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Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1α

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In response to decreased cellular oxygen concentrations the basic helix–loop–helix (bHLH)/PAS (Per, Arnt, Sim) hypoxia-inducible transcription factor, HIF-1α, mediates activation of networks of target genes involved in angiogenesis, erythropoiesis and glycolysis. Here we demonstrate that the mechanism of activation of HIF-1α is a multi-step process which includes hypoxia-dependent nuclear import and activation (derepression) of the transactivation domain, resulting in recruitment of the CREB-binding protein (CBP)/p300 coactivator. Inducible nuclear accumulation was shown to be dependent on a nuclear localization signal (NLS) within the C-terminal end of HIF-1α which also harbors the hypoxia-inducible transactivation domain. Nuclear import of HIF-1α was inhibited by either deletion or a single amino acid substitution within the NLS sequence motif and, within the context of the full-length protein, these mutations also resulted in inhibition of the transactivation activity of HIF-1α and recruitment of CBP. However, nuclear localization *per se* was not sufficient for transcriptional activation, since fusion of HIF-1α to the heterologous GAL4 DNA-binding domain generated a protein which showed constitutive nuclear localization but required hypoxic stimuli for function as a CBP-dependent transcription factor. Thus, hypoxia-inducible nuclear import and transactivation by recruitment of CBP can be functionally separated from one another and play critical roles in signal transduction by HIF-1α.

Keywords: gene regulation/hypoxia/nuclear translocation/signal transduction/transcriptional coactivator

Introduction

Oxygen plays a critical biological role as the terminal electron acceptor in the mitochondria of vertebrate cells. During evolution, these cells have developed ways to sense alterations in oxygen levels and, during this process, have acquired the ability to conditionally modulate the expression of genes involved in adaptive physiological responses to hypoxia including angiogenesis, erythropoiesis and glycolysis. These genes include vascular endothelial growth factor (VEGF), erythropoietin, several glycolytic enzymes and inducible nitric oxide synthase, and have all been shown to contain hypoxia-responsive elements (HREs) (for review, see Guillemin and Krasnow, 1997; Wenger and Gassmann, 1997). Under hypoxic conditions these response elements are recognized by a heterodimeric complex consisting of the hypoxia-inducible factor (HIF)-1α and Ah receptor nuclear translocator (Arnt) (Wang *et al*., 1995; Gradin *et al*., 1996). Both these transcription factors belong to the rapidly growing family of basic helix–loop–helix (bHLH)-PAS (Per, Arnt, Sim) proteins. bHLH/PAS transcription factors play diverse biological roles. For instance, Clock and Per proteins are involved in regulation of circadian rhythmicity in *Drosophila* and mammals (Antoch *et al*., 1997; Sun *et al*., 1997; Tei *et al*., 1997), WC proteins may function as photoreceptors in *Neurospora* (Crosthwaite *et al*., 1997), the dioxin receptor functions as an intracellular receptor for xenobiotics and is involved in cellular detoxification responses (Poellinger, 1995), whereas Sim and Trachealess are critical developmental factors in *Drosophila*, involved in neuronal and tracheal development, respectively (Zelzer *et al*., 1997; Crews, 1998).

The recent generation of HIF-1α and Arnt-deficient embryonal stem cells in mice have indicated critical roles of both these factors in cardiovascular development and regulation of HRE-driven target genes (Maltepe *et al*., 1997; Iyer *et al*., 1998). The mechanism of hypoxia-dependent formation and activation of the HIF-1α–Arnt complex is presently poorly understood. We and others have recently demonstrated that HIF-1α protein levels are specifically and massively upregulated under hypoxic conditions in most if not in all cells. Since HIF-1α mRNA levels are unaltered in response to hypoxia, this mode of regulation appears to occur via a post-transcriptional step involving stabilization of HIF-1α protein levels (Huang *et al*., 1996; Kallio *et al*., 1997; Salceda and Caro, 1997) preceding recruitment of Arnt and generation of a nuclear DNA-binding complex.

Import of transcription factors into the nucleus is frequently a conditionally regulated process that occurs in response to various internal and external stimuli as well as to developmental cues (Vandromme *et al*., 1996). Thus, this process can constitute a critical mechanism of regulation of transcription factor activity. Active, energy-dependent transport of proteins to the nucleus requires the presence of one or several nuclear localization signals (NLSs) within the transported protein (or its interaction partner). NLS motifs are short amino acid moieties that can generally be divided into two main groups: (i) the simian virus 40 (SV40) large T antigen type of NLS, characterized by a single cluster of four or more consecutive basic residues; and (ii) a bipartite NLS, consisting of two basic residues, a spacer of any ten amino acids, and

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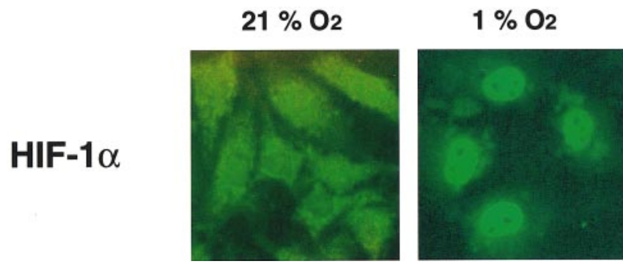

Fig. 1. HIF-1α shows hypoxia-inducible nuclear translocation. COS7 cells were transiently transfected with pCMV–HIF-1α and after 24 h expression incubated for an additional 6 h under either normoxic (21% O_2) or hypoxic (1% O_2) conditions before fixation for immunocytochemistry. Localization of expressed HIF-1α was determined by indirect immunofluorescence using anti-HIF-1α antiserum as described in Materials and methods.

a basic cluster where three of the next five residues are basic (Vandromme *et al*., 1996 and references therein).

Here we demonstrate that nuclear import of HIF-1α is regulated by hypoxia. Using green fluorescent protein (GFP) fusion protein constructs we have identified within the C-terminal portion of HIF-1α a functional NLS which is involved in conditionally regulated nuclear accumulation of the protein. However, nuclear localization *per se* is not sufficient for transcriptional activation by HIF-1α. In addition, HIF-1α requires hypoxia-dependent activation of its C-terminal transactivation domain within the nucleus to generate a functional form which recruits the CREB-binding protein (CBP)/p300 coactivator protein, demonstrating that the mechanism of signal transduction by HIF-1α is a multi-step process.

Results

HIF-1α shows hypoxia-inducible nuclear import

To study the intracellular localization of HIF-1α we transiently transfected simian COS7 cells with a cytomegalovirus (CMV) promoter-driven HIF-1α expression vector. Following 24 h of expression cells were incubated for a further 6 h under either normoxic (21% O_2) or hypoxic (1% O_2) conditions. Immunostaining with polyclonal anti-HIF-1α antibodies (Kallio *et al*., 1997) showed HIF-1α immunoreactivity predominantly localized in the cytoplasm of the normoxic cells with some reactivity also detected in the nuclear compartment. Interestingly, exposure of the cells to hypoxia resulted in a very striking nuclear accumulation of HIF-1α with hardly any detectable immunoreactivity remaining in the cytoplasm (Figure 1).

To investigate further the mechanism of activation of HIF-1α and its subcellular localization in living cells we constructed a vector carrying an in-frame GFP fusion of full-length HIF-1α (schematically shown in Figure 2A) expressed under the control of the CMV promoter. Following transient transfection of COS7 cells with either the parental GFP construct or the chimeric GFP–HIF-1α construct, fluorescence was observed in ~20–30% of the cells, reflecting the level of transfection efficiency. The fluorescence of GFP alone was uniformly distributed throughout the cell, and hypoxic treatment had no effect either on the intensity or the subcellular distribution of fluorescence activity (Figure 2B). Transient expression of the GFP–HIF-1α construct in normoxic cells resulted in a signal mimicking the picture observed with GFP alone, thus showing fluorescence activity throughout the cell (Figure 2B).

For quantitative purposes, 200–300 fluorescent cells were routinely analyzed for compartmentalization of HIF-1α and subdivided into four categories (Ylikomi *et al*., 1992): N, cells containing exclusively nuclear fluorescence; N > C, cells in which the nuclear fluorescence dominates over cytoplasmic fluorescence; N = C, cells having equal distribution of fluorescence in the nuclear and cytoplasmic compartments; and N < C, cells in which the intensity of fluorescence in the cytoplasm exceeds that in the nucleus. The percentage of cells belonging to each category is indicated in Table I. Under normoxic conditions 65% of cells transiently expressing the GFP–HIF-1α construct belonged to category N = C; whereas a limited number of cells (8%) showed exclusively nuclear compartmentalization of HIF-1α (category N). In contrast, exposure of the cells to hypoxia (1% O_2) resulted in almost complete nuclear accumulation of GFP–HIF-1α (Figure 2B), where 90% of the transfected cells exhibited exclusively nuclear fluorescence (category N; Table I). Moreover, treatment of the cells with chemicals known to mimic hypoxic induction of target gene expression (Wenger and Gassmann, 1997) and induction of HIF-1α DNA-binding activity (Wang *et al*., 1995; Gradin *et al*., 1996), e.g. $CoCl_2$ or the iron chelator 2,2′-dipyridyl (2,2′-DP), also induced nuclear import of GFP–HIF-1α. In fact, we did not observe any quantitative or qualitative differences in the potencies of any of these treatments to induce nuclear translocation of HIF-1α (Figure 2B).

We and others have recently demonstrated that a critical mechanism in activation of HIF-1α in response to hypoxia is the upregulation of HIF-1α protein levels. This response depends upon the stabilization of HIF-1α protein rather than being the result of increased HIF-1α mRNA expression levels or enhanced translation of HIF-1α mRNA (Huang *et al*., 1996; Kallio *et al*., 1997; Salceda and Caro, 1997). Since we did not detect any significant alterations in the relative intensity in fluorescence by GFP–HIF-1α upon hypoxic stimulation (Figure 2B), we examined by immunoblot analysis the expression levels of GFP–HIF-1α protein in transiently transfected normoxic cells or cells exposed to hypoxia (1% O_2) following transfection. As shown in Figure 2C, no increase was detected in the expression levels of GFP–HIF-1α protein in hypoxic cells compared with normoxic ones (compare Figure 2C, lanes 3 and 4). As a reference, however, endogenous HIF-1α levels were upregulated in extracts from cells exposed to hypoxia (Figure 2C, lanes 2 and 4, endogenous HIF-1α indicated by an asterisk). In fact, as documented earlier (Kallio *et al*., 1997), due to rapid turnover endogenous HIF-1α is virtually undetectable in extracts from normoxic cells (Figure 2C, lanes 1 and 3). These experiments therefore demonstrate that GFP efficiently stabilized HIF-1α in normoxic cells, and that the use of GFP fusion proteins enabled us to bypass the early level in regulation of HIF-1α function, and allowed us to study intracellular compartmentalization of HIF-1α without the added complexity of regulation of protein turn-over.

Upon exposure of COS7 cells to hypoxia or hypoxia-mimicking chemicals such as 2,2′-DP, the kinetics of

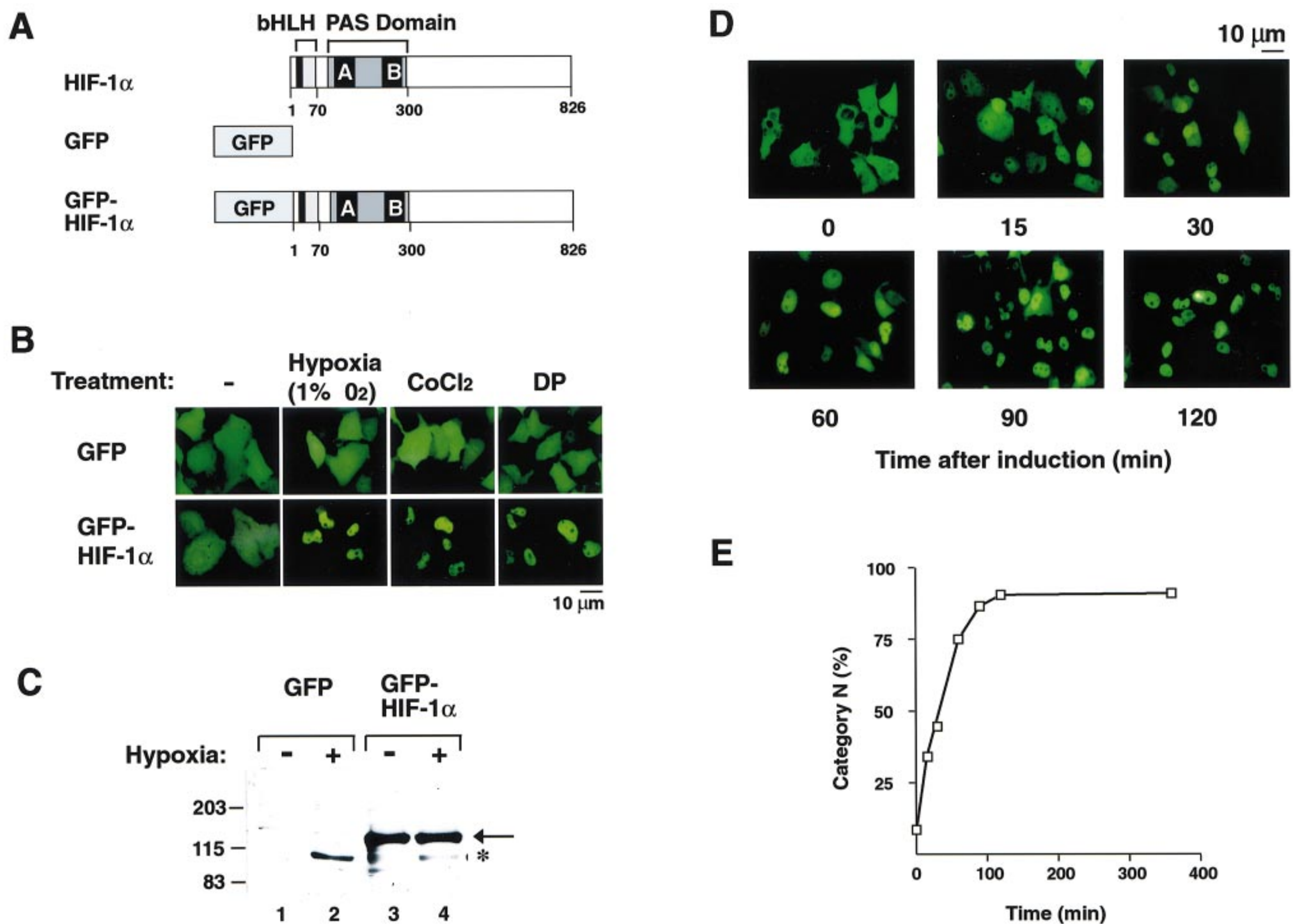

Fig. 2. Hypoxic regulation of nuclear translocation by GFP–HIF-1α. (**A**) Schematic representation showing the GFP and GFP–HIF-1α protein constructs. For HIF-1α, location of the basic helix–loop–helix and PAS (Per/Arnt/Sim) domains are also indicated. (**B**) Hypoxia and hypoxia-mimicking chemicals induce rapid nuclear translocation of GFP–HIF-1α fusion proteins. COS7 cells were transiently transfected with either GFP or GFP–HIF-1α expression vectors and after 24 h expression induced for 6 h either with 1% O_2, 100 μM $CoCl_2$ or with 100 μM 2,2′-dipyridyl (DP) before microscopy. (**C**) Endogenous HIF-1α, but not GFP–HIF-1α, protein levels are upregulated by hypoxic induction. COS7 cells were transfected with non-fusion GFP expression vector (lanes 1 and 2) or with GFP–HIF-1α (lanes 3 and 4). Following transfection cells were exposed to 21 or 1% (hypoxia) O_2 for 30 h as indicated by – and + signs. Whole-cell extracts were prepared as described in Materials and methods, and 50 μg aliquots were analyzed by SDS–PAGE and immunoblotting using anti-HIF-1α antiserum. GFP–HIF-1α protein is indicated by an arrowhead, whereas the asterisk denotes endogenous HIF-1α immunoreactivity. The positions of the mol. wt markers (in kDa) are indicated on the left. (**D**) Time-dependent nuclear translocation of GFP–HIF-1α. COS7 cells were transfected as in (A) with GFP–HIF-1α expression vectors and induced with 100 μM 2,2′-DP. Photographs were taken at the indicated timepoints after induction. (**E**) Graphical presentation of the nuclear entry of GFP–HIF-1α. Cells transfected with GFP–HIF-1α were induced with 100 μM 2,2′-DP and then a total of 250 cells were analyzed for distribution of the fluorescence at fixed timepoints. N (%) is the percentage of cells showing exclusively nuclear fluorescence.

nuclear accumulation of GFP–HIF-1α showed half-maximal time of nuclear transfer of ~30 min (Figure 2D and E) and a complete nuclear import of the protein following 1 h of exposure at 37°C. These kinetics are quite slow in comparison to other transcription factors which show signal-inducible nuclear import. For instance, a significantly more rapid time course of inducible nuclear transport has been reported for the glucocorticoid receptor (Ogawa *et al*., 1995; Carey *et al*., 1996; Htun *et al*., 1996), a ligand-dependent transcription factor showing hormone-dependent nuclear translocation.

To characterize further hypoxic signal-dependent regulation of compartmentalization of HIF-1α in living cells we next asked whether return of the cells to normoxia following exposure to hypoxia or withdrawal of hypoxia-mimicking chemicals would affect the intracellular localization of GFP–HIF-1α. To this end we first induced nuclear import of GFP–HIF-1α (84% of cells belonging to category N) by treatment of transfected cells with 2,2′-DP for 1 h. Subsequently 2,2′-DP was withdrawn by washing of the cells in medium, and the cells were further incubated at normal levels of atmospheric oxygen tension (schematically represented in Figure 3A). After 12 h of incubation under normoxic conditions, 75% of the transfected cells showed an equal distribution of GFP–HIF-1α in both the nuclear and cytoplasmic compartments (category N = C), and the exclusively nuclear pool of GFP–HIF-1α (category N) had been reduced from 84 to 5% (Figure 3B). An almost identical distribution of GFP–HIF-1α was observed after 24 h of withdrawal of 2,2′-DP (Figure 3B). Thus, hypoxia-induced nuclear retention of GFP–HIF-1α could be reversed following withdrawal of the hypoxic signal. To address the question whether this reversion could depend on inefficient nuclear import of *de novo* synthesized GFP–HIF-1α, we also performed this withdrawal experiment in the presence of the protein synthesis inhibitor cycloheximide. Under these conditions, reversal of 2,2′-DP-induced nuclear retention of GFP–HIF-1α was

Table I. Subcellular distribution of GFP–HIF-1α chimeras

	Normoxia				Hypoxia (dipyridyl)			
	N	N > C	N = C	N < C	N	N > C	N = C	N < C
GFP	0	0	100	0	0	0	100	0
GFP–HIF-1α	8	27	65	0	90	7	3	0
GFP–HIF-1α/1–74	69	20	11	0	56	35	9	0
GFP–HIF-1α/1–245	36	38	26	0	31	43	26	0
GFP–HIF-1α/1–330	0	9	91	0	0	5	95	0
GFP–HIF-1α/Δ178–390	57	23	20	0	42	29	29	0
GFP–HIF-1α/331–641	0	0	100	0	0	0	100	0
GFP–HIF-1α/526–641	0	0	100	0	0	0	100	0
GFP–HIF-1α/1–652	0	0	0	100	0	0	0	100
GFP–HIF-1α/526–826	100	0	0	0	100	0	0	0
GFP–HIF-1α/526–813	90	10	0	0	86	11	3	0

Cells were transfected as duplicates with indicated fusion constructs and 24 h after transfection induced with 100 μM 2,2′-DP or with vehicle only for 6 h before microscopic observation and cell counting. Cells were classified into four categories: N, cells containing exclusively nuclear fluorescence; N > C, cells in which the nuclear fluorescence exceeds the cytoplasmic fluorescence; N = C, cells having equal distribution of fluorescence; and N < C, cells having cytoplasmic fluorescence exceeding that in the nucleus. A total of 200–300 cells were analyzed for the distribution of fluorescence and the percentage of cells belonging to each category is indicated in the table.

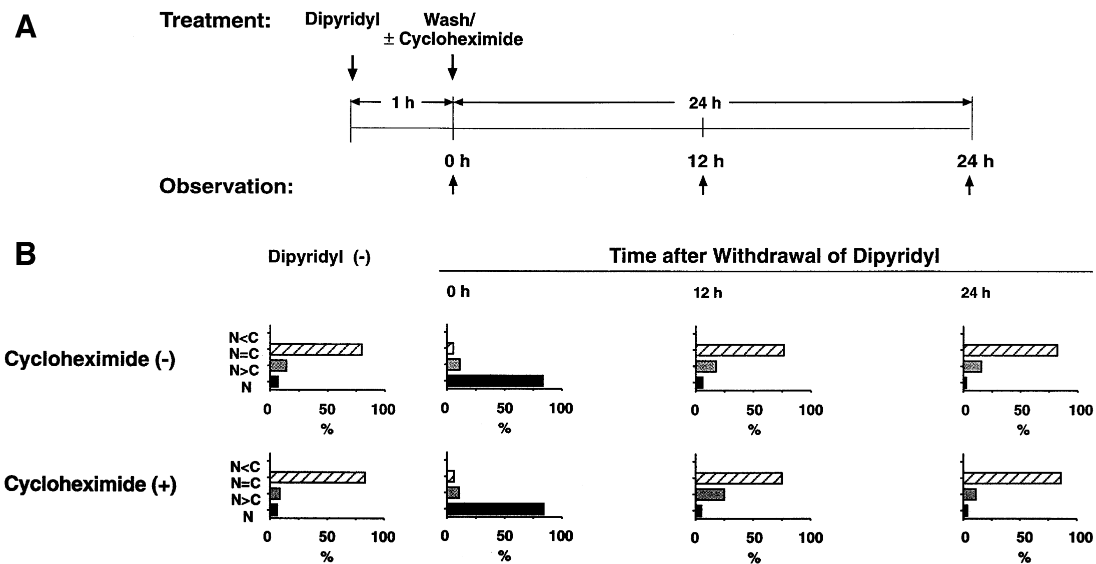

Fig. 3. Nuclear export of HIF-1α upon withdrawal of hypoxic signal. (**A**) Outline of the experimental strategy. COS7 cells were transfected with the GFP–HIF-1α expression vector (6 μg/60-mm dish) for 6 h and grown under atmospheric O_2 for 12 h, whereafter they were either induced with 100 μM 2,2′-DP or left uninduced for 1 h. Subsequently 2,2′-DP was withdrawn by changing the medium and washing the cells, and the cells were therefter incubated in the absence (–) or presence (+) of 25 μM cycloheximide for 24 h. During this time period cells were observed microscopically at different timepoints as indicated by arrows. For each timepoint, a total of 200 cells were counted and analyzed. (**B**) HIF-1α is redistributed from the nucleus upon withdrawal of 2,2′-DP. The subcellular distribution of GFP–HIF-1α is shown at different timepoints following withdrawal. The percentage of cells belonging to each category in indicated (for classification, see Table I). For reference the intracellular distribution of GFP–HIF-1α prior to induction with 2,2′-DP is also shown.

very similar to that observed in the absence of cycloheximide, i.e. reduction of the nuclear pool (category N) of GFP–HIF-1α from 84 to 3%, concomitant with an increase of the pool of GFP–HIF-1α belonging to category N = C from 6 to 82% (Figure 3B), indicative of an export of the originally nuclear pool of protein.

Role of the PAS domain in regulation of nuclear import of HIF-1α

In order to identify the structural motif(s) involved in nuclear import of HIF-1α, we generated a number of expression vectors containing GFP fused to different subdomains of HIF-1α (schematically represented in Figure 4A). Following transient expression of these constructs in COS7 cells, the cells were either exposed to 2,2′-DP or left untreated for 6 h, and subsequently analyzed for compartmentalization of the fusion proteins. As shown in Figure 4B, fusion of the most N-terminal region of HIF-1α spanning the bHLH motif (aa 1–74) to GFP resulted in a predominantly nuclear accumulation of the protein under normoxic conditions. Moreover, nuclear accumulation of this protein was not altered by exposure to 2,2′-DP (Figure 4B and Table I). It has previously been demonstrated that the basic DNA-binding region within the bHLH domain is sufficient for nuclear localization of the bHLH-PAS transcription factors Arnt and the dioxin

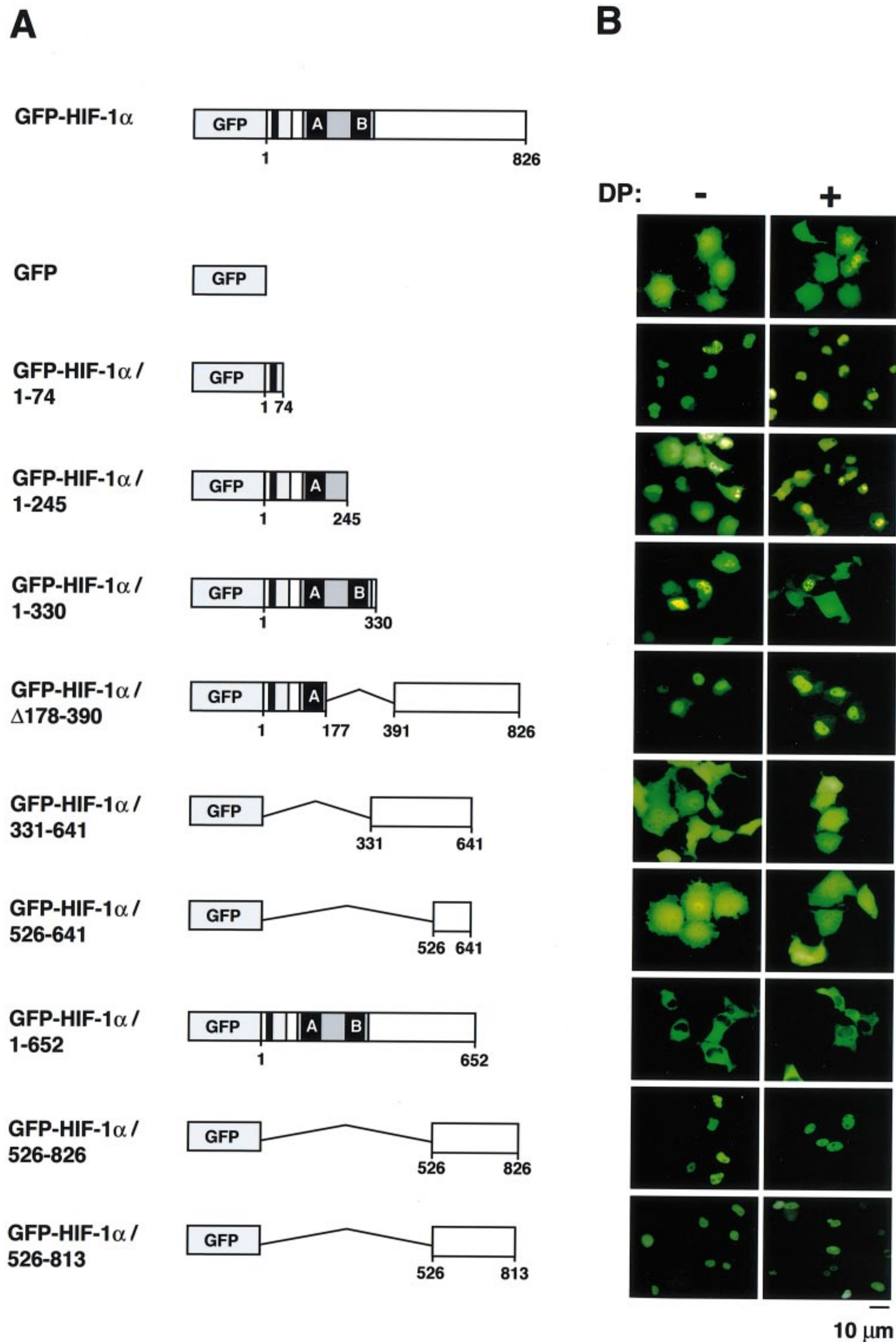

Fig. 4. Subcellular distribution of GFP–HIF-1α fusion proteins. (**A**) Schematic representation of fusion proteins. All expression constructs were assembled into pCMX–SAH/Y145F vector expressing a humanized GFP under the control of the CMV promoter. (**B**) Subcellular distribution of GFP–HIF-1α chimeric proteins. Fusion constructs corresponding to those presented (A) were transfected into COS7 cells and after 24 h expression either 100 μM 2,2′-DP or vehicle (H_2O) were added to the culture medium and incubated for 6 h before observation. Photographs were taken using a Zeiss fluorescent microscope.

(aryl hydrocarbon) receptor (Eguchi *et al*., 1997; Ikuta *et al*., 1998). Thus, in analogy to these proteins, the isolated bHLH domain of HIF-1α mediated constitutive nuclear import. In excellent agreement with these observations, HIF-1α contains between aa 17–33 a bipartite NLS motif similar to that of *Xenopus laevis* nucleoplasmin (Dingwall *et al*., 1987; Figure 5A).

Interestingly, extension of the N-terminal portion of HIF-1α to fragments spanning, in addition to the bHLH domain, the PAS-A motif (HIF-1α/1–245; Figure 4A) or the entire PAS domain (HIF-1α/1–330) generated GFP fusion proteins where the constitutive NLS motif within the bHLH domain appeared to be masked. Unlike HIF-1α/1–74, these two proteins showed significant cytoplasmic

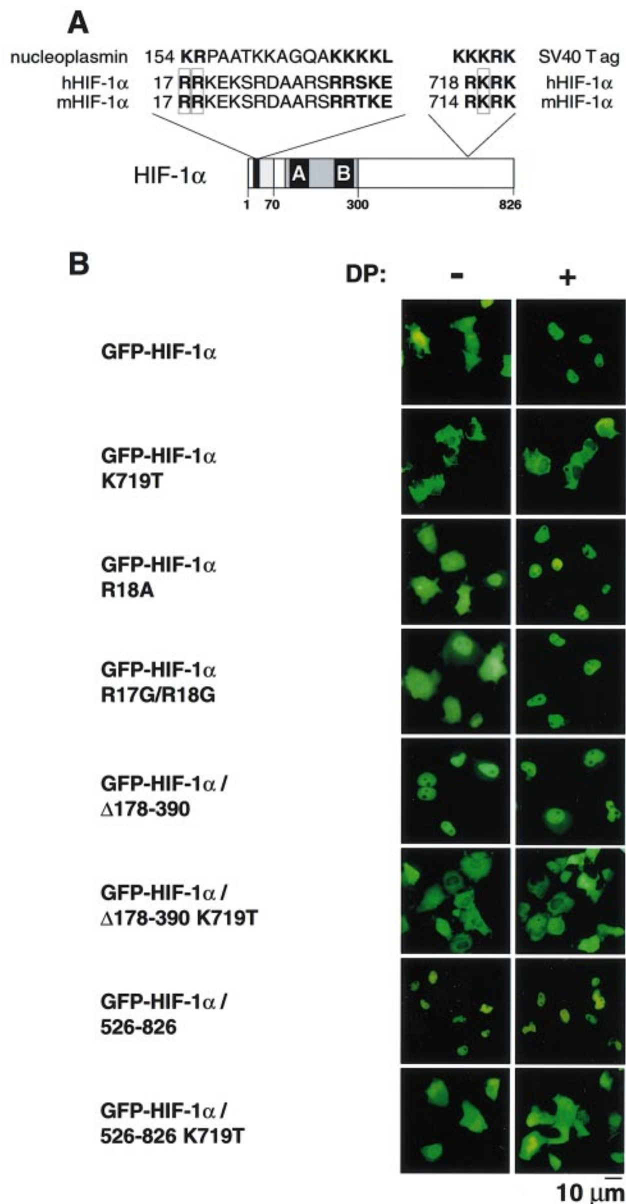

Fig. 5. Identification of an hypoxia-inducible NLS motif within the C-terminus of HIF-1α. (**A**) Analysis of human (h) and mouse (m) HIF-1α sequences reveals two conserved NLS motifs. The N-terminal NLS motif is showing homology to the bipartite-like NLS sequence as exemplified by *X.laevis* nucleoplasmin, whereas the C-terminal NLS motif is related to SV40 large T antigen-like NLS sequence. (**B**) Effect of point mutagenesis within the NLS motifs on nuclear accumulation of GFP–HIF-1α fusion proteins. COS7 cells were transfected with various GFP–HIF-1α fusion protein expression plasmids carrying either wild-type or mutated HIF-1α NLS sequences. The mutations were either single (Arg to Ala) or double (Arg to Gly) amino acid exchanges at residues 17 and 18, or a single amino acid exchange (Lys to Thr) at codon 719, respectively. After transfection, cells were treated with 100 μM 2,2′-DP or with vehicle only for 6 h before fluorescence microscopy.

fluorescence activity (Figure 4B) which was detectable both under normoxic and hypoxic conditions and generated a score of 26 and 95% of the transfected cells within the category N = C, respectively (Table I). These results suggest that the PAS domain harbors a structure repressing nuclear import mediated by the NLS motif within the bHLH domain of HIF-1α, resulting in cytoplasmic retention of the protein. In strong support of this model, in a GFP fusion protein containing a HIF-1α mutant lacking a significant portion of the PAS domain, most notably the PAS B motif (HIF-1α/Δ178–390; Figure 4A), fluorescence was already predominantly nuclear under normoxic conditions (Figure 4B), yielding a nuclear score (categories N and N > C) of 80% (Table I). Exposure to hypoxia had no further effect on the subcellular localization of GFP–HIF-1α/Δ178–390 (Figure 4B; Table I). Thus, removal of the inhibitory motif or cytoplasmic retention signal within the PAS domain of HIF-1α resulted in uncoupling of the protein from hypoxia regulation.

Identification of an NLS motif within the C-terminus of HIF-1α

To investigate further which structural motifs mediated hypoxia-inducible nuclear compartmentalization of HIF-1α and to map the functional architecture of HIF-1α we next examined the intracellular localization of GFP fusion proteins containing HIF-1α structures located C-terminally of the PAS domain, i.e. the fusion proteins GFP–HIF-1α/331–641 and GFP–HIF-1α/526–641 (Figure 4A). These two proteins demonstrated fluorescence both in the cytoplasm and nucleus (100% of the cells belonging to category N = C; Table I) which was unaltered by hypoxia and thus indistinguishable from the properties of the parental GFP protein (Figure 4B). Consistent with our observations above that the PAS domain represses nuclear import mediated by the constitutive NLS motif within the N-terminal bHLH domain, a fusion protein containing the bHLH/PAS domain and the C-terminal portion of HIF-1α extending to aa 652 (GFP–HIF-1α/1–652) showed exclusively cytoplasmic/perinuclear localization (Figure 4B) with 100% of the transfected cells falling into category N < C (Table I). Significantly, unlike GFP fused to full-length HIF-1α, GFP–HIF-1α/1–652 was non-responsive to hypoxia or dipyridyl treatment and thus unable to undergo nuclear translocation under these conditions, indicating that the very C-terminus of HIF-1α harbors sequences which may function as important determinants for inducible nuclear accumulation. Strikingly, fusion to GFP of the C-terminal portion of HIF-1α spanning aa 526–826 generated a protein demonstrating exclusively nuclear fluorescence (Figure 4B). Thus, as shown schematically in Figure 5A, HIF-1α contains a second NLS motif within this region of the protein. In fact, sequence analysis indicated the presence within this portion of HIF-1α at aa 718 a bona fide SV40 large T antigen-type NLS motif characterized by a single cluster of four consecutive basic residues, RKRK. This motif is also conserved in the mouse HIF-1α protein (Figure 5A). Nuclear fluorescence by GFP–HIF-1α/526–826 containing this motif was detected both under normoxic and hypoxic conditions, demonstrating that the domain of HIF-1α harboring this second, SV40 large T antigen-type of NLS motif mediated constitutive nuclear import (Figure 4B; Table I).

A single amino acid mutation in the C-terminal NLS motif abolishes hypoxia-inducible nuclear import of full-length HIF-1α

Given the presence of a second NLS motif within the C-terminus of HIF-1α and the inability of the N-terminal

Table II. Subcellular distribution of GFP–HIF-1α chimeras carrying point mutations in the NLS motifs

	Normoxia				Hypoxia (dipyridyl)			
	N	N > C	N = C	N < C	N	N > C	N = C	N < C
GFP–HIF-1α	8	27	65	0	90	7	3	0
GFP–HIF-1α K719T	0	0	4	96	0	0	19	81
GFP–HIF-1α R18A	37	15	48	0	82	6	12	0
GFP–HIF-1α R17G/R18G	50	24	26	0	84	2	14	0
GFP–HIF-1α/Δ178–390	57	23	20	0	42	29	29	0
GFP–HIF-1α/Δ178–390 K719T	0	0	18	82	0	0	15	85
GFP–HIF-1α/526–826	100	0	0	0	100	0	0	0
GFP–HIF-1α/526–826 K719T	26	24	50	0	15	14	71	0

Cells were transfected as duplicates with indicated fusion constructs and 24 h after transfection induced with 100 μM 2,2′-DP or with vehicle only for 6 h before microscopic observation and cell counting. Cells were classified into four categories as outlined in Table I. A total of 200–300 cells were analyzed for the distribution of fluorescence and the percentage of cells belonging to each category is indicated in the table.

portions of HIF-1α to show hypoxia-inducible nuclear accumulation, we investigated the role of the N- and C-terminal NLS motifs in inducible nuclear import by mutation analysis. To achieve this we introduced within the context of full-length HIF-1α either single or double exchanges of the N-terminal Arg residues of the N-terminal bipartite NLS motifs of HIF-1α, or mutated Lys719 to Thr within the C-terminal NLS motif (Figure 5A). As shown in Figure 5B, point mutations of the N-terminal NLS motif did not affect hypoxia-inducible nuclear translocation of HIF-1α. Importantly, however, the single amino acid exchange within the C-terminal NLS motif resulted in a dramatic reduction of the ability of the HIF-1α to accumulate in the nucleus in response to hypoxia (Figure 5B) yielding a score of predominantly cytoplasmically localized protein (category N < C) of 96% during normoxia versus 81% under hypoxic conditions (Table II). In conclusion, these experiments demonstrate that the C-terminal NLS motif of HIF-1α mediates hypoxia-inducible nuclear import of the protein.

Introduction of the identical amino acid exchange within the C-terminal NLS motif severely impaired the constitutive nuclear accumulation of the isolated C-terminus of HIF-1α fused to GFP (compare GFP–HIF-1α/526–826 in Figures 4B and 5B with GFP–HIF-1α/526–826K719T in Figure 5B). Thus, whereas the isolated C-terminal NLS motif mediates constitutive activity, it shows hypoxia-inducible activity within the context of full-length HIF-1α. Since GFP–HIF-1α/Δ178–390 lacking a major part of the PAS domain, most notably the PAS-B motif (Figure 4A), shows constitutive nuclear localization (Figures 4B and 5B), it appears that the C-terminal NLS motif functions in close partnership with the PAS domain in mediating the inducible nuclear import response. To test this notion, we also introduced the single amino acid exchange of Lys719 to Thr into the PAS B deletion mutant GFP–HIF-1α/Δ178–390. Interestingly, this double mutant failed to show any significant nuclear import under either normoxic or hypoxic conditions (Figure 5B), generating scores of protein predominantly localized in the cytoplasmic compartment (category N < C) of 82 and 85%, respectively (Table II). In summary, these results demonstrate that the N-terminal basic region does not function as an NLS motif in the context of the full-length protein and that the C-terminal NLS motif plays a critical role in mediating inducible nuclear import of HIF-1α.

Evidence for a multi-step activation pathway of HIF-1α in response to hypoxia

We next examined the ability of GFP–HIF-1α fusion proteins in functional cotransfection assays where GFP–HIF-1α expression vectors were introduced into COS7 cells together with a hypoxia-responsive gene reporter construct carrying three tandem copies of the erythropoietin HRE in front of the Herpes simplex thymidine kinase promoter and the luciferase gene. Reporter gene activity was not significantly altered by hypoxic treatment (1% O_2; Figure 6A) or incubation with 2,2′-DP (data not shown) either in the absence of coexpressed proteins or in the presence of transiently expressed parental GFP, most probably due to low levels of endogenous HIF-1 activity in COS7 cells (unpublished observations). However, coexpression of GFP–HIF-1α resulted in stimulation (~2-fold) of reporter gene activity already at normoxia, possibly due to stabilization of HIF-1α levels by the GFP moiety and, as described above, a small but detectable pool of HIF-1α localized in the nucleus under these conditions. This reporter gene activity was further stimulated ~4-fold by hypoxia (Figure 6A). In control experiments, transient expression of wild-type HIF-1α also yielded a similar pattern of reporter gene activation, albeit with a slightly lower potency, generating an ~2-fold activation response upon exposure to hypoxia (Gradin *et al*., 1996; data not shown). Thus, fusion of GFP to the immediate proximity of the N-terminal basic (DNA-binding) region did not interfere with transcriptional activation. In fact, stabilization of the fusion protein (Figure 2C) by the GFP moiety may rather have enhanced the potency of the activation response produced by HIF-1α. As expected, in this functional assay GFP–HIF-1α fusion proteins lacking the DNA-binding bHLH region (GFP–HIF-1α/331–641, GFP–HIF-1α/526–641 and GFP–HIF-1α/526–826) did not induce any reporter gene activity (data not shown).

Although ectopic expression of wild-type Arnt did not significantly enhance hypoxia-induced transcriptional activation by GFP–HIF-1α, transient expression of a dominant negative Arnt mutant, ArntΔb, lacking the DNA-binding basic (b) domain (Lindebro *et al*., 1995), resulted in potent inhibition of the the hypoxia-dependent activation response (Figure 6B). Thus, these results demonstrate that hypoxia-induced transcriptional activation of HRE-driven reporter gene expression by GFP–HIF-1α was critically dependent on interaction with the bHLH–PAS partner

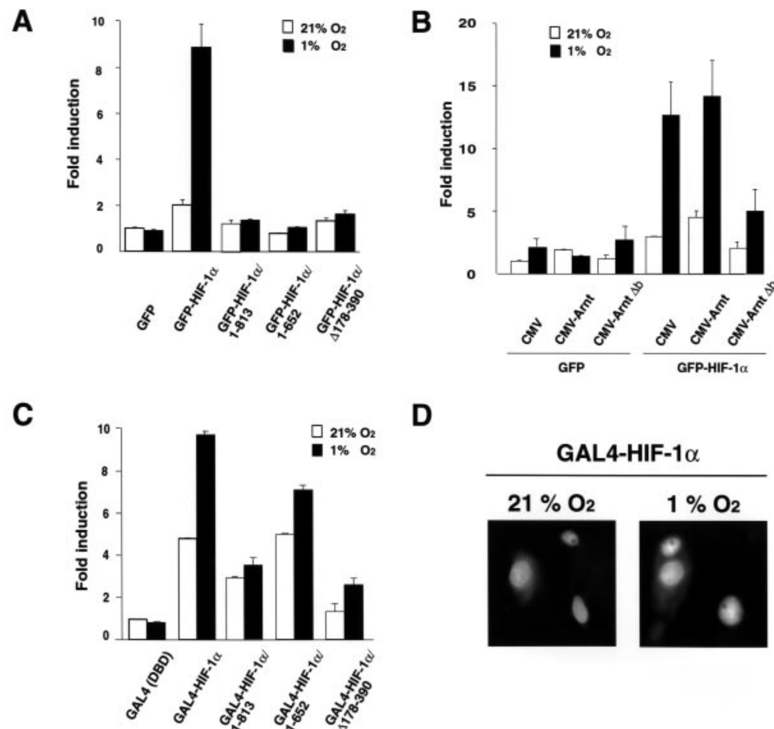

Fig. 6. Overlapping C-terminal structures mediate inducible nuclear translocation and transcriptional activation by HIF-1α. (**A**) Transcriptional activation of a hypoxia-responsive gene by GFP–HIF-1α chimeras. COS7 cells were cotransfected either with GFP or GFP–HIF-1α fusion constructs together with a hypoxia-responsive reporter gene (HRE-luc). Six h after transfection cells were exposed to either 21 or 1% O_2 for 30 h before harvest. Normalized reporter gene activities are expressed relative to that of non-fusion GFP in normoxia. The results of two independent experiments performed in triplicate ± SEM are shown. (**B**) Transcriptional activation by GFP–HIF-1α is Arnt-dependent. COS7 cells were cotransfected with either GFP or GFP–HIF-1α expression vectors and a hypoxia-responsive reporter gene (HRE-luc) in the presence of either wild-type Arnt or a dominant negative Arnt mutant, ArntΔb, lacking the DNA-binding b domain. Cells were exposed to 21 or 1% O_2 for 30 h before harvest and reporter gene assays. Normalized reporter gene activities are expressed relative to that of non-fusion GFP in normoxia. The results of two independent experiments performed in triplicate ± SEM are shown. (**C**) Functional analysis of GAL4–HIF-1α fusion proteins. The same subregions of HIF-1α as characterized in (A) were fused to the GAL4 DBD and transfected into COS7 cells together with a reporter plasmid expressing the luciferase gene driven by the thymidine kinase minimal promoter under the control of five copies of GAL4-binding sites. Cells were exposed for 21 or 1% O_2 for 30 h before harvest and reporter gene assays. Normalized reporter gene activities are expressed relative to that of the non-fusion GAL4 DBD in normoxia. The results of three independent experiments performed in triplicate ± SEM are shown. (**D**) Constitutive nuclear localization of GAL4–HIF-1α. COS7 cells were transiently transfected with pCMX-GAL4–HIF-1α and after 24 h expression exposed to normoxic (21% O_2) or hypoxic (1% O_2) conditions for 6 h before immunocytochemistry. Indirect immunofluorescence of expressed GAL4–HIF1α fusion protein was conducted with anti-GAL4 DBD antiserum, biotinylated anti-rabbit Ig antibody and Texas Red conjugated with streptavidin.

factor Arnt. In line with these results the mutant GFP–HIF-1α/Δ178–390 lacking the PAS-B domain, an important dimerization interface with Arnt (Lindebro *et al.*, 1995), failed to significantly induce HRE-dependent reporter gene expression in response to hypoxia (Figure 6A).

To analyze functional activities of HIF-1α independently of the endogenous DNA-binding and dimerization domains and the Arnt partner, we next fused HIF-1α to the heterologous DNA-binding domain of GAL4 (GAL4 DBD). Functional activity of the fusion protein was monitored in COS7 cells by a cotransfection assay using a reporter gene construct carrying five GAL4 DNA-binding elements in front of a thymidine kinase promoter and the luciferase gene. Six h after transfection the media was changed and cells were either left uninduced or exposed to 1% O_2 for 30 h prior to harvest. In the presence of either empty expression vector or a vector expressing the minimal GAL4 DBD, reporter gene activity was unaltered by reduced oxygen levels. Transient expression of GAL4–HIF-1α at normoxia resulted in moderate (~4- to 5-fold) reporter gene activation (Figure 6C). However, when cells were incubated at 1% O_2 (Figure 6C; or with 100 μM 2,2′-DP; data not shown), the functional activity of GAL4–HIF-1α was further enhanced ~2-fold, resulting in ~8- to 10-fold stimulation of reporter gene activity over the background levels (Figure 6C). The GAL4 DBD is known to harbor an NLS motif capable of taking fusion proteins to the nucleus (Ma and Ptashne, 1987). We therefore used antibodies directed against the DNA-binding domain of GAL4 to examine in both normoxic and hypoxic cells the intracellular localization of the GAL4–HIF-1α fusion protein by immunofluorescence. As shown in Figure 6D, the GAL4 DBD mediated constitutive nuclear localization of the GAL4–HIF-1α fusion protein (Figure 6D). Given the fact that GAL4–HIF-1α showed hypoxia-inducible functional activity, these data establish that inducible nuclear import of HIF-1α does not suffice for transcriptional activation, indicating a multi-step pathway that determines activation of HIF-1α upon exposure to hypoxia.

Overlapping C-terminal structures mediate inducible nuclear translocation and transcriptional activation by HIF-1α

Based on deletion analysis, HIF-1α has been reported to harbor two transactivation domains (Jiang *et al.*, 1997;

Pugh *et al.*, 1997). Whereas conflicting data exist with regard to hypoxia inducibility of the expression levels of these two domains, the minimal transactivation domains have been mapped to amino acid residues 531–582 and 775–826 (Jiang *et al.*, 1997; Pugh *et al.*, 1997). Consistent with these observations, GFP–HIF-1α fragments lacking transactivation domains but showing constitutive nuclear localization (e.g. GFP–HIF-1α/1–74) did not induce reporter gene activity. In a similar fashion the fusion proteins GFP–HIF-1α/1–245 and GFP–HIF-1α/1–330 were devoid of any functional activity (data not shown). Moreover, transient expression of GFP–HIF-1α/1–652 failed to stimulate HRE-dependent reporter gene activity over background values (Figure 6A), although this protein contains one of the transactivation domains of HIF-1α, resulting in transcriptional activation when fused to the GAL4 DBD (Figure 6C), and was expressed at levels similar to those of transiently expressed GFP–HIF-1α (data not shown). This result is in excellent agreement with the inability of GFP–HIF-1α/1–652 to enter the cell nucleus (Figure 4B). In conclusion, these data demonstrate that the inducible nuclear localization and transactivation functions, although contained within the C-terminus of HIF-1α, represent distinct functional entities which can be separated from one another. This conclusion is further strengthened by the finding that deletion of the 13 most C-terminal amino acid residues from HIF-1α resulted in significant reduction of transcriptional activity, both with GFP–HIF-1α/1–813 and GAL4–HIF-1α/1–813 (Figures 6A and 6C), although both of these proteins were capable of entering the nucleus (data not shown).

Transcriptional activation by HIF-1α—hypoxia-dependent recruitment of the CBP coactivator is functionally uncoupled from the inducible NLS motif

CBP is a transcriptional coactivator protein known to interact with a number of constitutively active or inducible DNA-binding transcription factors including, among others, CREB, c-Fos, c-Jun, various members of the steroid receptor superfamily and p53 (Shikama *et al.*, 1997). CBP has also been demonstrated to physically associate with HIF-1α and to play a role in hypoxia-dependent transactivation of erythropoietin promoter in Hep3B cells (Arany *et al.*, 1996). We therefore initially examined the effect of overexpression of CBP on hypoxia-dependent activation by GFP–HIF-1α of the minimal HRE-driven reporter gene. As expected, transient expression of GFP–HIF-1α under hypoxic conditions resulted in ~4- to 5-fold stimulation of the HRE-dependent reporter gene compared with the activation response observed under normoxic conditions (Figures 6A and 7A). Coexpression of GFP–HIF-1α with CBP further enhanced the hypoxia-dependent activation response by ~3-fold, resulting in ~15-fold stimulation of reporter gene activity over the levels observed at normoxia, thereby establishing that CBP supports transcription by the HIF-1α/Arnt complex (Figure 7A). Moreover, coexpression of GFP–HIF-1α K719T with CBP did not produce any significant stimulation of reporter gene activity under hypoxic conditions, indicating the inability of the single amino acid mutant HIF-1α protein to functionally interact with CBP. This is in excellent agreement with the data

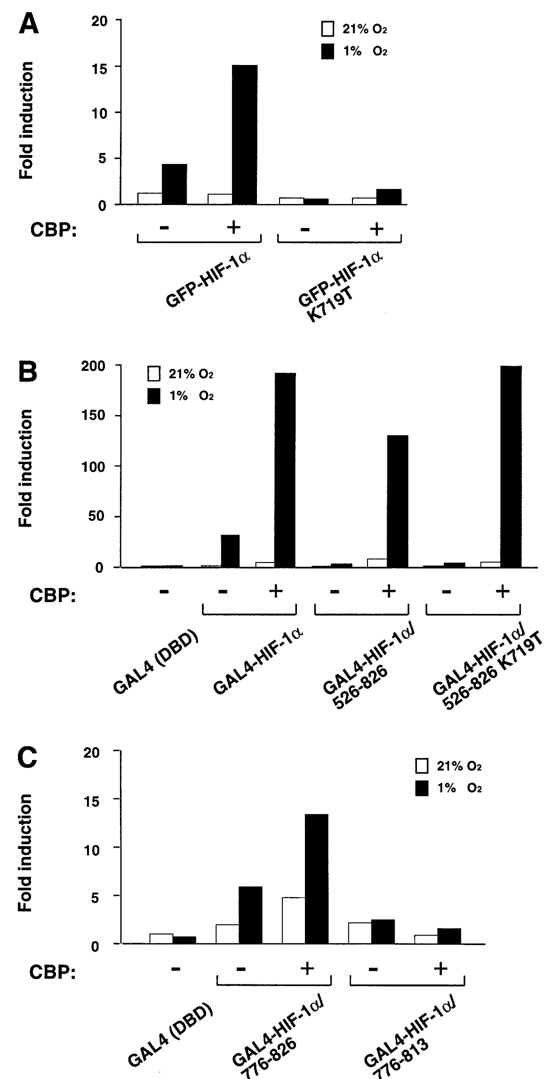

Fig. 7. Hypoxia-dependent recruitment of the CBP coactivator by HIF-1α. (**A**) Stimulation of hypoxia-responsive gene expression by CBP. COS7 cells were cotransfected with either GFP–HIF-1α or GFP–HIF-1α/K719T together with HRE-luciferase reporter and Rous sarcoma virus (RSV)-driven CBP expression vector (or empty expression plasmid). Six h after transfection cells were exposed to either 21 or 1% O_2 for 30 h before harvesting the cells. Luciferase values were normalized for transfection efficiency by cotransfection of alkaline phosphatase expressing pRSV-AF. The data are expressed relative to that of GFP–HIF-1α in normoxia. (**B**) NLS mutation eliminating inducible nuclear import does not affect hypoxia-dependent recruitment of CBP. Expression vectors for GAL4–HIF-1α or GAL4–HIF-1α transactivation domain fusion proteins were transfected into COS7 cells together with a GAL4-responsive reporter plasmid with or without CBP expression plasmid. Cells were exposed to either 21 or 1% O_2 for 30 h before harvest and reporter gene assays. (**C**) The C-terminal transactivation domain of HIF-1α is targeted by CBP. Expression vectors for GAL4 fusion protein containing the minimal C-terminal transactivation domain (amino acids 776–826) or a deletion mutant thereof were transfected into COS7 cells together with a GAL4-responsive reporter plasmid in the absence or presence of the CBP expression vector. Cells were exposed for 21 or 1% O_2 for 30 h before harvest and reporter gene assays. After normalization for transfection efficiency using alkaline phosphatase activity, reporter gene activities are expressed relative to that of GAL4 in normoxia.

demonstrating that GFP–HIF-1α K719T protein was unable to undergo inducible nuclear accumulation (Figure 5B and Table II). As a further control, we examined

whether exposure to hypoxia affected the function of other transcription factors which are targets for regulation by CBP. In these experiments we observed in reporter gene assays no effect of hypoxia on ligand-dependent enhancement by CBP of the functional activity of the retinoic acid receptor α (data not shown), a well-characterized target of CBP (for a recent review see Torchia *et al*., 1998).

As outlined above, fusion of a HIF-1α to the GAL4 DBD generated a chimeric protein which produced a modest (~2-fold) hypoxia-dependent activation of reporter gene activity (Figures 6C and 7B). In the presence of CBP, however, a further 19-fold stimulation of reporter gene activity was observed under hypoxic conditions, whereas, under normoxic conditions, CBP only slightly (2- to 3-fold) enhanced the activity of GAL4–HIF-1α (Figure 7B). We next wanted to examine the transactivation capacity of a GAL4 DBD fusion protein containing the C-terminal subregion 526–826 which harbors both transactivation domains of HIF-1α (Jiang *et al*., 1997; Pugh *et al*., 1997) and showed exclusive nuclear localization when fused to GFP (Figures 4B and 5B). This chimeric protein produced a modest (~2-fold) hypoxia-dependent activation of reporter gene activity but was potently enhanced in its inducible activation function following overexpression of CBP (Figure 7B). Thus, these data demonstrate that the C-terminus of HIF-1α recruits CBP in a hypoxia-dependent manner. Interestingly, CBP was also able to enhance dramatically the activity of the GAL4–HIF-1α/526–826 K719T fusion protein carrying a point mutation in the hypoxia-inducible NLS motif of HIF-1α. In fact, the effect of CBP to support inducible transcription by either GAL4–HIF-1α/526–826 or GAL4–HIF-1α/526–826 K719T was very similar (Figure 7B), thus demonstrating that inactivation of the hypoxia-inducible NLS motif does not alter the ability of this domain to respond to hypoxia by coactivator recruitment and subsequent transactivation. We also examined the ability of CBP to support the function of the isolated C-terminal transactivation domain (amino acids 776–826) fused to the GAL4 DBD. Interestingly, whereas this domain mediated activation in the presence of CBP in hypoxic cells, it failed to respond to CBP following deletion of its 13 most C-terminal amino acids (Figure 7C; compare GAL4–HIF-1α/776–826 with GAL4–HIF-1α/776–813), strongly suggesting that these residues constitute a target for hypoxia-dependent regulation by CBP.

Discussion

In this report we have demonstrated a complex mode of signal transduction by the transcription factor HIF-1α in response to hypoxia. This signaling pathway involves induced nuclear translocation of the protein in hypoxic cells, and, within the nucleus, activation of the C-terminal transactivation domain of HIF-1α enabling it to recruit the CBP/p300 coactivator protein.

In the case of a number of transcription factors mediating inducible gene regulation in response to extracellular signals, translocation of the factor from the cytoplasm to the nucleus constitutes an early and important regulatory event. For instance, NF-κB represents a well-studied model system where NF-κB is maintained in a latent form in the cytoplasm of non-stimulated target cells associated with the inhibitory protein IκB. Upon exposure to an inducing signal, e.g. TNF, NF-κB is released from its inhibitor by phosphorylation of IκB, and ensuing proteosomal degradation of IκB, enabling nuclear import of NF-κB (for a recent review see Stancovski and Baltimore, 1997 and references therein). In another well-studied model system certain steroid hormone receptors, including the glucocorticoid, androgen and progestrone receptors, show inducible nuclear translocation upon binding of hormone (Guiochon-Mantel *et al*., 1991; Ylikomi *et al*., 1992; Carey *et al*., 1996; Htun *et al*., 1996; Georget *et al*., 1997). For example, the glucocorticoid and progesterone receptors harbor hormone-inducible NLS motifs in the vicinity of the ligand-binding domain which mediates repression and derepression of receptor function (Picard and Yamamoto, 1987; Ylikomi *et al*., 1992). In the absence of hormone, this NLS motif appears to be masked, possibly due to the conformation of the non-occupied ligand-binding domain or proteins associated with the ligand-binding domain. Three-dimensional studies of a number of ligand-binding domains of steroid receptors have documented conformational changes (Brzozowski *et al*., 1997 and references therein) which may possibly result in unmasking of the NLS. In agreement with these regulatory properties, deletion of the ligand-binding domain of the glucocorticoid receptor generates a protein which shows constitutive nuclear localization (Picard and Yamamoto, 1987).

In the present study we have observed that HIF-1α was present to a considerable degree in the cytoplasm of normoxic cells. Upon exposure to hypoxia GFP–HIF-1α was targeted to the nucleus, albeit with slower kinetics of nuclear translocation than those described, for example, in a similar GFP-fusion protein construct with the glucocorticoid receptor (with a time of half-maximal nuclear transfer of HIF-1α of ~30 min in comparison to 5–10 min for the glucocorticoid receptor; Carey *et al*., 1996; Htun *et al*., 1996), possibly suggesting a more complex mode of induction of nuclear import of HIF-1α. We and others have previously demonstrated that the ubiquitin–proteasome proteolytic pathway determines a very rapid turn-over of HIF-1α protein levels under normoxic conditions. In contrast, there is a dramatic stabilization by an as yet unknown mechanism of HIF-1α protein in hypoxic cells, resulting in up-regulation of HIF-1α protein levels (Huang *et al*., 1996; Kallio *et al*., 1997; Salceda and Caro, 1997). Consistent with the general ability of GFP to stabilize fusion partner proteins due to its long half-life (Abrams, 1998), we observed in the present experiments no differences in the levels of GFP–HIF-1α fusion proteins in normoxic cells in comparison to hypoxic cells. Thus, as pointed out above, the generation of GFP fusion proteins efficiently uncoupled HIF-1α from this presumably very early level of regulation of HIF-1α function, facilitating the study of subsequent regulatory steps in the hypoxia signal transduction pathway.

What determines inducible nuclear translocation of HIF-1α? Within the family of bHLH/PAS transcription factors the property of inducible nuclear import is not unique for HIF-1α. In analogy to HIF-1α, the non-activated, ligand-free form of the dioxin receptor is present in the cytoplasmic compartment of target cells, and, upon binding certain environmental pollutants such as dioxin,

the receptor translocates to the nucleus (Ikuta *et al.*, 1998 and references therein). In further analogy to HIF-1α, the ligand activated form of the dioxin receptor dimerizes with Arnt to recognize asymmetric response elements which are distinct from the HREs. Whereas both HIF-1α and the dioxin receptor show inducible nuclear import, however, Arnt has been demonstrated to be a constitutively nuclear protein (Pollenz *et al.*, 1994; Eguchi *et al.*, 1997) showing constitutive DNA-binding and transcriptional activities on the symmetric E box motif CACGTG (Antonsson *et al.*, 1995; Sogawa *et al.*, 1995; Swanson *et al.*, 1995). Unlike HIF-1α, a detailed search of sequences mediating nuclear import of the dioxin receptor and Arnt have only demonstrated a single NLS motif within or close to the highly charged basic region of the DNA-binding bHLH domain (Eguchi *et al.*, 1997; Ikuta *et al.*, 1998). Interestingly however, two factors most closely related to HIF-1α, *Drosophila* Similar and Trachealess (Trh) proteins, both share in their C-terminal region SV40-like NLS sequences. Whereas the intracellular localization of Similar has not been analyzed, both endogenous and ectopically expressed Trh are localized in cell nuclei (Ward *et al.*, 1998).

Our data demonstrate that a small but significant pool of HIF-1α was partially localized in the nucleus already under non-stimulated (normoxic) conditions with 8% of the GFP fusion proteins falling into category N. Thus, these data indicate that the C-terminal NLS motif of HIF-1α is partially masked or repressed in normoxic cells. Although the single N-terminal NLS motif of the dioxin receptor mediates ligand-inducible nuclear import in the context of the full-length protein, it functions as a constitutive NLS when removed from remaining C-terminal structures of the receptor (Ikuta *et al.*, 1998). In a similar fashion the NLS motif of HIF-1α mediated constitutive nuclear import when separated from surrounding HIF-1α structures. Thus, inducible nuclear import is not an inherent property of these NLS motifs themselves but determined by neighboring functional domains. In this context it is interesting to note that deletion of the PAS B region of HIF-1α resulted in constitutive nuclear localization of the protein, and in turn, this nuclear import was dependent on the C-terminal NLS motif (Figure 5B). Thus, these results strongly implicate the PAS B domain in repression of nuclear import of HIF-1α. Strikingly, the PAS B domain of the dioxin receptor harbors the minimal ligand-binding domain (Whitelaw *et al.*, 1993; Coumailleau *et al.*, 1995). In addition, this domain mediates interaction with the molecular chaperone hsp90 (Whitelaw *et al.*, 1993) which has been implicated in maintaining the dioxin receptor in a repressed, ligand-binding conformation (Pongratz *et al.*, 1992; Carver *et al.*, 1994; Whitelaw *et al.*, 1995). We have previously demonstrated that HIF-1α is associated with hsp90 (Gradin *et al.*, 1996), and it will now be important to elucidate the role, if any, of this molecular chaperone and associated proteins in modulation of HIF-1α function and potential repression of nuclear import of HIF-1α. Obviously, it is an attractive model that, in the hypoxic cell, derepression of HIF-1α function may occur via destabilization of the HIF-1α–hsp90 complex, resulting in unmasking of the NLS motif of HIF-1α.

CBP/p300 has previously been reported to enhance activation of the VEGF and erythropoietin promoters

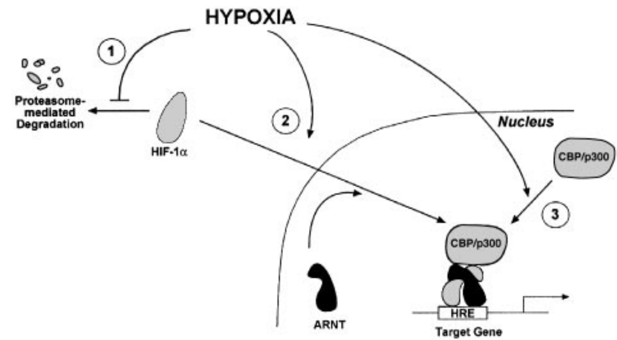

Fig. 8. Model of multiple levels of regulation of HIF-1α activity. Hypoxia leads to inhibition of proteasome-mediated degradation and consequently to stabilization of the protein (1), and stimulates the nuclear import of HIF-1α by unmasking its NLS (2). Subsequently, the Arnt partner factor is recruited to HIF-1α in the nucleus and the resulting HIF-1α/Arnt heterodimer recognizes HREs of target genes, and functionally interacts with transcriptional coactivators (exemplified by CBP/p300) to activate target promoters (3). The order of events within the nucleus (Arnt dimerization/DNA binding and recruitment of CBP) is not yet known.

under hypoxic conditions (Arany *et al.*, 1996). Here we demonstrate that CBP, in a strictly hypoxia-dependent manner, stimulates transcription by the HIF-1α/Arnt-heterodimer on a minimal HRE-containing promoter. By using chimeric proteins containing the HIF-1α transactivation domain fused to the DNA-binding domain of GAL4 we also show that the stimulatory response is likely to involve hypoxia-dependent recruitment of CBP by the HIF-1α transactivation domain. Furthermore, as illustrated in Figure 8, our data strongly indicate that hypoxia-induced nuclear import and gene activation in concert with CBP represent two distinct and separable steps in the signaling pathway by HIF-1α. Most notably, the mutant GAL4–HIF-1α/526–826 K719T containing the nuclear translocation-deficient mutant C-terminal NLS motif but nevertheless targeted to the nucleus by the GAL4 DBD, showed full hypoxia-responsiveness and maintained its ability to interact functionally with CBP in an inducible manner.

CBP has been implicated in regulation of various aspects of cell proliferation and differentiation (Shikama *et al.*, 1997). The precise mechanism(s) by which CBP stimulates the functional activity of the transcriptional machinery is uncertain. Acetylation of the N-terminal portion of histones has been implicated to be a characteristic feature of transcriptionally active chromatin. Several recent discoveries have shown that CBP/p300 and its interacting protein P/CAF both carry intrinsic acetyltransferase activities and, moreover, their acetyltransferase activity is directly involved in stimulating gene transcription (Martínez-Balbás *et al.*, 1998 and references therein). However, the substrate specificity of the histone acetyltransferase activity of CBP is not restricted to histones but has recently been reported to include, at least *in vitro*, other target proteins such as p53, TFIIE-β and the RAP30/74 subunits of TFIIF, suggesting that these enzymes can stimulate transcription also via non-histone pathways (Gu and Roeder, 1997; Imhof *et al.*, 1997). Whereas the C-terminal transactivation domain of HIF-1α appears to be the target of CBP, CBP can acetylate the C-terminal domain of p53 both *in vitro* and *in vivo*, thereby stimulating sequence-

specific DNA-binding activity by p53 (Gu and Roeder, 1997). Thus, p53 and HIF-1α functions appear to be regulated differently by CBP, and both p53 and HIF-1α appear to be able to independently activate transcription following recruitment of CBP. However, under near anoxic conditions, it has recently been reported that p53 and HIF-1α form a physical complex with one another (An *et al.*, 1998), adding further complexity to the mode of combinatorial regulation of functional properties of HIF-1α, and suggesting that a convergence of CBP-dependent, p53- and HIF-1α-mediated pathways may be required for certain functions. The present study has enabled us to identify critical early regulatory steps in signal transduction by HIF-1α in hypoxic cells. To develop our understanding of the mechanisms that determine biological responses to hypoxia, e.g. angiogenesis, it will now be important to dissect further the complex mode of multi-step regulation of HIF-1α function.

Materials and methods

Reagents

pCMX–SAH/Y145F expression vector encoding a modified and highly chromophoric form of GFP under the control of CMV immediate early promoter was a generous gift from Dr Kazuhiko Umesono (Kyoto University, Japan). This humanized GFP (SAH/Y145F) contains an S65A mutation which confers a wavelength shift and temperature resistance to the protein as well as a Y145F substitution increasing the intracellular stability of GFP. pRc/RSV-mCBP-HA construct expressing full-length mouse CBP was a gift from Dr Richard H.Goodman (Vollum Institute, OR). The vector expressing a GFP fusion of full-length HIF-1α was generated by cutting the HIF-1α coding region from pGEX-4T3–HIF-1α as a *Bam*HI–*Not*I fragment (*Not*I site filled-in with Klenow polymerase) and ligating this in-frame into *Bam*HI–*Nhe*I opened pCMX–SAH–Y145F where *Nhe*I site had been blunt-ended with Klenow. Deletion mutant HIF-1α/1–652 was assembled by inserting a *Bam*HI–*Spe*I fragment of HIF-1α into *Bam*HI–*Nhe*I-digested pCMX–SAH/Y145F. GFP fusions encoding HIF-1α subfragments (corresponding to amino acids 331–641, 526–641 and 526–826) were generated by amplifying the equivalent DNA sequences by PCR using Pfu DNA polymerase (Stratagene) together with primer pairs carrying *Bam*HI or *Nhe*I ends. The resulting products were inserted into *Bam*HI–*Nhe*I opened pCMX–SAH/Y145F. GFP fusions carrying N-terminal sequences HIF-1α (1–74, 1–245, 1–330) were cut out from corresponding bacterial glutathione-*S*-transferase (GST) fusion expression vectors (I.Pongratz and L.Poellinger, in preparation) and inserted in-frame to *Bam*HI–*Nhe*I site (*Nhe*I blunt-ended) of pCMX–SAH/Y145F. GFP–HIF-1α fusion proteins ending at amino acid 813 were generated by cleaving the C-terminal 13 amino acids by *Pst*I digestion. Corresponding GAL4 DBD fusion proteins were assembled by cleaving the HIF-1α inserts as *Bam*HI–*Nhe*I fragments and religating them into pCMX expression vector.

Site-directed mutagenesis of the C-terminal NLS was performed by overlap PCR (Ausubel *et al.*, 1994) where the desired mutation (codon 719 AAG→ACA was introduced into a PCR product and then inserted as an *Eco*RI–*Pst*I subfragment into pGFP–HIF-1α/526–826. A GFP fusion of full-length HIF-1α carrying K719T mutation was thereafter assembled by inserting the N-terminal *Bam*HI–*Spe*I fragment of HIF-1α into pGFP–HIF-1α/526–826 K719T. PCR-based mutagenesis with specific oligonucleotides was employed to generate mutations to the N-terminal NLS motif. Fidelity of PCRs and identity of constructs was confirmed by sequencing the inserts.

Cell culture and transfections

COS7 cells (from ATCC) were routinely maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) plus penicillin (50 IU/ml) and streptomycin (50 μg/ml). For analysis of nuclear translocation of HIF-1α in living cells, we transiently expressed GFP-tagged HIF-1α and its various mutant plasmids in COS7 cells. The cells were cultured on the silane-coated coverslips in 6-cm diameter plastic dishes, and the medium was changed to OPTI-MEM medium lacking phenol red (Life Technologies, Inc.) before transfection. A plasmid cocktail containing 6 μg of the expression plasmids for GFP-tagged HIF-1α and its various mutants was mixed with 12 μl of TransIT-LT1 reagent (Panvera Corp., Madison, WI) and added to the culture. After 6 h of incubation, the medium was replaced with DMEM with 10% FCS. Cells were induced 24 h later with 1% O_2, 100 μM 2,2′-DP or 100 μM $CoCl_2$, or treated with vehicle only.

Transcriptional activity of GFP and GAL4 fusion constructs was analyzed in a cotransfection assay where effector plasmids (0.2 μg/30-mm dish) together with a reporter gene (0.5 μg/30-mm dish) were introduced into COS7 or HeLa cells. The reporter plasmid encoded firefly luciferase gene under the control of thymidine kinase minimal promoter and either five or three copies of GAL4 or HIF-responsive elements, respectively.

Immunocytochemical analysis

COS7 cells grown on fibronectin-coated coverslips were transiently transfected with the expression plasmid for full-length HIF-1α (pCMV–HIF-1α) or HIF-1α fused to the GAL4 DBD (pCMX–GAL4–HIF-1α). After various treatments, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 30 min. Immunostaining of the cells was carried out using anti-HIF-1α antiserum (Kallio *et al.*, 1997) or anti-GAL4 DBD antiserum (Upstate Biotechnology) and the appropriate biotinylated-second antibodies and streptavidin-conjugated fluorescein isothiocyanate (FITC) or Texas Red (Amersham). The coverslips were mounted on glass slides and subjected to microscopic analysis.

Visualization of intracellular trafficking of GFP-tagged proteins in living cells

Transiently expressed GFP fusion proteins were expressed at detectable levels between 24 and 72 h after transfection. Routinely, cells were used for further experiments 48 h after transfection. After various treatments, cells were examined using a Zeiss Axiovert 135 microscope enclosed by an incubator and equipped with a heating-stage, a FITC-filter set, and epifluorescence with illumination from a Gixenon burner (Carl Zeiss Jena GmbH, Jena, Germany). Photographs were taken using Kodak Echtachrome 400, and semi-quantitative assessment of the subcellular localization of the GFP-tagged proteins was performed according to the methods described by Ylikomi *et al.* (1992). In brief, subcellular localization of GFP-tagged proteins was determined by counting ~200 cells in which GFP fluorescence was detected. The GFP fluorescence-positive cells were classified into four different categories: N $<$ C for cytoplasmic dominant fluorescence; N $=$ C, cells having equal distribution of fluorescence in the cytoplasmic and nuclear compartments; N $>$ C for nuclear-dominant fluorescence; and N for exclusive nuclear fluorescence.

Immunoblotting and detection

For the detection of the HIF-1α fusion protein expression, whole cell extracts were prepared essentially as described (Kallio *et al.*, 1997). Briefly, cells were harvested in TEN buffer (40 mM Tris–HCl pH 7.9, 10 mM EDTA, 150 mM NaCl). The cell pellet was frozen in liquid nitrogen and thawed by resuspending in 80 μl of cell extraction buffer [10 mM HEPES pH 7.9, 400 mM NaCl, 0.1 mM EDTA, 5% (v/v) glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride], followed by centrifugation for 30 min at maximal velocity. Fifty μg of the total cell proteins were blotted after SDS–polyacrylamide gel electrophoresis onto nitrocellulose filter and blocked overnight with 5% non-fat milk in PBS. Anti-HIF-1α antiserum (Kallio *et al.*, 1997) was used as a primary antibody as 1:500 dilution in PBS containing 1% non-fat milk for 2 h. After washes, 1:750 dilution of anti-rabbit IgG-horseradish peroxidase conjugate (Amersham) in PBS, 1% non-fat milk was used as a secondary antibody. After extensive washing with PBS the complexes were visualized using enhanced chemiluminescence (Amersham) according to the manufacturer's instructions.

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