Hypoxia-inducible factor-1 α (HIF-1 α) escapes O₂-driven proteasomal degradation irrespective of its subcellular localization: nucleus or cytoplasm

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Eukaryotic cells sense oxygen and adapt to hypoxia by regulating a number of genes. Hypoxia-inducible factor 1 (HIF-1) is the 'master' in this pleiotypic response. HIF-1 comprises two members of the basic helix-loop-helix transcription factor family, HIF-1 α and HIF-1 β . The HIF-1 α protein is subject to drastic O₂-dependent proteasomal control. However, the signalling components regulating the 'switch' for 'escaping' proteasomal degradation under hypoxia are still largely unknown. The rapid nuclear translocation of HIF-1 α could represent an efficient way to escape from this degradation. We therefore asked, where in the cell is HIF-1 α degraded? To address this question, we trapped HIF-1 α either in the cytoplasm, by fusing HIF-1 α to the cytoplasmic domain of the Na⁺-H⁺ exchanger (NHE-1), or in the nucleus, by treatment with leptomycin B. Surprisingly, we found that HIF-1 α is stabilized by hypoxia and undergoes O₂-dependent proteasomal degradation with an identical half-life (5–8 min) in both cellular compartments. Therefore, HIF-1 α entry into the nucleus is not, as proposed, a key event that controls its stability. This result markedly contrasts with the mechanism that controls p53 degradation via MDM2.

INTRODUCTION

Hypoxia-inducible factor 1 (HIF-1) plays a central role in oxygen homeostasis by inducing the expression of a broad range of genes including *VEGF*, *VEGFR1*, and almost every gene of the glycolytic pathway in response to a decrease in pO₂ (Guillemin and Krasnow, 1997; Semenza, 1998). HIF-1 is a heterodimer composed of the constitutively expressed HIF-1 β , and the rate limiting factor HIF-1 α (Wang and Semenza, 1995). HIF-1 β is the already characterized aryl hydrocarbon receptor nuclear translo-

cator (ARNT), previously shown to heterodimerize with the aryl hydrocarbon receptor (AHR) (Hoffman *et al.*, 1991). In contrast, HIF-1 α specifically mediates hypoxic responses. HIF-1 α is a short-lived protein, the levels of which are tightly regulated by oxygen concentration via the ubiquitin-proteasome system (Salceda and Caro, 1997; Huang *et al.*, 1998; Kallio *et al.*, 1999). In normoxia, HIF-1 α is maintained at low and often undetectable levels, whereas hypoxia rapidly and strongly increases the amounts of HIF-1 α by relaxing its proteasome-dependent degradation. It has been demonstrated previously that the product of the von Hippel-Lindau tumour suppressor gene (pVHL) directly interacts with HIF-1 α as a component of an E3 ubiquitin-protein ligase complex responsible for the degradation of HIF-1 α (Maxwell *et al.*, 1999; Cockman *et al.*, 2000; Ohh *et al.*, 2000; Tanimoto *et al.*, 2000). pVHL/HIF-1 α association has been reported to take place even in hypoxic conditions. This finding however, unexpected for a ligase/substrate complex, was in fact invalidated by two recent reports demonstrating that pVHL/HIF-1 α interaction is indeed regulated by the intracellular O₂ levels. The key enzyme controlling this O₂-dependent step is a specific HIF-1 α -proline hydroxylase (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001). In addition to stabilizing HIF-1 α , hypoxia also induces the nuclear translocation of the protein (Kallio *et al.*, 1998). This relocalization of HIF-1 α could be a way by which the protein escapes from proteasomal degradation. In fact, HIF-1 α nuclear localization has been recently proposed as a regulatory step involved in its stabilization (Tanimoto *et al.*, 2000). To test this hypothesis, and get more insight into the mechanism of HIF-1 α degradation, we impaired the cytoplasmic-nuclear shuttling of HIF-1 α . We first trapped HIF-1 α in the cytoplasmic compartment by fusing HIF-1 α to the Na⁺-H⁺ exchanger (NHE-1) and secondly we blocked nuclear export by using

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Fig. 1. Schematic representation of the NHE-1>>HIF-1 α chimera. The figure outlines the main features of both the plasma membrane-bound Na⁺-H⁺ exchanger and transcription factor HIF-1 α .

leptomycin B (Fornerod *et al.*, 1997; Ossareh-Nazari *et al.*, 1997). Interestingly, and in marked contrast to the previously proposed model (Tanimoto *et al.*, 2000), our data unequivocally demonstrate that: (i) nuclear translocation is not necessary for HIF-1 α stabilization, and (ii) both 'nuclear' and 'cytoplasmic' proteasomes are fully competent for HIF-1 α degradation in an oxygen-dependent manner. This result also contrasts with the mechanism regulating the stability of p53, a transcription factor that is required to exit the nucleus for degradation via MDM2.

RESULTS AND DISCUSSION

As schematized in Figure 1, we fused the entire human HIF-1 α protein to the cytoplasmic domain of the NHE-1. NHE-1 is an integral plasma membrane protein that exchanges intracellular protons at the expense of the inwardly-directed Na⁺ gradient (Sardet *et al.*, 1989). The protein (815 aa) is composed of two domains, the transporter *per se* (500 aa) which spans the plasma membrane 10 times followed by a cytoplasmic domain of about 315 residues, dispensable for transport activity but essential for conveying extracellular signals to the H⁺ modifier site (Counillon and Pouyssegur, 2000; for review). The NHE-1 sequence used has been engineered by point mutations to confer resistance to amiloride analogues allowing the direct selection of the transgene by H⁺ suicide-selection (Pouyssegur and Roux, 1999). Therefore, transfectants resistant to H⁺ suicide, in the presence of the amiloride analogue, must express HIF-1 α fused in-frame with a functional, amiloride-resistant NHE-1. Because NHE-1 is exclusively targeted to the plasma membrane or membrane vesicles, this approach ensures the sequestration of HIF-1 α to the cytoplasmic compartment. Hamster lung fibroblasts were transfected with either NHE-1 alone (control) or the NHE-1>>HIF-1 α chimera and populations resistant to H⁺ suicide were selected and independent clones isolated for further study.

Figure 2A and C show no HIF-1 α immunoreexpression in normoxic conditions, whereas hypoxia (1–2% O₂ for 3 h) is sufficient to result in increased expression of HIF-1 α in both

clones. As expected, HIF-1 α accumulated in the nucleus of control cells (Figure 2B) and chimerical HIF-1 α was restricted to the cytoplasmic compartment (Figure 2D). The cytoplasmic localization of HIF-1 α seen in Figure 2D is identical to that of NHE-1 in cells that overexpress the transporter. Confocal analysis indicates a localization in both the plasma membrane and the cytosolic network of membrane vesicles (data not shown). At this stage we could say that the chimerical HIF-1 α protein appears to be regulated as endogenous HIF-1 α : degraded in normoxia and stabilized at low O₂ tension. CoCl₂ is well known to mimic hypoxia. Indeed Figure 2E shows, by immunoblotting of total cellular extracts, a stabilization of both, endogenous (115 kDa) and chimerical (160–180 kDa) HIF-1 α proteins.

As we could identify in total extracts both the endogenous and chimerical HIF-1 α proteins by their different apparent molecular weight, we next investigated their respective half-life following reoxygenation. Figure 3, left panel, shows that following hypoxia (1–2% O₂ for 4 h), reoxygenation leads rapidly to dramatic destruction of the protein in control cells. Interestingly, the same experiment conducted in cells expressing the chimerical HIF-1 α protein demonstrated that reoxygenation, following hypoxia, rapidly leads to degradation of both proteins with parallel kinetics (Figure 3, middle panel). We obtained a half-life of 5–8 min for both HIF-1 α proteins. It is well established that this rapid oxygen-driven degradation is mediated via the ubiquitin-dependent proteasomal system, an event which is prevented by the proteasomal inhibitor, lactacystin (Fenteany *et al.*, 1995). Figure 3, right panel, shows a drastically increased half-life for both HIF-1 α proteins in cells pre-treated with this proteasome inhibitor. Note, in the presence of lactacystin, the increased level in normoxic conditions and the steady-state level of both HIF-1 α proteins upon reoxygenation (compare middle and right panels of Figure 3). Altogether, these results clearly establish that cytoplasmic proteasomes are fully competent for O₂-dependent degradation of HIF-1 α .

Fig. 2. Stable expression of the chimerical NHE-1>>HIF-1 α protein. NHE-1 or NHE-1>>HIF-1 α cells incubated either in normoxia (20% O₂) (A and C) or hypoxia (1–2% O₂) (B and D) for 3 h were analysed by immunofluorescence using the anti-HIF-1 α antiserum. In addition, control and chimerical protein expressing cells were incubated in the absence or in the presence of CoCl₂ (200 μ M) for 3 h and whole cellular extracts (50 μ g) were separated by SDS–PAGE and revealed by immunoblot analysis using the same anti-HIF-1 α antibody (E). Note that the experiment shown in (E) was done with the stable transfected population whereas (D) is representative of a subclone expressing high levels of the chimera. Results are representative of three independent experiments.

The next step was to investigate whether HIF-1 α entry and retention in the nucleus facilitates in some way its escape from proteasomal degradation upon reoxygenation. HIF-1 α and p53 levels were followed in parallel since p53, another transcription factor stabilized by a variety of stresses, must exit the nucleus to undergo MDM2-dependent proteasomal degradation (Freedman and Levine, 1998). Thus, we prevented HIF-1 α nuclear export by treatment with leptomycin B. Figure 4 shows that addition of leptomycin B has no effect on the basal level (normoxic) of HIF-1 α nor on the rate of HIF-1 α degradation upon reoxygenation. Leptomycin B, however strongly stabilizes p53 either in basal

conditions or after hypoxic stress. This result, which validates the action of leptomycin, strongly indicates that the pVHL-dependent degradation of HIF-1 α is totally independent of its subcellular localization. Leptomycin B was used here as a ‘forcing device’ to prevent possible HIF-1 α nuclear export. Upon reoxygenation of cells treated with leptomycin B, and lactacystin to abolish degradation, HIF-1 α remained in the nucleus (see Supplementary data).

In this communication, we have firmly established that HIF-1 α escape from proteasomal degradation, in response to hypoxia, could not be explained by the rapid entry of HIF-1 α into the

Fig. 3. Oxygen-driven proteasome-mediated regulation of cytoplasmic HIF-1α. Control cells (left panel) or cells expressing the chimerical protein NHE-1>>HIF-1α either untreated (middle panel) or pre-treated with 20 μM of the proteasome inhibitor lactacystin for 30 min (right panel), were incubated under normoxia (20% O₂; N) or hypoxia (1–2% O₂; H) for 4 h after which hypoxic cells were returned to 20% O₂ for 0–40 min. Total cell lysates were analysed by immunoblotting with the antibody to HIF-1α. Essentially identical results were obtained in three independent experiments.

Fig. 4. Blocking nuclear export does affect O₂-dependent degradation of HIF-1α. HeLa cells cultured either in the absence or in the presence of leptomycin B (20 nM) for 16 h were incubated under normoxia (20% O₂; N) or hypoxia (1–2% O₂; H) for an additional 4 h after which hypoxic cells were returned to 20% O₂ for 0–20 min. Total cell lysates were analysed by immunoblotting with antibodies to HIF-1α and p53. Results are representative of three independent experiments.

nucleus. A previous study has eliminated the possible role of heterodimerization of HIF-1α/HIF-1β in this process (Chilov *et al.*, 1999). The authors reported that the HIF-1α protein translocated and accumulated in the nucleus of cells lacking HIF-1β. We fully agree with this finding. Indeed the cytoplasmic chimerical HIF-1α protein when stabilized by hypoxia was not found to be associated with the HIF-1β partner (data not shown), therefore excluding this association as a mechanism for proteasomal escape. Our findings however, contradict a recent report from Poellinger's group (Tanimoto *et al.*, 2000). These authors propose that hypoxia-induced protection of HIF-1α against regulation by pVHL involves two distinct and successive steps: nuclear translocation of HIF-1α and a hypoxic signal leading to escape from pVHL-induced proteolysis. We agree on the need for a hypoxic signal but not for the nuclear translocation of HIF-1α.

Now one could argue that the HIF-1α proteasomal degradation that we reported here with the NHE-1>>HIF-1α chimera is not regulated by pVHL. It is important to clarify this point because it has been suggested that HIF-1α could be sent for proteasomal degradation via at least two E3-ubiquitin ligases, pVHL and MDM2 (Maxwell *et al.*, 1999; Ravi *et al.*, 2000). The experiment conducted with leptomycin B (Figure 4) shows that sequestration of the MDM2–p53 complex in the nucleus, away from cytoplasmic proteasomes, has no impact on HIF-1α degradation. This result suggests that the contribution of MDM2 to O₂-induced HIF-1α degradation, if it exists, remains minimal. Our findings also indicate that the E3-ligase pVHL is fully competent in targeting HIF-1α to both cytoplasmic and nuclear proteasomes, a result in marked contrast with the E3 ligase, MDM2. Although there are many striking similarities between p53 and HIF-1α, two transcription factors auto-regulated post-transcriptionally via proteasomal degradation and maintained at very low levels in normal and unstressed cells (Berra *et al.*, 2001), p53 must exit the nucleus for degradation, while HIF-1α is destroyed in both compartments. This 'attack' in both sub-cellular locations could explain the extraordinary short half-life of HIF-1α protein. Although this area of research has rapidly progressed, many steps in the adaptive response to hypoxia remain to be deciphered, in particular the hypoxic signal leading to HIF-1α stabilization. Now with the implication of the HIF-1α-proline hydroxylase as a key enzyme controlling the O₂-dependent ubiquitylation (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001), our results imply that proline hydroxylation of HIF-1α and subsequent ubiquitylation must take place in both cellular compartments. We are currently testing this hypothesis.

METHODS

Materials and plasmids. For the cloning of NHE-1>>HIF-1α, human HIF-1α (pcDNA3-HA-HIF-1α) was initially digested with *Hind*III and *Xba*I and subcloned into pBluescript KS (Stratagene). The

N-terminal region of HIF-1 α was amplified by PCR using the primers: 5'-CCCCGGGGCCCCAGGGCGCCGGCGCG-3' (sense) and 5'-GTTCATAGTTCTTCTCGGC-3' (antisense), digested with *Apal* and *HindIII* and subcloned into the same vector. The NHE-1>>HIF-1 α chimera was finally generated by subcloning of the *Apal*-*XbaI* fragment of HIF-1 α into pECE-NHE-1 previously digested with the same enzymes (as shown in Figure 1, HIF-1 α is linked to the residue 698 of NHE-1). Details for the two plasmids expressing either the amiloride-resistant NHE-1 or human HIF-1 α have been described previously (see references Pouyssegur and Roux, 1999 and Richard *et al.*, 1999; Gothié *et al.*, 2000, respectively). The anti-HIF-1 α antibody (antiserum 2087) was raised in our laboratory in rabbits immunized against the last 20 amino acids C-terminal of the human HIF-1 α (Richard *et al.*, 1999). The monoclonal antibody against human p53 was from DAKO (DO-7).

Cell culture and transfections. CCL39, NHE-1 and NHE-1>>HIF-1 α cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal calf serum (FCS), penicillin G (50 U/ml), and streptomycin (50 μ g/ml) (Gibco-BRL) in an incubator (5% CO₂) at 37°C. HeLa cells were incubated in the same medium but supplemented with inactivated FCS. Hypoxic conditions were performed by incubation of cells in a sealed 'Bug-Box' anaerobic workstation (Ruskin Technologies, Jouan). The oxygen in this workstation was maintained at 1–2% with a residual gas mixture being 93–94% nitrogen, and 5% carbon dioxide.

Clones expressing the amiloride-resistant form of NHE-1 or NHE-1>>HIF-1 α were obtained by stable transfection of CCL39 cells with pECE-NHE-1 or pECE-NHE-1-HIF-1 α plasmids, respectively. Transfection was performed by the calcium phosphate method. Three H⁺-suicide selections were applied as previously described (Pouyssegur and Roux, 1999) to obtain a stable cellular population. From the population expressing the chimera, independent clones were isolated and after selection for HIF-1 α expression, three different clones were selected for further study. We notice that high levels of NHE-1>>HIF-1 α had a tendency to decline with passage because this expression slows down the growth rate.

Immunofluorescence studies. Cells grown on glass coverslips were analysed using the anti-HIF-1 α antiserum as previously described (Richard *et al.*, 2000). Immunofluorescence was then analysed with a Leica DM-R microscope equipped with a DC-100 digital camera.

Western blot analysis. Subconfluent cells were lysed in Laemmli buffer. The protein concentration was determined using the Lowry assay and 50 μ g of whole cellular extracts were resolved by SDS-PAGE (7.5%) and electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon-P). HIF-1 α and p53 were revealed with specific antibodies. The immunoreactive bands were visualized with the ECL system (Amersham Pharmacia Biotech).

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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