Constitutive/Hypoxic Degradation of HIF-α Proteins by the Proteasome Is Independent of von Hippel Lindau Protein Ubiquitylation and the Transactivation Activity of the Protein*§

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The transcriptional activator complex HIF-1 plays a key role in the long term adaptation of cells and tissues to their hypoxic microenvironment by stimulating the expression of genes involved in angiogenesis and glycolysis. The expression of the HIF-1 complex is regulated by the levels of its HIF-α subunits that are degraded under normoxic conditions by the ubiquitin-proteasome system. Whereas this pathway of HIF-α protein degradation has been well characterized, little is known of their turnover during prolonged hypoxic conditions. Herein, we describe a pathway by which HIF-1α and HIF-2α proteins are constitutively degraded during hypoxia by the proteasome system, although without requirement of prior ubiquitylation. The constitutive/hypoxic degradation of HIF-α proteins is independent of the presence of VHL, binding to DNA, or the formation of a transcriptionally active HIF-1 complex. These results are further strengthened by the demonstration that HIF-α proteins are directly degraded in a reconstituted in vitro assay by the proteasome. Finally, we demonstrate that the persistent down-regulation of HIF-1α during prolonged hypoxia is mainly caused by a decreased production of the protein without change in its degradation rate. This constitutive, ubiquitin-independent proteasomal degradation pathway of HIF-α proteins has to be taken into account in understanding the biology as well as in the development of therapeutic interventions of highly hypoxic tumors.

Changes in gene expression in response to decreased oxygen availability are largely regulated by the activity of the HIF-1 complex, a transcriptional activator complex constituted with two types of subunits: HIF-β that is constitutively expressed and HIF-α, whose expression is regulated by oxygen (reviewed in Refs. 1–3). Under normoxic conditions, the HIF-α subunits are rapidly degraded by the proteasome system following its ubiquitylation by the von Hippel Lindau (VHL)2 protein that acts as its E3 ligase (4). The interaction of HIF with VHL is greatly enhanced by the hydroxylation of prolyl residues located in the oxygen-dependent degradation domain (ODD) of the α proteins (5, 6). In the HIF-1α isoform, Pro-402 and Pro-564 residues are the substrates for hydroxylation that are mediated by specific prolyl-hydroxylase enzymes. These enzymes belong to a family of iron-containing hydroxylases (i.e. PHD1, PHD2, and PHD3) that have low affinity for oxygen and can act as oxygen sensors (7, 8). Under conditions of oxygen sufficiency, the HIF-α proteins are hydroxylated, ubiquitylated, and degraded by the proteasome system. Thus, in normoxia the HIF-α protein half-life is extremely short, and very low or undetectable levels are found in well oxygenated cells. During hypoxia, HIF-1α accumulates, dimerizes with HIF-1β, and forms the transcriptionally active HIF-1 complex. Similar increases in HIF-1 complexes are found in normoxic VHL-deficient cells because of their inability to degrade HIF-α proteins (9). Whereas the above described mechanism provides a tight control of HIF-α levels under normoxic conditions, little is known about the mechanisms that may control the fate of HIF-α proteins in hypoxic cells. Several transcription factors have more than one mechanism of control that operates under variable conditions of stimulation. Moreover, there is evidence that transcriptional activity by itself may regulate the survival of transcription factors as a way to control its unlimited activity (10). For some of these factors, there is a functional overlap between protein sequences that activate transcription and those required for signal-dependent ubiquitin-mediated proteolysis (11). By having the destruction of transcription factors “hard wired” into the transcription process, cells ensure that repeated rounds of transcription are constantly replenished by newly synthesized factors, and furthermore, it ensures that activators do not persist longer than are required. A consequence of these mechanisms of control is the requirement that degradation complexes be formed close to the site of transcription. Indeed, there is now clear evidence that proteasomal components interact with chromatin and can influence transcription at multiple levels (12–14). The proteasome is composed of a 20S proteolytic core capped by two 19S regulatory complexes

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‡ The abbreviations used are: VHL, von Hippel Lindau; ODD, oxygen-dependent degradation domain; Chx, cycloheximide; Z, benzotrioxycarbonyl; FMK, fluoromethylketone; AA, amino acids; ts, temperature-sensitive.

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The preparation of cell extracts for Western blots was as described previously (19). Briefly, cells were homogenized in 8 M urea buffer (8 M urea in 10 mM Tris, pH 6.8, 1% SDS, 5 mM dithiothreitol in the presence of a 1× protein inhibitor mix) with a portable Ultra-Turrax homogenator. 60 μg of total cell extract for each sample were resolved by SDS-PAGE using either 7.5 or 4–20% gradient gels (Bio-Rad), transferred to polyvinylidene difluoride membrane and immunoblotted with primary and subsequently with horseradish peroxidase-conjugated secondary antibodies. An ECL-plus kit (Amersham Biosciences) was used for development.

Immunoprecipitations were performed as described (19). Cells were lysed in lysis buffer (1% Triton, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride) in the presence of a 1× protease inhibitor mix. The primary antibody incubation was performed for 4 h at 4 °C followed by precipitation with protein A-Sepharose (Pierce). Precipitates were extensively washed in lysis buffer for subsequent immunoblots.

Plasmids and Transfections—Full-length HIF-1α, HIF-1αΔ1–27, HIF-1αΔ392–575 (originally provided by Dr. Semenza, Johns Hopkins), HIF-1α P402A, P564G (originally provided by Dr. P. Ratcliffe, Oxford), HIF-1αΔ1–391, HIF-1α393–580, and HIF-1α393–580 P402A, P564G were cloned into pCMV-3FLAG-1A vector containing FLAG tag (Stratagene) and PCDNA3 vector (Invitrogen) by PCR-based strategies; mutant constructs including K532R, Δ695–785 were generated using the QuikChange Site-directed mutagenesis kit (Stratagene). The ODD-nl plasmid, containing a nuclear localization signal, was based on the pShooter vector from Invitrogen. All plasmids were validated by DNA sequence analysis.

Transfections were performed as previously described using Lipofectamine 2000 (Invitrogen) (19).

Protein Degradation in in Vitro Reconstituted Proteasomal Assays—Rat liver 20S proteasome was purified as described (21, 22). The different HIF-1α constructs were in vitro transcribed with T7 RNA polymerase, and translated in the presence of [35S]methionine/cysteine (Amersham Biosciences) using the TNT system (Promega) according to the manufacturer’s instructions. Degradation reactions were performed essentially as described (21), and contained in a final volume of 20 μl: 20 mM Hepes pH 7.4, 2 mM EDTA, 1 mM EGTA, 1 μl of the respective 35S-labeled HIF-1α construct and 0.5–1 μg of purified rat liver proteasome (35–70 nM). Reactions were incubated at 37 °C for the times indicated and stopped with concentrated SDS-PAGE sample buffer. Control reactions contained 10 μM MG132. Samples, after boiling for 5 min, were loaded onto 10% SDS-PAGE gels. Gels were stained, destained, dried under vacuum, and exposed to x-ray film at −70 °C for 14–24 h as required. Quantitation was performed using the Quantity-One Software (Bio-Rad).

RNA Extraction and RT-PCR—Total RNA was extracted using a kit (RNeasy) from Qiagen (Valencia, CA) and utilized for reverse transcription (RT) as described (19). RT-PCR against mouse HIF-1α utilized primers as follows: forward, 5′-TCTCGGCGAAAGAAGAGGTCTGAA-3′; reverse, 5′-CACAAATCAGCAGCAAGCAGTCA-3′.

MATERIALS AND METHODS

Cell Culture—Cell lines HT1080, Hela, A549, 786-0, Caki-1, HTB-26, C4, 1C1C7 were from ATCC. RCC4 VHL (−/−) and VHL (+/+) were generously provided by Dr. Ratcliffe (Oxford, UK), and ts20 cells were from Dr. H. L. Ozer (Rutgers). Cells, with the exception of ts20, were cultured at 37 °C, 5% CO2 in recommended medium supplemented with 10% fetal bovine serum. ts20 cells were cultured at 35 or 39 °C according to the experimental requirements (20). For hypoxia treatment, cell dishes were placed in an oxygen station (In VIVO2, Ruskin Tech), flushed with gas containing 1% O2, 5% CO2, and 94% N2 and incubated for the indicated times.

Chemicals and Reagents—Common chemicals, solvents, and general reagents were from Sigma. Cycloheximide, desferroxamine, E64, leupeptin, lactacystin were from Sigma; MG132, Epoxomicin, ALLN, ALLM, and Z-VAD-FMK were from BioMol (Plymouth Meeting, PA).

ImmunobLOTS and Immunoprecipitations—Antibodies were purchased as follows: monoclonal anti-hHIF-1α from BD Biosciences (San Diego, CA); polyclonal anti-HIF-2α from Novus Biologicals (Littleton, CO); monoclonal anti-mHIF-1α from R&D Systems; monoclonal anti-FLAG M2 from Sigma; monoclonal anti-α-tubulin from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal anti-ubiquitin from Stressgen (Ann Arbor, MI). p21 rabbit polyclonal antibodies from Santa Cruz Biotechnology, and anti-cyclin B1 monoclonal from BD Biosciences.
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**RESULTS**

**HIF-1α and HIF-2α Proteins Are Constitutively Degraded by a VHL-independent Mechanism**—We first evaluated the role of the proteasome system in degrading HIF-α proteins in the absence of VHL by treating VHL-deficient (−/−) cells with the proteasome inhibitor Z-LLL-CHO (MG132) and measuring the accumulation of proteins following exposure for up to 12 h. Fig. 1A shows that MG132 treatment induced a steady accumulation of HIF-1α and HIF-2α in RCC4 cells, while no significant changes were observed in tubulin levels. HIF-2α also accumulated in 786-O cells, which do not express HIF-1α. No changes were observed in the levels of HIF-1β (ARNT) protein (not shown). Similar results to those obtained with MG132 were observed with more specific proteasome inhibitors like lactacystin and epoxomycin, while other protease inhibitors like E64, leupeptin, and calpain inhibitors (Z-VAD-FMK and ZLLL-CHO) were not effective (data not shown). At the concentrations used, there was no evidence of cell toxicity or apoptosis.

The steady accumulation of HIF-α proteins in cells treated with proteasome inhibitors indicated that there is a continuous turnover of these proteins in VHL-deficient cells. To further study this turnover, we treated VHL (−/−) cells with cycloheximide, a protein synthesis inhibitor, and measured the rate of HIF-α protein decay. Fig. 1B shows that in the absence of VHL, both HIF-1α and HIF-2α proteins are degraded with a half-life of about 3–4 h. More importantly, a similar pattern of degradation was observed in HT1080 cells stimulated by hypoxia plus desferroxamine (Dfx) and exposed to cycloheximide (Fig. 1C). Similar results were obtained in several other cell lines including HeLa, HTB-26, and RCC4 (VHL (+/+)) cells, as well as in RCC4 (VHL (−/−)) cells equally treated with Hx and Dfx. In all cases, the effect of cycloheximide was completely abrogated in the presence of the proteasomal inhibitors MG132, epoxomycin, and lactacystin, but not by calpain inhibitors, clearly suggesting that the observed degradation of the proteins is due to the proteolytic activity of the proteasome. These results indicated that HIF-α proteins are continuously degraded, even during severe hypoxic conditions, by the proteasome system in a VHL-independent way.

**VHL-independent Degradation of HIF Proteins Does Not Require Ubiquitylation**—Because in the absence of VHL or under the condition where PHD enzymes are highly inhibited (hypoxia plus desferroxamine) the “normal” HIF-α ubiquitylation system is impaired, we investigated whether ubiquitylation was at all necessary for the VHL-independent degradation. For this purpose we utilized ts20 cells that have a temperature-sensitive E1 enzyme and therefore, at the non-permissive temperatures, ubiquitylation is almost completely impaired (20). Fig. 2A shows that in ts20 cells exposed to 39 °C for 18 h, exposure to cycloheximide induces the decay of HIF-1α with a half-life of about 3–4 h, and that this effect is completely reversed by proteasome inhibitors MG132, epoxomycin, and lactacystin (Fig. 2B). When the same cell line was cultured at 35 °C and exposed to hypoxia for 18 h, it was found that the HIF-1α decay was similar to the 39 °C-treated cells and equally prevented by MG132 treatment (Fig. 2C). Furthermore, we confirmed the inactivation of the E1 enzyme at 39 °C by finding that p21, a degradation ubiquitin-independent protein, was equally degraded at 35 or 39 °C, while cyclin B1, a degradation ubiquitin-independent protein, was only degraded at 35 °C (supplemental Fig. S1). Unfortunately, the degradation of HIF-2α in ts20 cells could not be studied because its level of expression was below the sensitivity of the assay. To confirm that ubiquitylation was not involved in HIF-α protein degradation, we exposed RCC4 VHL-competent (+/+)- and -incompetent (−/−)- cells to MG132, immunoprecipitated HIF-1α, and performed immunoblotting with anti-ubiquitin antibodies. As shown in Fig. 2D, HIF-1α accumulated in VHL (−/−) cells is not ubiquitylated whereas it is highly ubiquitylated in similarly MG132-treated RCC4-VHL-competent cells. The immunoprecipitation data are consistent with the pattern of HIF-1α migration in SDS PAGE (7.5%) shown in Fig. 2E, where accumulated HIF-1α in VHL (+/+)- cells display the typical polyubiquitylated forms, while only a single HIF-1α band is observed in VHL (−/−) cells.

**Sequences Involved in the Hypoxic Degradation of HIF-1α in Cultured Cells**—To further characterize the sequences and possible mechanisms involved in the constitutive/hypoxic degradation of HIF-1α, we utilized FLAG-tagged constructs con-
taining various deletions or mutations of HIF-1α in transient transfection assays in HT1080 cells. Following exposure to hypoxia plus desferroxamine, transfected cells were treated with cycloheximide, and the HIF-1α half-life was determined by Western blots using anti-FLAG antibodies. As shown in Fig. 3A, the full-length protein was degraded like the endogenous one, and its degradation was not affected by mutations involving a putative acetylation site (K532R) or the ODD domain. In contrast, the N-terminal 391 amino acid construct (1–391) was quite stable, whereas the HIF-1α construct 1–575 was rapidly degraded. The degradation rate of the ODD-deleted construct was intermediate between the wild-type HIF-1α and the stable N-terminal fragment. With the same proteasome preparations used in HIF in vitro degradation and under the same assay conditions, we performed control experiments of degradation of in vitro transcribed and translated IκB and p65, as previously reported (21). IκB was degraded (50% degradation in 30 min), whereas p65 was not significantly degraded after 3 h of incubation (data not shown).

Is the Constitutive Pathway Regulated?—Many transcription factors are regulated by their own transcriptional activity. This process could be secondary to the activation of genes that enhance their degradation, such as the case of p53 up-regulating Mdm-2 or by accelerating its degradation during their interactions with the basal transcriptional machinery (10). Of importance, protein sequences that are involved in degradation are usually contained or overlap transcriptional active domains (11). Recently, Demidenko et al. (18) reported that the HIF-1 complex would regulate its own activity through a negative feedback loop that would enhance HIF-1α destruction. To study the role of HIF-1 complex transcriptional activity on HIF-1α degradation, we utilized a mouse hepatoma cell line (HEPA) that is deficient in HIF-1β (ARNT (−/−)) and therefore cannot form active HIF-1 complexes (23). Measurements of HIF-1α decay following exposure to hypoxia plus desferroxamine for 4 h revealed that the HIF-1α half-life was similar in ARNT (−/−) and ARNT (+/+) cells (Fig. 4A). Moreover, it was not affected by a more prolonged hypoxic stimulation up to 48 h. To further study the relationship between HIF-1α tran-
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A

Transfection assays

Hox + Df

HIF1 (1-826)

HIF1 (K539R)

HIF1 (Δ402/2AP564G)

HIF1 (1-391)

HIF1 (1-575)

HIF1 (4-695)

HIF1 (Δ392-575)

HIF2 (1-870)

Tub

B

In vitro degradation assays

20S Proteasome

Time (hours)

0

1

2

4

4

HIF1 (1-826)

HIF1 (1-391)

HIF1 (1-575)

HIF1 (A392-575)

HIF2 (1-870)

FIGURE 3. Sequences involved in hypoxic degradation of HIF-1α. A, degradation rates of HIF-1α constructs in hypoxic cells. HT1080 cells were transiently transfected with plasmids containing FLAG-tagged constructs of the HIF-1α wild-type sequence (1–826) or with constructs containing mutations in the putative acetylation site (K539R) or hydroxylation sites (P402A, P564G). Other transfections included deletions of the C terminus-half (1–391, 1–575, and 1–695) and a deletion of the ODD (Δ392–575). Following transfection, cells were stimulated with hypoxia plus desferroxamine for 12 h, treated with Chx and total cell extracts analyzed by Westerns using anti-human HIF-1α antibodies. Representative figures from two or three independent experiments are shown.

B

FIGURE 4. Role of HIF-1 activity on HIF-1α degradation: role of HIF-1 complex formation. A, HEPA cells that are either ARNT-deficient (ARNT (−/−)) or competent ARNT (+/+)) were subjected to hypoxia plus desferroxamine for either 4 or 48 h, treated with Chx, and total cell extracts analyzed by Westerns using anti-FLAG antibodies. B, DNA binding does not affect HIF-1α survival. HEPA (ARNT (+/+)) and HT1080 cells were transfected with plasmids containing the full-length HIF-1α sequences (1–826) or a construct that lacks the N-terminal DNA binding domain (28–826). Following transfection, cells were exposed to hypoxia plus desferroxamine and treated with cycloheximide. Westerns were performed with anti-human HIF-1α (HEPA cells) or anti-FLAG antibodies (HT1080 cells). Representative figures from two or three independent experiments are shown.

Prolonged Hypoxia Decreases HIF-1α Levels but Does Not Affect Degradation—Several studies have indicated that HIF-1α levels steadily decrease during prolonged hypoxia, whereas the levels of HIF-2α are minimally affected (24, 25). The mechanism for the decline in HIF-1α levels is not clear, and both increased degradation (18) and decreased production of the protein have been proposed (36). Results utilizing HT1080, A549, and Caki-1 cells (Fig. 5A) confirmed that following a prolonged hypoxia, HIF-1α levels decrease substantially. Interestingly, HIF-2α was much less or not affected at all. Of note, we also observed a great variation in the degree of HIF-1α down-regulation between various cell lines. HIF-1α levels decay more rapidly in A549 and Caki-1 than in HT1080, HeLa, or RCC4 cells (not shown). Notably, despite the significant decrease in HIF-1α levels, prolonged hypoxia did not significantly affect the half-life of the protein, as shown in Fig. 5B. These results in HT1080 and A549 cells are similar to the findings in HEPA cells, already shown in Fig. 4A. Similarly, prolonged hypoxia did not affect the half-life of HIF-2α (not shown). Thus, the decrease in HIF-1α protein observed during prolonged hypoxia appeared more likely mediated by a decrease in its rate of synthesis rather than its increased degradation. To evaluate HIF-1α biosynthetic rates, we treated cells with MG132 and measured the accumulation of the protein during short periods of treatment. Fig. 5C shows that the accumulation of HIF-1α during 3 h of exposure to MG132 is markedly decreased in cells pre-exposed to hypoxia for 21 h compared with that found in normoxic cells or cells concomitantly exposed to hypoxia and MG132 for only 3 h. Hypoxia had no significant effect on the levels and accumulation of HIF-2α. The role of the HIF-1 complex on the decreased production of HIF-1α protein during prolonged hypoxia was evaluated in HEPA ARNT (+/+).
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ARNT (−/−) cells. As shown in Fig. 5D, prolonged hypoxia reduced the levels of HIF-1α only in ARNT (+/+) cells, while no effect was found in ARNT (−/−) cells. The effect of prolonged hypoxia is not secondary to changes in mRNA because no significant changes in HIF-1α messenger were observed between 8 and 24 h of hypoxia. As it was found in the other cell lines studied, the levels of HIF-2α were not affected by more prolonged hypoxia and were not affected by the presence or absence of ARNT (supplemental Fig. S2). These results indicate that the down-regulation of HIF-1α during prolonged hypoxia is secondary to an HIF-1 complex-dependent decreased production of the protein.

DISCUSSION

Hypoxia-inducible complex-1 is the major transcriptional activator of genes that respond to the absence or inadequacy of oxygen supply. Among them are genes involved in glucose metabolism, angiogenesis, and apoptosis; all of them of importance in adaptation to hypoxia and in cancer growth (26). The HIF-1 complex is formed by two types of subunits: HIF-β that is constitutively expressed and HIF-α that is regulated by oxygen tension (reviewed in Refs. 1–3). The two major components of the α-type, HIF-1α and HIF-2α have been extensively studied, whereas HIF-3α is still poorly characterized. Multiple studies have indicated that the expression, regulation, and targeted genes of both subunits may be quite different as may be their role in tumor development (27, 28). Of relevance, a recent report indicates that in neuroblastoma HIF-2α is the major subunit involved in HIF-1 complex formation and thus conferring a more malignant phenotype (25). Under adequate oxygenation, the HIF-α subunits are rapidly degraded by the ubiquitin-proteasome system by a mechanism that involves their hydroxylation and recognition by the VHL protein that acts as their ubiquitin-ligase (E3). During hypoxia, this process is disrupted, and HIF-α proteins accumulate and form the transcriptionally active HIF-1 complex. Likewise, in VHL deletions or mutations, HIF-1α proteins accumulate and activate their target genes (9). Whereas this process of degradation that assures a tight control during normoxia has been extensively characterized, little is known about the mechanisms that control the levels of HIF-α proteins during continuous hypoxia. Our results indicate that during hypoxia, HIF-1α...
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...tions with the α2 (C3) subunit of the core proteasome (22). Similarly, the retinoblastoma protein also appears to be degraded by the 20S proteasome in a process that requires no ubiquitylation, but is greatly enhanced by its interaction with MDM2, which facilitates the binding to the C8 subunit of the 20S proteolytic complex (31). Although the mechanisms involved in the constitutive/hypoxic degradation of HIF-α proteins are still not clear, our results indicate that this process is not mediated by acetylation or hydroxylation, though the odd sequences appear to have a predominant role. The transfection studies clearly demonstrate that the N terminus of the protein is quite stable and addition of ODD promotes degradation. However, these experiments do not rule out that other downstream sequences may also be involved. In vitro studies showed that HIF-α proteins were efficiently degraded by the 20S proteasome. Most importantly, the in vitro results conformed to the in vivo data showing that the N-terminal 391 amino acids were resistant to degradation, while addition of the ODD enhanced degradation. Targeting of proteins to the 20S proteasome appears to require the presence of an unstructured or disordered protein region; that is, regions that lack secondary structure (16, 17). These regions are believed to promote “gating” of the proteasome and allow the penetration of the target protein inside the proteolytic core. In some cases accessory molecules are involved as in the case of the human cytomegalovirus protein pp71, which targets the hypophosphorylated members of the retinoblastoma family for ubiquitin-independent degradation (33). Recent structural studies of the HIF-ODD region (AA403–603) have shown that under physiological conditions this region of the protein is highly unstructured, thereby accounting for the high versatility of this area, i.e. interactions with PHDs, VHL, p53, and ARD1 (34). It is yet unclear how HIF-α proteins interact with the 20S proteasome; however, an earlier report indicated that HIF-α can interact with the α-4 subunit through binding to the HIF-α inhibitory domain (695–785) or the ODD (403–603) (35).

One important mechanism by which cells can control the level and activity of transcription factors is by having their degradation “hard wired” to the transcription process itself. This process ensures that the transcription factors are in the proper conformation and do not linger longer than when they are needed. The exact nature of how transcriptional activity and destruction are linked is not completely clear, but several mechanisms have been proposed. Lipford et al. (13) have classified transcription factors as class A if degraded regardless of binding DNA, and class B and C if targeted only once they have bound DNA. In the case of HIF-α proteins, degradation was independent of DNA binding as the deleted N-terminal protein was degraded at the same rate as the wild-type form. Moreover, degradation rates were independent of the interaction with ARNT or the presence or absence of the C-terminal transcriptional domain. Notably, the rate of degradation was not significantly affected by prolonged hypoxia exposure. These results indicate that in contrast to p53 (10), HIF-1α appears not to have a feedback mechanism to control its own degradation rate. However, as already noted by others, prolonged hypoxia decreases HIF-1α levels, affecting less HIF-2α (22, 23). Of note, this effect varies significantly between different cell lines and may depend also on cell confluence or cell proliferation. The decrease in HIF-1α levels during prolonged hypoxia is mediated by a decrease in its production rather than by an increase in its degradation rate. This decrease in production, in turn, depends on the presence of an active HIF-1 complex, because it does not occur in the ARNT (−/−) cells. At least two mechanisms mediated by HIF-1 transcriptional activity that would inhibit HIF-1α production during prolonged hypoxia have been described. One involves the induction of REDD1, which acting through the mTOR pathway would inhibit HIF-1α mRNA translation (36). The other involves the transcriptional activation of a natural antisense messenger that would inhibit HIF-1α mRNA translation or accelerate its decay (22, 37). Of interest, this last mechanism appears not to affect the HIF-2α mRNA (22). It is unclear whether those mechanisms operate in every cell type and which one predominates under different physiological or pathological conditions.

In summary we describe a novel pathway of constitutive/hypoxic degradation of HIF-α proteins that is independent of VHL, ubiquitylation, and does not depend on the transactivation activity of the HIF-α proteins. This pathway is likely very important in the regulation of HIF-α proteins during sustained hypoxia. In this respect, it likely has more importance in the long term regulation of HIF-2α rather than HIF-1α, because the latter would autoregulate its expression by decreasing its production. Importantly, recent studies in neuroblastomas have shown that HIF-2α may be the main regulator of long term hypoxic gene expression. This finding may be true for other tumors where sustained hypoxic conditions are encountered. Thus, inhibition of this pathway may be relevant for the inhibition of HIF-1 complex in tumor therapy.

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