct association between HIF-1α, Hsp90, and Hsp70 in control cells.

However, under conditions of LY294002 treatment we precipitated less HIF-1α because the HIF-1α protein amount decreased as seen in Fig. 4. Therefore, it was without surprise that less Hsp90 or Hsp70 co-immunoprecipitated with HIF-1α under those conditions. Decreased co-immunoprecipitation of HIF-1α with either Hsp90 or Hsp70 under LY294002 treatment reflects less input, i.e. HIF-1α loading rather than allowing the assumption that the protein-protein interaction is attenuated.

Hsp70 Interacts with the ODD Domain of HIF-1α—Previous studies have shown that Hsp90 interacts with the bHLH-PAS domain of HIF-1α (7). Having established that Hsp70 co-immunoprecipitates with HIF-1α, it was our intention to locate the potential binding site. Therefore, we transfected the RCC4 cell with pGST or pGST-HIF1αODD plasmids, and used GSH-agarose to pull-down HIF-1α and its potentially associated protein, i.e. Hsp70. Western analysis proved that Hsp70 directly associated with the ODD domain of HIF-1α (Fig. 6A). PI3K Inhibitor Attenuated HIF-1α Accumulation in HEK 293 Cells under Hypoxia—To confirm inhibition of PI3K in close association with HIF-1α degradation in cells other than RCC4 we employed human embryonic kidney (HEK 293) cells that contain a functional pVHL/ubiquitination/degradation system. HEK 293 cells, exposed to hypoxia (0.5% oxygen) for 8 h, responded with a robust HIF-1α accumulation signal. At this time we added 30 μM LY294002 or 100 nM wortmannin and continued incubations under hypoxic conditions for an additional 16 h (Fig. 7). Western analysis showed that inhibition of PI3K down-regulated Hsp90 and Hsp70 expression and caused disappearance of HIF-1α, whereas in controls prolonged hypoxia for a total of 24 h allowed high expression of HIF-1α. It was noticed that hypoxia up-regulated Hsp90 as well as Hsp70 expression, an effect completely antagonized by inhibition of PI3K.

The Impact of PI3K Inhibition on Hsp90/Hsp70 Expression—Having established that PI3K inhibitors decreased basal expression of Hsp90 as well as Hsp70 in RCC4 cells, we went on to explore the impact of PI3K inhibition during heat treatment (Fig. 8A). RCC4 cells were heat shocked at 42 °C for 8 h, followed by Western analysis of Hsp90 and Hsp70 expression. As expected, Hsp70 responded with increased protein synthesis toward heat treatment. Interestingly, pretreatments for 30 min with either LY294002 or wortmannin largely attenuated this response. Although Hsp90 was not induced by heat treatment, inhibition of PI3K decreased basal Hsp90 expression as seen before. In addition, we investigated whether hypoxia (0.5% oxygen, 8 h) might affect heat shock protein expression (Fig. 8B). Hypoxia induced both Hsp90 and Hsp70. Preincubations with LY294002 for 30 min largely attenuated hypoxia-evoked...
Hsp90 and Hsp70 induction, which pointed to a demanding role of PI3K in heat shock protein synthesis.

Consistent with results obtained by employing chemical inhibitors such as LY294002 and wortmannin, transient transfection of a dominant negative p85-PI3K subunit revealed inhibition of Hsp90 as well as Hsp70 expression (Fig. 8C). In contrast, expression of wild-type p85 left Hsp protein appearance unchanged. The role of Akt as a downstream target of PI3K signaling in promoting Hsp90 and Hsp70 expression was confirmed by transfecting a kinase-dead mutant of Akt (Fig. 8D). Whereas the kinase-dead mutant of Akt (pCMV5-m-p-PKBK197A) versus 1 μg of empty vector plasmid (pCMV5), Hsp90 and Hsp70 expression were determined by Western analysis as described under “Experimental Procedures.” Blots are representative of three independent experiments.

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PI3K Regulates Hsp90 and Hsp70 Protein Synthesis—To further substantiate an essential role of PI3K signaling in affecting Hsp90 as well as Hsp70 protein synthesis we performed [35S]methionine pulse labeling experiments (Fig. 9). Within 6–12 h substantial protein synthesis of Hsp90 and Hsp70 occurred, as detected by incorporation of radioactivity. In the presence of 30 μM LY294002, [35S] labeling of either Hsp90 or Hsp70 was drastically attenuated compared with
controls. This suggests protein synthesis of Hsp90 and Hsp70 to be under the control of the PI3K pathway.

**DISCUSSION**

Our study corroborates earlier observations on the involvement of PI3K in affecting HIF-1α protein appearance. In pVHL-negative RCC4 cells pharmacological inhibition of PI3K or a molecular approach to eliminate PI3K as well as Akt activity largely attenuated HIF-1α expression although HIF-1α mRNA expression and basal protein translation remained unchanged. PI3K inhibition not only decreased HIF-1α expression but also lowered protein amounts of Hsp70 and Hsp90 and effectively antagonized heat-elicted up-regulation of Hsp70 or hypoxia-induced Hsp90 and Hsp70 expression. Our study shows direct Hsp70-HIF-1α binding and suggests that an active PI3K/Akt pathway is required for heat shock protein, i.e., Hsp70 and Hsp90 synthesis. Long lasting PI3K inhibition decreased Hsp expression and thus favored vHLH-independent HIF-1α proteasomal degradation rather than blocking protein translation.

**PI3K/Akt Signaling via Hsp90 and Hsp70 Contributes to HIF-1α Protein Stability**—More recently it became apparent that HIF-1α and Hsp90 physically interact (6, 7). In addition, it was noticed that geldanamycin, a Hsp90 inhibitor, promoted efficient proteasomal, however, vHLH-independent degradation of HIF-1α (10, 11). These results are confirmed in our study by using geldanamycin to decrease expression of HIF-1α. Unexpectedly, inhibition of PI3K/Akt not only repressed HIF-1α but also constitutive expression of both, Hsp70 as well as Hsp90, whereas geldanamycin left heat shock protein expression unaltered. The impact of PI3K inhibition and Hsp expression became further evident when LY294002 or wortmannin attenuated heat-induced Hsp70 expression or hypoxia-evoked Hsp90 and Hsp70 up-regulation. Along that line dominant negative p85 or a kinase-dead mutant of Akt lowered Hsp90 and Hsp70 expression and LY294002 blocked Hsp90 as well as Hsp70 protein synthesis as shown by a [35S]methionine pulse labeling experiment. It must be concluded that basal as well as stimulated expression of heat shock proteins demands a functional PI3K/Akt pathway. Stress proteins, i.e., heat shock proteins, regulate fundamental cellular processes, such as folding, sorting, degradation, resolubilization of proteins, and assembly of proteins into larger aggregates. At least expression of Hsp70 is transcriptionally regulated by heat shock transcription factor 1, which seems to require phosphorylation for full activity (20). There are indications that PI3K either upstream of PKCδ or upstream of Rac/PKA contributes to heat shock transcription factor 1 activation and Hsp70 expression (20). This may explain why inhibition of PI3K down-regulated Hsp expression.

The interaction of Hsp90 with HIF-1α requires the PAS-bHLH structure of HIF-1α. Making use of a HIF-1α peptide array on a spot membrane and confirmatory results from HIF-1α-Hsp70 pull-down assays, allowed us to assign the binding region of Hsp70 to the ODD domain of HIF-1α. This interaction may be relevant for stabilizing HIF-1α. Association of HIF-1α with individual heat shock proteins or a combination of heat shock proteins as known for steroid hormone receptors may promote stabilization of HIF-1α along the observation that heat stabilized HIF-1α (9). In contrast, counteracting binding of Hsp90 to client proteins by geldanamycin, or by down-regulating Hsp expression by blocking the PI3K/Akt pathway will provoke degradation of those clients. The impertinent role of Hsp was further analyzed in pVHL-containing HEK cells. Hypoxia clearly stabilized HIF-1α, presumably by blocking pVHL-evoked hydroxylation and subsequent proteasomal degradation. Taking into account that translational regulation does not participate in HIF-1α stabilization under hypoxia it was interesting to see decreased protein amounts with PI3K being blocked in close association with decreased Hsp70 and Hsp90 expression. We may conclude that blocking PI3K for longer time periods interferes only indirectly with HIF-1α accumulation by attenuating Hsp70 and/or Hsp90 expression.

**PI3K/Akt in Translational Regulation of HIF-1α**—The use of chemical inhibitors of PI3K such as wortmannin or LY294002, or kinase-dead mutants of PI3K/Akt were shown to attenuate growth factor-, hormone-, or cytokine-stimulated HIF-1α protein accumulation (27, 28, 30). Reducing the HIF-1α protein amount resulted in attenuated DNA binding and a failure to stimulate transcription of reporter constructs or endogenous downstream HIF-1 target genes (27, 28). Similarly, inhibition of PTEN (phosphatase and tensin homolog), a negative regulator of the PI3K pathway, increased HIF-1α and activated HIF-1 transcriptional activity (27). Mechanistically, PI3K/Akt is linked to an increased rate of HIF-1α protein synthesis, rather than inhibition of degradation as known from the action of hypoxia. Heregulin, insulin-like growth factor-1, or insulin stimulate PI3K/Akt to further activate FRAP (FK506 binding protein-rapamycin associated protein; also known as mammalian target for rapamycin), which promotes increased translation of HIF-1α mRNA into protein (24, 28, 37). In detail, FRAP de-represses the translational regulatory protein eIF4E by phosphorylating and inactivating its binding protein 4E-BP1. FRAP also activates p70S6K, which stimulates the 40 S ribosomal protein S6. Thus, in a PI3K/Akt and rapamycin-sensitive manner increased translation from the 5′-untranslated region of HIF-1α mRNA leads to an increase in HIF-1α protein (24, 38). To explain a functional cellular response, e.g. growth factors, one has to assume that under these conditions the endogenous degradation pathway, i.e. prolyl-hydroxylase, is treated out and that transcriptional repression because of asparagine hydroxylation is relieved.

**The Role of PI3K/Akt in Hsp and HIF-1α Degradation Versus HIF-1α Translational Control**—Interfering with protein translation by attenuating PI3K signaling to block HIF-1α protein accumulation is different than the situation seen here. We repressed the PI3K/Akt pathway for at least 8–16 h and more importantly, followed disappearance of an already stabilized protein by using RCC4 cells. Our observation on LY294002-provoked pVHL-independent degradation of HIF-1α supports an earlier observation on the role of PI3K and HIF-1α appearance (39, 40). We went on to show that once HIF-1α disappeared its accumulation is regained by blocking proteasomal degradation with MG132. This implies ongoing basal translation of HIF-1α mRNA into protein as blocking of protein turnover again causes protein accumulation. As a consequence our experiments suggest a new role of the PI3K/Akt pathway in
HIF-1α protein degradation besides its established role in enforced HIF-1α mRNA translation.

Our observations may help to resolve existing controversy on the role of PI3K in coordinating a HIF-1α response. Under conditions of translational regulation of HIF-1α as achieved by growth factors, hormones, or cytokines, PI3K inhibitors will suppress assembly of the translational machinery and thus block protein appearance. Basal production of the protein as occurring in RCC4 cells or the classic hypoxic accumulation of HIF-1α because of blocked proteasomal degradation may turn out insensitive to PI3K inhibition as long as PI3K inhibition remains short. An exception may be hypoxia or NO under conditions where they stimulate Akt (41, 42), a situation when transcriptional and blocked degradation pathways might overlap in accumulating HIF-1α. With long lasting PI3K inhibition for periods extending 8 h, the mechanism of HIF-1α destabilization may involve altered Hsp expression. Blocking Hsp90 function with geldanamycin or blocking Hsp expression by PI3K/Akt inhibition eliminates the ability to stabilize the client HIF-1α. Heat shock proteins apparently are required to maintain HIF-1α stabilization even under conditions of hypoxia. Detailed knowledge on the functional interplay between HIF-1α and heat shock proteins will help to understand the full dynamic range of HIF-1α stability regulation.

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