HIF-1-dependent transcriptional activity is required for oxygen-mediated HIF-1α degradation

Edurne Berra*, Darren E. Richard, Emmanuel Gothié, Jacques Pouysségur

Institute of Signaling, Developmental Biology and Cancer Research, CNRS-UMR 6543, Centre Antoine Lacassagne, 33 Avenue Valombrose, 06189 Nice, France

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Abstract Hypoxia-inducible factor-1α (HIF-1α) plays a central role in oxygen homeostasis. In normoxia, HIF-1α is a short lived protein, whereas hypoxia rapidly increases HIF-1α protein levels by relaxing its ubiquitin–proteasome-dependent degradation. In this study, we show that the p42/p44 MAP kinase cascade, known to phosphorylate HIF-1α, does not modulate the degradation/stabilization profile of HIF-1α. However, we present evidence that the rate of HIF-1α degradation depends on the duration of hypoxic stress. We demonstrate that degradation of HIF-1α is suppressed by: (i) inhibiting general transcription with actinomycin D or (ii) specifically blocking HIF-1-dependent transcriptional activity. In keeping with these findings, we postulate that HIF-1α is targetted to the proteasome via a HIF-1α proteasome targetting factor (HPTF) which expression is directly under the control of HIF-1-mediated transcriptional activity. Although HPTF is not yet molecularly identified, it is clearly distinct from the von Hippel–Lindau protein (pVHL).

Key words: Hypoxia; HIF-1; Proteasome; pVHL

1. Introduction

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor composed of the HIF-1α (120 kDa) and HIF-1β subunits (94 kDa) [1]. Both HIF-1 subunits belong to the subfamily of basic-helix-loop-helix transcription factors containing a PAS (PER-ARNT-SIM) motif [2]. HIF-1β is the already characterized aryl hydrocarbon nuclear translocator (ARNT) previously shown to heterodimerize with the aryl hydrocarbon receptor, whereas the HIF-1α subunit is specific for the hypoxic responses [3]. Recently, in addition to HIF-1α and HIF-1β, cloning experiments have identified several new members of the HIF family of transcription factors [4]. HIF-1 plays a central role in oxygen homeostasis by inducing the expression of a broad range of genes in a hypoxic-dependent manner: erythropoietin (EPO), vascular endothelial growth factor (VEGF), VEGFR-1, glucose transporter-1 (GLUT-1), inducible nitric oxide synthase (i-NOS), heme oxygenase (HO-1), transferrin, tyrosine hydroxylase, and almost every gene in the glycolytic pathway [5,6]. All these genes contain at least one hypoxia response element that specifies the binding of HIF-1.

Several studies have demonstrated that the hypoxic activation of HIF-1 is a multistage and a complex process. However, hypoxia-mediated induction of HIF-1α appears to be the primary mode of regulation since the expression of the rate limiting α subunit determines the activity of the HIF-1 complex [7,8]. HIF-1α is a short lived protein that is maintained at low and often undetectable levels in normoxia, whereas it is strongly induced in hypoxic cells [2,7]. Indeed, as quickly as HIF-1α is induced, it is even more rapidly degraded upon return to normoxic conditions [2,7]. Previous results have shown that, in oxygenated cells, HIF-1α is quickly ubiquitinated and degraded by the proteasome system, whereas hypoxia induces HIF-1α by relaxing its ubiquitin–proteasome-mediated degradation [8–10]. Hence, regulation of the proteasome-dependent degradation of HIF-1α appears to be a pivotal step in the hypoxic induction of HIF-1.

In spite of extensive studies on the molecular mechanisms underlying the proteasome-dependent degradation of HIF-1α, it is only recently that a major breakthrough has been done. The product of the von Hippel–Lindau tumor suppressor gene (pVHL) has been demonstrated to interact directly with HIF-1α as a component of an E3 ubiquitin–protein ligase complex responsible for the degradation of HIF-1α [11–14]. However, the association of pVHL and HIF-1α has been reported to be constitutive and independent of oxygen levels. Indeed, the signaling components regulating the 'switch' to proteasomal degradation in normoxic conditions are not still fully characterized. In this work, we show that p42/p44 MAP kinases, known to phosphorylate HIF-1α, are not implicated in the stabilization of HIF-1α protein or the regulation of its ubiquitin–proteasome-dependent degradation. We present evidence that HIF-1α degradation depends on HIF-1-mediated transcriptional activity. We propose a model in which a HIF-1α proteasome targetting factor, referred to here as HPTF, is involved in this process. We demonstrate that HPTF is distinct from the previously characterized pVHL, and we propose that a hypoxia-regulated gene encodes HPTF. This working model provides an autoregulatory feedback mechanism for HIF-1α degradation.

2. Materials and methods

2.1. Material and plasmid construction

The transcriptional inhibitor, actinomycin D, was from Boehringer Mannheim. PD 98059 was from New England Biolabs. Estradiol and monoclonal anti-phospho-p42/p44 MAP kinases antibody were from Sigma. The anti-HIF-1α antibody (antiserum 2087) was raised in our laboratory in rabbits immunized against the last 20 amino acids C-terminal of the human HIF-1α. Monoclonal antibody against pVHl

*Corresponding author. Fax: (33)-4-92 03 12 25.
E-mail: berra@unice.fr

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was from Pharmingen (Ig32). The HIF-1α expression vector pcDNA3-HA-DN-HIF-1α has previously been described [15].

2.2. Cell culture and transfections

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% inactivated fetal calf serum (FCS), penicillin G (50 U/ml), and streptomycin (50 μg/ml) (Gibco-BRL) in a CO_2 incubator (5% CO_2) at 37°C. CCL39-Raf-1:ER cells were cultured in the same media but in the absence of phenol red in order to reduce the basal activity of the chimera. Cells were arrested by total serum deprivation for 24 h. Hypoxic conditions were performed by incubation of cells in a sealed 'Bug-Box' anaerobic workstation (Ruskinn Technologies, Jouan, France). The oxygen in this workstation was maintained at 1–2% with residual gas mixture being 93–94% nitrogen, and 5% carbon dioxide.

Subconfluent HeLa cells on 60 mm culture dishes were transfected with 10 μg of either pcDNA3 or pcDNA3-HA-DN-HIF-1α by the calcium phosphate method. After 16 h, plasmid DNA was removed and cells were incubated in DMEM medium containing 7.5% FCS. At 48 h post-transfection, cells were incubated under normoxia (20% O_2) or hypoxia (1–2% O_2) for 3 h after which hypoxic cells were returned to 20% O_2 for different times. Under these conditions and using GFP as a co-marker, we usually transfected about 70–80% of cells.

2.3. Immunoprecipitation and immunoblot analysis

In order to analyze HIF-1α expression, cellular proteins were extracted with Triton X-100 lysis buffer (0.1% Triton X-100, 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, 40 mM β-glycerophosphate, 200 μM sodium orthovanadate, 5 μg/ml aprotinin, 0.7 μg/ml pepstatin, 0.5 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride) and total cellular extracts (50 μg) were resolved in sodium dodecyl sulfate (SDS)–polyacrylamide (7.5%) gels. For the pVHL protein, proteins from HeLa cells were extracted by using the previously described lysis buffer with addition of 0.5% sodium deoxycholate. Clarified lysates (500 μg) were incubated for 2 h with the anti-HIF-1α antiserum (5 μl). Immunocomplexes were collected on protein A Sepharose (Pharmacia), washed three times with the lysis buffer, boiled in Laemmli loading buffer, and separated by SDS–PAGE (12%). Afterwards, proteins were electrophoretically transferred onto a PVDF membrane (Immobilon-P, Millipore) and revealed with the specific antibodies as indicated. The bands were visualized with the ECL system (Amersham Pharmacia Biotech).

3. Results

3.1. p42/p44 MAP kinase activity does not affect the stabilization/degradation pattern of HIF-1α

Phosphorylation and dephosphorylation activities have often been implicated in the stabilization and/or degradation of a number of transcription factors, and other proteins regulated by the proteasome system [16]. Interestingly, results from our laboratory have demonstrated that HIF-1α is a phosphoprotein that is specifically phosphorylated by p42/p44 MAP kinases in vitro and in vivo [17]. In order to specifically evaluate whether p42/p44 MAP kinase activity affects ubiquitin–proteasome-mediated degradation of HIF-1α, we used a derivative of the CCL39 cell line stably expressing the Raf-1:ER chimera. This protein is a fusion between the catalytic domain of Raf-1, the MAPKKK upstream activator of the p42/p44 MAP kinase pathway, and the ligand binding domain of the estradiol receptor. Addition of estradiol to these arrested cells induces a rapid and strong stimulation of p42/p44 MAP kinases that persists as long as estradiol is present in the medium [18–20]. Therefore, CCL39-Raf-1:ER starved cells were incubated either in normoxic (20% O_2) or hypoxic (1–2% O_2) conditions as indicated in the legend in the presence of either estradiol or the MEK inhibitor, PD 98059, for the last 30 min. HIF-1α protein levels were determined by immunoblot analysis of total cell extracts. Results from Fig. 1A showed that HIF-1α is strongly and rapidly induced by hypoxia either in PD 98059-treated or estradiol-stimulated cells. Of note the marked differences in the migration pattern of HIF-1α corresponding to varying levels of phosphorylation. As we have previously demonstrated, HIF-1α migrates as a single band in quiescent cells, whereas the activation of the p42/p44 MAP kinase pathway induces the phosphorylation and the 'shift-up' of HIF-1α (Fig. 1). We next evaluated the profile of HIF-1α degradation upon reoxygenation in the same experimental conditions. As shown in Fig. 1B, this profile remained very similar in control or estradiol-stimulated cells. Interestingly, the rate of degradation seems to be identical for the hypophosphorylated and upper form of HIF-1α (Fig. 1B, right panel). These results demonstrate that p42/p44 MAP kinase cascade, in CCL39 cells, does not significantly modulate the stabilization/degradation kinetics of HIF-1α.

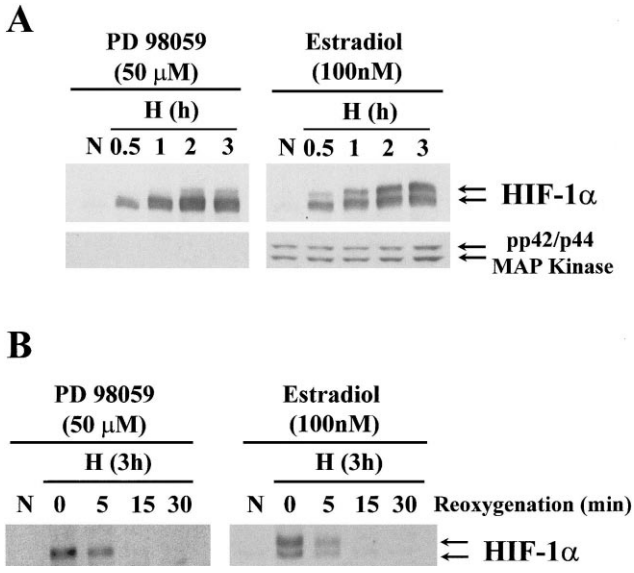

Fig. 1. p42/p44 MAP kinase activation does not affect the expression patterns of HIF-1α. (A) CCL39-Raf-1:ER cells were starved for 24 h, and then incubated under normoxia (20% O_2; N) or hypoxia (1–2% O_2; H) for different times. Before the end of each treatment (30 min), cells were stimulated with either 50 μM PD 98059 or 100 nM estradiol. (B) Starved CCL39-Raf-1:ER cells were incubated under normoxia (20% O_2; N) or hypoxia (1–2% O_2; H) for 3 h after which hypoxic cells were returned to 20% O_2 for 0–30 min. 30 min before the end of the hypoxic stress, Raf-1:ER cells were stimulated with either 50 μM PD 98059 or 100 nM estradiol. Total cell lysates were analyzed by immunoblotting with the antibodies to HIF-1α and anti-phospho-p42/p44 MAP kinase. Essentially identical results were obtained in three independent experiments.

3.2. Degradation of HIF-1α depends on the duration of hypoxic stress

HIF-1α is highly up-regulated by hypoxia in almost every cell type tested. Interestingly, in HeLa cells as compared to the CCL39 cell line, induced HIF-1α migrates with a pattern showing the different phosphorylated forms of the protein [17]. Therefore, for the rest of the study we used HeLa cells as our cellular model. Thus, HeLa cells were incubated under hypoxia for 1 h or 8 h after which they were returned to an oxygenated atmosphere for periods of time varying between 0 and 30 min. As previously demonstrated, results from Fig. 2A

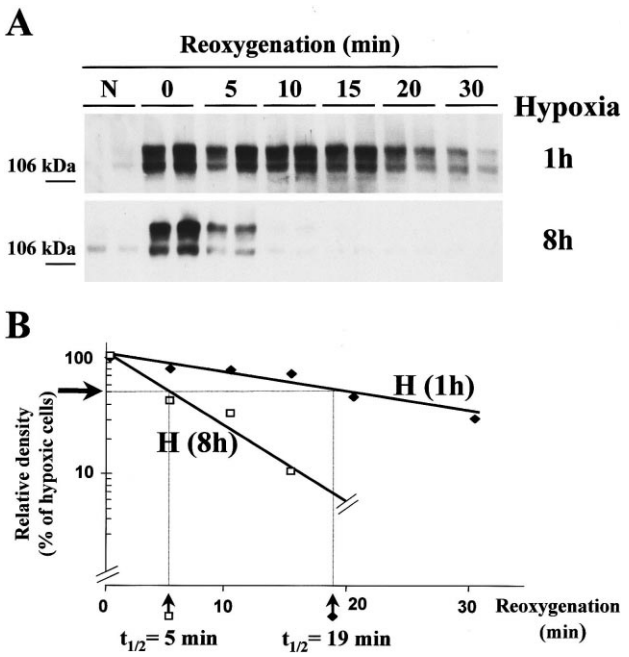

Fig. 2. HIF-1α half-life is inversely proportional to the duration of hypoxic stress. HeLa cells were incubated under normoxia (20% O_2; N) or hypoxia (1–2% O_2; H) for 1 h (♦) or 8 h (□) after which hypoxic cells were returned to 20% O_2 for 0–30 min. Total cell lysates were immunoblotted with anti-HIF-1α (A). The intensity of HIF-1α signals in A was quantified and plotted (B). Essentially identical results were obtained in three independent experiments performed in duplicate.

showed that HIF-1α is strongly induced by hypoxia and rapidly degraded upon reoxygenation of hypoxic cells. However and more interestingly, these results revealed that the rate of HIF-1α degradation depends on the duration of hypoxic stress. The increase of the hypoxic treatment from 1 h to 8 h reduced the half-life of HIF-1α by 5-fold. Cells which underwent the shortest hypoxic stress (1 h) had the longest HIF-1α half-life (19 min), whereas the $t_{1/2}$ of HIF-1α drastically fell to 5 min in cells that were maintained in hypoxia for 8 h (Fig. 2B). Indeed, cells stressed for 3 h showed an intermediate $t_{1/2}$ (data not shown). As a control, the same cellular lysates were analyzed by Western blot with an antibody against p42 MAP kinase. The protein levels of this kinase, which is not affected by O_2 tension, remained unmodified (data not shown). These findings suggest that the degradation of HIF-1α is dependent on hypoxia-induced transcription and/or translation.

3.3. Actinomycin D inhibits HIF-1α degradation

We wanted to evaluate whether transcriptional or translational activities were implicated in the regulation of HIF-1α breakdown. Unfortunately, since HIF-1α induction involves on-going protein synthesis, the use of cycloheximide was ruled out. To test whether oxygen-mediated HIF-1α degradation depends on a hypoxia-induced transcriptional activity, HeLa cells were incubated in 1–2% O_2 for 8 h, either in the absence or in the presence of actinomycin D, and then reoxygenated for the indicated periods of time. In agreement with previous studies showing that the HIF-1α mRNA is constitutively expressed and is not significantly affected by hypoxia, results from Fig. 3A demonstrated that inhibition of transcription did not affect the induction of HIF-1α protein upon hypoxic stress. The addition of actinomycin D, however, had a significant inhibitory effect on HIF-1α degradation upon reoxygenation, whereas the p42 MAP kinase protein levels remained unaffected. The same result was obtained when the experiment was performed in the presence of another transcriptional inhibitor, 5,6-dichlorobenzimidazole riboside (data not shown). These findings suggest that hypoxia induces the transcription of a gene(s) encoding a regulatory protein(s), which controls the oxygen-mediated degradation of HIF-1α.

3.4. HIF-1-dependent transcriptional activity is required for HIF-1α degradation

As we mentioned in Section 1, HIF-1 is a key mediator of the hypoxic induction of many hypoxia-regulated genes. We therefore wanted to evaluate the possibility that HIF-1-dependent transcriptional activation controls oxygen-mediated HIF-1α degradation. If we are right, the inhibition of the transcriptional activity of HIF-1 should be able to slow down HIF-1α degradation. Hence, we transfected HeLa cells with a dominant negative mutant of HIF-1α. This mutant (pcDNA3-HA-DN-HIF-1α) was obtained by a carboxyl-terminal truncation of HIF-1α that eliminates the transactivating domains (amino acids 390–826). Accordingly, the expression of this construct has been shown to inhibit induction of reporter genes by endogenous HIF-1 in a dose-dependent manner (Gothié, E. and Pouysségur, J., unpublished data). At 48 h post-transfection, cells were incubated either in normoxia or hypoxia for 3 h after which hypoxic cells were returned to 20% O_2 for 0, 15 or 30 min. Total cell extracts were prepared, and HIF-1α

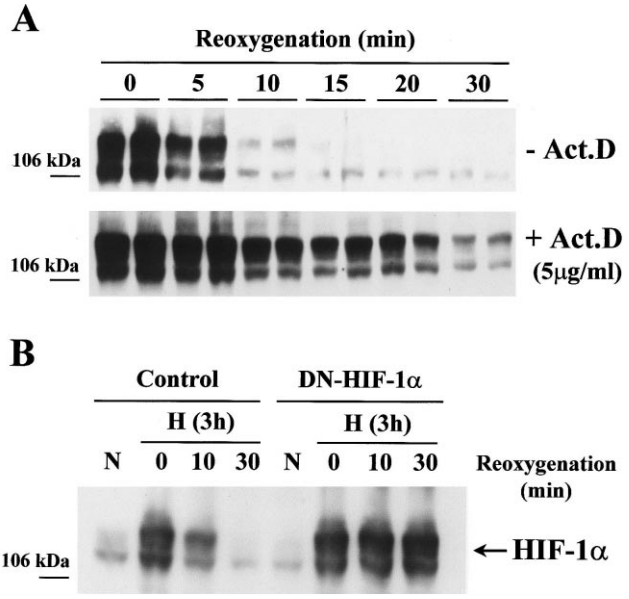

Fig. 3. HIF-1-dependent transcriptional activity controls oxygen-mediated degradation of HIF-1α. (A) HeLa cells, either untreated or treated with 5 μg/ml of actinomycin D for 30 min, were incubated under normoxia (20% O_2; N) or hypoxia (1–2% O_2; H) for 8 h after which hypoxic cells were returned to 20% O_2 for 0–30 min. Total cell lysates were immunoblotted with anti-HIF-1α (B). Subconfluent HeLa cells were transfected with 10 μg of either empty vector or HA-DN-HIF-1α expression vector. At 48 h post-transfection, cells were incubated under normoxia (20% O_2; N) or hypoxia (1–2% O_2; H) for 3 h after which hypoxic cells were returned to 20% O_2 for 0, 15, or 30 min. Total cell lysates were analyzed by immunoblotting with the antibody to HIF-1α. Results are representative of three independent experiments.

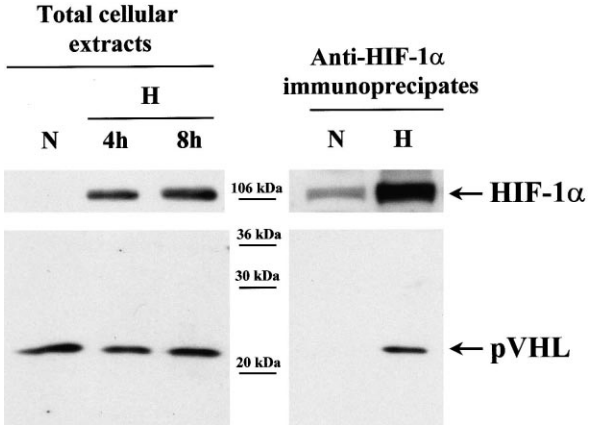

Fig. 4. Hypoxia does not modulate the expression of the von Hippel–Lindau protein (pVHL). HeLa cells were incubated under normoxia (20% O_2; N) or hypoxia (1–2% O_2; H) for 4 h or 8 h. Total cell lysates were immunoblotted with anti-HIF-1α or anti-pVHL antibodies (left panel). The same cellular extracts (N and H (8 h)) were immunoprecipitated using the anti-HIF-1α antibody. These immunoprecipitates were analyzed by SDS–PAGE and immunoblotted with the antibody to HIF-1α or pVHL (right panel). Essentially identical results were obtained in three independent experiments.

protein levels analyzed by immunoblotting. Interestingly, results from Fig. 3B demonstrated that the ability of oxygen to induce HIF-1α degradation was strongly inhibited by expression of the HIF-1α dominant negative mutant. Of note, HIF-1α construct was expressed at similar levels (data not shown). Taken together, these results clearly show that during hypoxia, HIF-1 is critically involved in the induction of a regulatory protein(s), which is necessary for oxygen-mediated HIF-1α degradation.

3.5. VHL is not a hypoxia-inducible gene

Ratcliffe's group has nicely demonstrated that the product of the von Hippel–Lindau tumor suppressor gene (pVHL) plays an essential role on HIF-1α degradation [11]. In addition, our above results lead to the notion that hypoxia via HIF-1 controls the expression of a factor(s) which is critical for the oxygen-induced degradation of HIF-1α. Is pVHL this hypoxia-inducible regulatory protein? To answer this question, cellular lysates from HeLa cells incubated for different periods in normoxia or hypoxia were analyzed by immunoblot using an antibody against pVHL. This antibody detects a single protein migrating in a 12% SDS–polyacrylamide gel as a band which corresponds to a molecular mass of 24 kDa. This band has been validated as pVHL by a coimmunoprecipitation assay using the anti-HIF-1α specific antibodies (Fig. 4, right panel). Results from Fig. 4 (left panel) clearly demonstrated that the expression level of pVHL in whole cellular extracts is not affected even after 8 h of incubation in 1–2% O_2, whereas the same hypoxic treatment was found to drastically accelerate the rate of HIF-1α degradation (Fig. 2A). In addition, we showed that the interaction between HIF-1α and pVHL, by immunoprecipitation assays, was not modified by the length of the hypoxic stress (data not shown). Hence, we conclude that pVHL is distinct from the regulatory protein, actinomycin D-sensitive and HIF-1-dependent, which would be implicated in the regulatory mechanism here presented.

4. Discussion

HIF-1 plays an essential role in embryonic development, cellular and systemic physiology [5,6]. HIF-1 activity is critically dependent on the expression of HIF-1α [7,8], which protein levels are tightly regulated by oxygen concentrations via the ubiquitin–proteasome system [11–14]. In this work, we

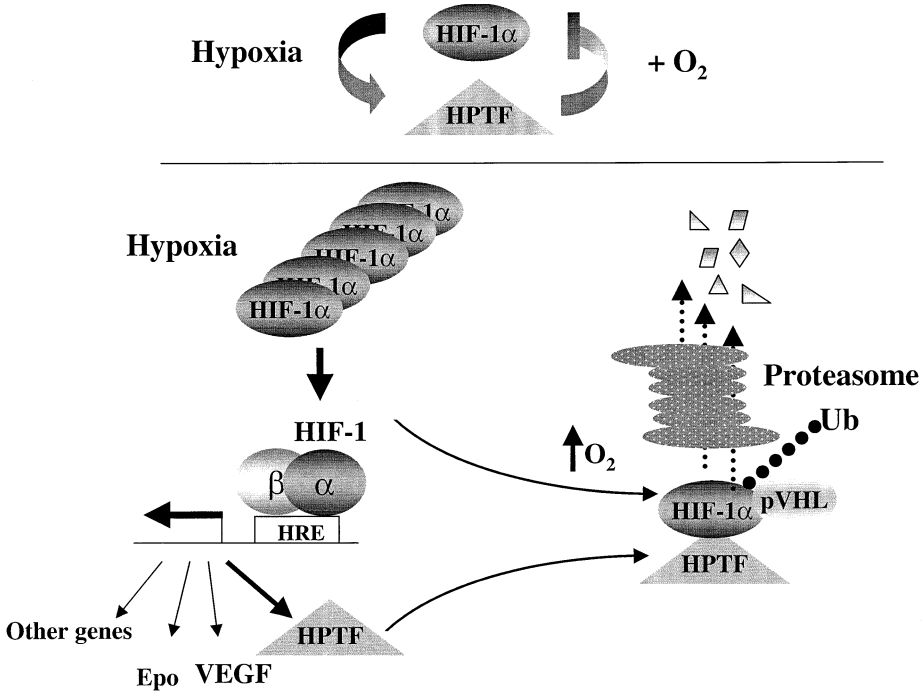

Fig. 5. Working model depicting a potential mechanism by which HIFα degradation can be regulated. During hypoxia, HIFα is induced and HIF-1-dependent transcriptional activation leads to the expression of HPTF. Upon normoxia, HPTF associates with the HIFα/pVHL complex and targets HIF-1α to the proteasome. HIF-2α should also be subjected to a similar regulatory mechanism.

focused our attention on the characterization of the mechanisms controlling proteasome-mediated degradation of HIF-1α.

The ubiquitin-dependent proteolysis is a highly complex, temporally controlled, and acutely regulated enzymatic process [16]. Regulation of certain proteins by the proteasome machinery may be dependent on post-translational modifications such as phosphorylation [21–24]. We and others have previously demonstrated that p42/p44 MAP kinases phosphorylate HIF-1α/HIF-2α, in vitro and in vivo [17,25–27]. Hence, we attempted to investigate the possible role of the p42/p44 MAP kinase cascade in HIF-1α degradation/stabilization. In this study, we have demonstrated that either strong and persistent activation of the p42/p44 MAP kinase pathway or its inhibition with the MEK inhibitor, PD 98059, does not significantly modify the induction/degradation pattern of HIF-1α. These results correlate well with the previously demonstrated data showing that the basal and growth factor-stimulated activity of p42/p44 MAP kinases is not modified by hypoxia in the cellular model we used [17]. Recently, Giaccia's and Semenza's groups have shown that PI-3kinase/PTEN/Akt cascade regulates hypoxia- and growth factor-induced HIF-1α stabilization in human glioblastoma-derived cells and prostate cancer cells, respectively [28,29]. However, in the cellular systems we use to induce HIF-1α in response to a moderate or drastic hypoxic stress, the PI-3kinase/Akt pathway is not activated (Berra, E. and Pouysségur, J., unpublished observations).

Alternatively, association with an ancillary protein may control the targetting of some proteins through the proteasome. A relevant example is the promoted instability of p53 by association with either papilloma virus E6 protein or MDM2 [30–32]. p53/MDM2 form an autoregulatory feedback loop, in which p53 can control its own levels by inducing the expression of its negative regulator, MDM2. By analogy, we hypothesized that the latter mechanism could also take place in the case of HIF-1α. To test this hypothesis, we performed the series of experiments summarized in this work. Taken together, our findings greatly support an autoregulatory feedback loop controlled by the hypoxic stress. We first demonstrate that the length of the hypoxic stress affects HIF-1α half-life upon reoxygenation. Second, we present evidence that HIF-1α degradation is dependent on hypoxia-induced transcription since actinomycin D is able to reduce the degradation rate. Finally, and more specifically, we demonstrate that HIF-1-dependent transcription is required for the induction of a regulatory factor(s) targetting HIF-1α degradation. Furthermore, the second isoform HIF-2α, which expression like for HIF-1α is also controlled by O_2 tension, appears to be subjected to the same regulatory mechanism (data not shown). Therefore, in keeping with the results presented here, we would like to propose the model depicted in Fig. 5 for the degradation of HIF-1α. In this model we propose that, under normal pO_2, HIF-1α is targetted to the proteasome by a HPTF. According to this model, HPTF is a hypoxia-inducible gene product which accumulates as a function of the length of hypoxia, and therefore of HIF-1 activity. Along this work, two proteins, pVHL and MDM2, have been suggested to target the proteasomal degradation of HIF-1α [11,33]. If some uncertainty remains for the case of MDM2, the role of pVHL in the control of HIF-1α and HIF-2α has been clearly established. It was therefore crucial to answer whether HPTF is either pVHL or MDM2. pVHL was rapidly eliminated, among these candidates, since hypoxia does regulate neither the level of this protein nor its association with HIF-1α (Fig. 4 and data not shown). As far as MDM2 is concerned, it does not appear to be a hypoxia-regulated gene although p53 tends to accumulate in response to severe hypoxia [34]. Moreover, it has been reported that MDM2-mediated p53 degradation requires nuclear export [35]. Indeed, leptomycin B, known to prevent p53 degradation by blocking nuclear export, does not affect HIF-1α degradation (Berra, E., Roux, D. and Pouysségur, J., manuscript in preparation). Furthermore, it is important to note that pVHL associates constitutively with HIF-1α, even in hypoxic conditions suggesting that there necessary exists a complementary mechanism(s) modulating the oxygen-dependent pVHL-mediated degradation of HIF-1α. We speculate that HPTF is responsible for the conditional 'switch' of HIF-1α to O_2-mediated degradation. Upon return to an oxygenated environment, interaction of HPTF with the HIF-1α/pVHL complex could 'switch on' the ubiquitination of HIF-1α ensuring rapid degradation, and maintenance of low levels during normoxia. We do not yet know the molecular nature of HPTF nor the mechanisms regulating its interaction with the HIF-1α/pVHL complex. Is there a direct interaction of HPTF and HIF-1α or is this association mediated by pVHL? HIF-2α is also subjected to the same regulatory mechanism, and essentially identical results were obtained in every cellular system tested (HeLa, HepG2, and 293) but how generalizable is this model? Further research will help us to clarify these important questions.

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