Introduction and nuclear translocation of hypoxia-inducible factor-1 (HIF-1): heterodimerization with ARNT is not necessary for nuclear accumulation of HIF-1α

Dmitri Chilov1, Gieri Camenisch1, Ivica Kvietikova1,*, Urs Ziegler2, Max Gassmann1 and Roland H. Wenger1,‡

1Institute of Physiology and 2Institute of Anatomy, University of Zürich-Irchel, CH-8057 Zürich, Switzerland
*Present address: Institut de Biochimie, Université de Lausanne, Ch. des Boveresses 155, CH-1066 Epalinges, Switzerland
‡Author for correspondence (e-mail: labbauer@physiol.unizh.ch)

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SUMMARY

Hypoxia-inducible factor-1 (HIF-1) is a master regulator of mammalian oxygen homeostasis. HIF-1 consists of two subunits, HIF-1α and the aryl hydrocarbon receptor nuclear translocator (ARNT). Whereas hypoxia prevents proteasomal degradation of HIF-1α, ARNT expression is thought to be oxygen-independent. We and others previously showed that ARNT is indispensable for HIF-1 DNA-binding and transactivation function. Here, we have used ARNT-mutant mouse hepatoma and embryonic stem cells to examine the requirement of ARNT for accumulation and nuclear translocation of HIF-1α in hypoxia. As shown by immunofluorescence, HIF-1α accumulation in the nucleus of hypoxic cells was independent of the presence of ARNT, suggesting that nuclear translocation is intrinsic to HIF-1α. Co-immunoprecipitation of HIF-1α together with ARNT could be performed in nuclear extracts but not in cytosolic fractions, implying that formation of the HIF-1 complex occurs in the nucleus. A proteasome inhibitor and a thiol-reducing agent could mimic hypoxia by inducing HIF-1α in the nucleus, indicating that escape from proteolytic degradation is sufficient for accumulation and nuclear translocation of HIF-1α. During biochemical separation, both HIF-1α and ARNT tend to leak from the nuclei in the absence of either subunit, suggesting that heterodimerization is required for stable association within the nuclear compartment. Nuclear stabilization of the heterodimer might also explain the hypoxically increased total cellular ARNT levels observed in some of the cell lines examined.

Key words: Dioxin receptor, Erythropoietin, Gene expression, Oxygen, Protein stability

INTRODUCTION

In response to reduced oxygenation, activation of the hypoxia-inducible factor-1 (HIF-1) regulates transcription of several genes involved in oxygen homeostasis (reviewed in Bunn and Poyton, 1996; Semenza, 1998; Wenger and Gassmann, 1997). The transcription factor HIF-1 was originally discovered as a critical factor binding to the hypoxia-inducible 3′ enhancer of the erythropoietin (Epo) gene. Subsequently, HIF-1 was shown to be involved in oxygen-dependent expression of many other genes, including vascular endothelial growth factor (VEGF), glycolytic enzymes, glucose transporter-1 (Glut-1), transferrin, inducible nitric oxide synthase and heme oxygenase-1 (Wenger and Gassmann, 1997). HIF-1 is a heterodimeric complex composed of the two basic-helix-loop-helix (bHLH) Per-ARNT-Sim (PAS) subunits HIF-1α and HIF-1β (Wang et al., 1995a). As determined in HeLa cells, highest HIF-1α protein levels are reached at 0.5% oxygen (Jiang et al., 1996b) by a process that involves redox-dependent proteolytic stabilization to prevent HIF-1α ubiquitinylation and rapid degradation in proteasomes (Huang et al., 1998, 1996; Salceda and Caro, 1997).

HIF-1β is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT), which was first cloned as a heterodimerization partner of the aryl hydrocarbon receptor (AhR), also known as the dioxin receptor (reviewed in Gassmann and Wenger, 1997; Hankinson, 1995; Schmidt and Bradfield, 1996). By using an ARNT-mutant cell line (Hepa1C4) derived from Hepa1 mouse hepatoma cells, we (Gassmann et al., 1997; Gradin et al., 1996) and others (Forsythe et al., 1996; Salceda et al., 1996; Wood et al., 1996) have shown that ARNT is indispensable for HIF-1 DNA binding and transactivation.

Mice homozygous for a targeted deletion in the gene encoding HIF-1α are not viable and die around midgestation, mainly due to defective vascularization, heart malformations and failure in neuronal tube closure (Iyer et al., 1998). In addition, embryonic stem (ES) cell-derived solid tumour formation is also affected in the absence of HIF-1α (Carmeliet et al., 1998; Ryan et al., 1998). Similar to HIF-1α, ARNT
deficiency is also embryonic lethal (Kozak et al., 1997; Maltepe et al., 1997), indicating that the heterodimeric HIF-1 complex is a non-redundant master regulator of oxygen homeostasis. Interestingly, targeted deletion of HIF-1α also results in embryonic lethality, because the impaired function of the organ of Zuckerkandl results in reduced catecholamine synthesis (Tian et al., 1998).

HIF-1α protein stabilization in hypoxia is generally considered to be the rate-limiting step in HIF-1 activation. However, a (weaker) concomitant increase in ARNT protein levels has also been observed by immunoblot analyses of nuclear extracts derived from many different cell types (Iyer et al., 1998; Jiang et al., 1997a,b, 1996a,b; Lee et al., 1997; Liu et al., 1998; Martin et al., 1998; Wang et al., 1995a,b,c). The significance of these observations has not been further investigated, but hypoxically increased ARNT levels in nuclear extracts might be related to HIF-1α nuclear translocation. To clarify the role of ARNT in HIF-1α regulation, we analyzed HIF-1α and ARNT levels and subcellular localization following exposure to hypoxia in cell lines that are either wild type or deficient for ARNT.

MATERIALS AND METHODS

Cell culture and hypoxic induction

The human HeLa epithelial carcinoma and Hep3B hepatoma cell lines were obtained from American Type Culture Collection (ATCC numbers CCL-2 and HB-8064, respectively). The human LN229 glioblastoma cell line (Wenger et al., 1997) was a kind gift of E. G. Van Meir (Lausanne, Switzerland). The mouse L929 fibroblast (ATCC CCL-1), and the Hepal (also termed Hepa1C1c7), Hepa1C4 and VT(2) hepatoma cell lines, originally developed by O. Hankinson and co-workers (Hoffman et al., 1991), were kindly provided by V. O'Donnell (Bern, Switzerland), L. Poellinger (Stockholm, Sweden) and J. Caro (Philadelphia, PA), respectively. Wild-type and ARNT-deficient (Maltepe et al., 1997) R1 ES cells were kind gifts of C. Simon (Chicago, IL). All cells were cultured in DMEM medium (high glucose, Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS, Boehringer-Mannheim), 100 i.u./ml penicillin, 100 μg/ml streptomycin, 1X MEM non-essential amino acids, 2 mM L-glutamine and 1 mM sodium pyruvate (all Gibco-BRL) in a humidified atmosphere containing 5% CO2 at 37°C. Oxygen tensions in the incubator (Forma Scientific, model 3319) were either 140 mm Hg (20% O2 v/v, normoxia) or 7 mm Hg (1% O2 v/v, hypoxia). The cells were exposed to hypoxia for 4 hours. Where indicated, lactacystin (10 μM in DMSO, Calbiochem) or N-(2-mercaptobenzoyloxy)glycine (NMPG, 1 μM in H2O, Fluka) was added to normoxic media.

Protein extractions

Nuclear extracts and cytoplasmic fractions were prepared as described previously (Kvietikova et al., 1995). Briefly, 1×10⁸ cells were washed twice, collected in ice-cold PBS and pelleted. After incubation in buffer A (10 mM Tris-HCl, pH 7.8, 1.5 mM MgCl2, 10 mM KCl) on ice for 10 minutes, the cells were lysed by 10 strokes of a Dounce homogenizer and centrifuged for 10 minutes at 15,000 g. The supernatant was collected and stored frozen at −80°C. Protein concentrations were determined by the Bradford protein assay (BioRad) or the BCA assay (Pierce) using bovine serum albumin as a standard.

Immunoblot analysis

Protein extracts were electrophoresed through SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher and Schuell) using standard procedures. Equal protein loading and transfer was verified by Ponceau staining (Sigma). The membrane was blocked with 4% (w/v) instant non-fat milk powder and incubated for 2 hours with the indicated antibodies diluted in PBS containing 4% milk powder. An anti-HIF-1α chicken polyclonal IgY and a mouse monoclonal IgG1 antibody (designated mgc3) were raised against a bacterially expressed GST-HIF-1α fusion protein containing amino acids 530-825 of human HIF-1α. The generation and purification of these antibodies are described in more detail elsewhere (Camenisch et al., 1999). Rabbit anti-ARNT polyclonal IgG antibodies and the mouse anti-ARNT monoclonal antibody 2B10 (Hord and Perdew, 1994) were kindly provided by L. Poellinger and G. H. Perdew (Pennsylvania, PA) or purchased from Affinity BioReagents. Rabbit-anti Sp1 polyclonal IgG antibodies were purchased from SantaCruz Biotechnology. The respective horseradish peroxidase-coupled secondary antibodies were: rabbit anti-chicken (Promega), goat anti-mouse (Pierce) or goat anti-rabbit (Sigma), and Super Signal Chemiluminescent Substrate (Pierce) was used for their detection.

Immunoprecipitation

Nuclear extracts (200 μg) and cytoplasmic fractions (2 mg) from mouse hepatoma cell lines were incubated with the affinity-purified anti-HIF-1α IgY antibody (30 ng) overnight on ice followed by incubation with a rabbit anti-chicken antibody (Promega), or with a rabbit anti-ARNT antibody. Protein A-Sepharose (Pharmacia) was added and incubated with rotation for 30 minutes at 4°C. Following centrifugation of the precipitate at 15,000 g, the pellet was washed three times with either buffer A or buffer C (see above) for cytoplasmic and nuclear fractions, respectively, and twice with 10 mM Tris-HCl, pH 7.5. Finally, the precipitates were analyzed by immunoblotting using either the anti-HIF-1α IgY or the anti-ARNT 2B10 antibody.

Electrophoretic mobility shift assay (EMSA)

An oligonucleotide probe derived from the Epo 3′ enhancer was purified and labelled as described previously (Kvietikova et al., 1995). DNA-protein binding reactions were carried out for 20 minutes at 4°C in a total volume of 20 μl containing 4-5 μg of nuclear extract, 0.1-0.4 μg of sonicated, denatured calf thymus DNA (Sigma) and 1×10⁴ cpm of oligonucleotide probe in 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 50 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 5 mM DTT and 5% glycerol and run on 4% non-denaturing polyacrylamide gels. Electrophoresis was performed at 200 V in TBE buffer (89 mM Tris, 89 mM boric acid, 5 mM EDTA) at 4°C and dried gels were autoradiographed. In vitro transcription and translation of ARNT were performed using the plasmid pBSArntKS+ (Hoffman et al., 1991) and the TNT® coupled transcription-translation rabbit reticulocyte lysate system (Promega) under conditions recommended by the manufacturers. For supershift analysis, 1 μl of anti-HIF-1α mgs3 of leupeptin, pepstatin and aprotinin (all obtained from Sigma). The nuclear extract was centrifuged and the supernatant was dialyzed twice against buffer D (20 mM Tris-HCl, pH 7.8, 100 mM KCl, 0.2 mM EDTA, 20% glycerol). For total cellular lysates, the culture medium was removed, the cells were washed twice with ice-cold PBS and incubated in lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS plus protease inhibitor cocktail) for 10 minutes on ice. The lysates were sonicated and centrifuged at 700 g for 20 minutes. The supernatant was collected and stored frozen at −80°C. Protein concentrations were determined by the Bradford protein assay (BioRad) or the BCA assay (Pierce) using bovine serum albumin as a standard.
hybridoma supernatant was added to the completed EMSA reaction mixture and incubated for 16 hours at 4°C prior to loading.

**Indirect immunofluorescence microscopy**
Adherent cells were fixed with 4% formaldehyde in PBS (pH 8.0) for 10 minutes, washed three times with PBS, permeabilized with 0.5% Triton X-100 in PBS for 5 minutes and rinsed again three times with PBS (all at room temperature). After blocking nonspecific binding by adding 10% FCS in PBS for 30 minutes, the cells were incubated with either the affinity-purified anti-HIF-1α IgY (1-2 ng/ml in PBS) or the monoclonal anti-ARNT 2B10 antibody at 37°C for 1 hour. Primary antibodies were detected by incubation with either an FITC-conjugated rabbit anti-chicken antibody (Promega) or Cy3-conjugated donkey anti-mouse antibody (Jackson Immunoresearch Laboratory) at room temperature for 30 minutes. Following extensive washing in PBS and mounting in Dabco solution (Sigma), cells were analyzed by confocal laser scanning microscopy (CLSM-310, Zeiss). For antibody depletion experiments, the IgY antibody was incubated with a 100-fold excess of GST-HIF-1α fusion protein for 30 minutes on ice. The solution was centrifuged at 10,000 g for 30 minutes and the supernatant was used to stain the cells as described above.

**RESULTS**

**ARNT is induced in nuclear extracts but not in total cellular lysates from cells exposed to hypoxia**
HIF-1α and ARNT protein levels were examined in cytoplasmic fractions and nuclear extracts derived from HeLa, Hep3B, LN229, L929 and Hepa1 cells that were cultured at either normoxia or hypoxia. The samples were analyzed by immunoblotting using an affinity-purified chicken anti-HIF-1α IgY or a rabbit anti-ARNT antibody. As shown in Fig. 1A, HIF-1α protein was detected exclusively in nuclear extracts of hypoxically induced cells. No additional bands were observed, confirming the specificity of the IgY antibody (not shown). While ARNT was not detectable in normoxic nuclear extracts of HeLa, Hep3B and Hepa1 cells, it was found in LN229 and L929 cells. Unexpectedly, following exposure to hypoxia, increased ARNT protein levels were found in nuclear extracts of all cell lines. No HIF-1α and ARNT could be detected in the same amount of cytoplasmic protein derived from the same cell lines (Fig. 1A). Next we tested whether nuclear ARNT accumulation in hypoxia is due to increased cellular ARNT protein concentrations. As shown in Fig. 1B, ARNT levels in total cellular lysates remained unchanged after hypoxic induction of the human cell lines HeLa, Hep3B and LN229, whereas ARNT was repeatedly induced in the mouse L929 and Hepa1 cell lines. HIF-1α was not detectable in normoxic total cellular lysates (not shown).

Taken together, these results imply that hypoxic nuclear ARNT accumulation was a result of translocation from the cytoplasm to the nucleus. At least in some cell lines, increased ARNT production and/or reduced degradation also contributes to the elevated ARNT levels in nuclear extracts. However, the fact that no cytoplasmic ARNT could be detected in normoxia

**Fig. 1.** Immunoblot analysis of HIF-1α and ARNT expression in subcellular fractions of various human and mouse cell lines. (A) The cell lines were cultured under normoxic (20% O2) or hypoxic (1% O2) conditions for 4 hours, and 25 μg of protein from nuclear extracts (n) or cytoplasmic fractions (c) were separated by SDS-PAGE followed by immunoblotting and detection with the monoclonal anti-HIF-1α IgY antibody (upper panel) or the rabbit anti-ARNT antibody (lower panel). (B) ARNT expression analyzed in total cellular lysates (50 μg). Ponceau staining of the membrane prior incubation with antibodies confirmed equal loading and blotting efficiency.

**Fig. 2.** Immunoblot analysis of HIF-1α and ARNT expression in wild-type and ARNT-mutant mouse hepatoma cells. (A) Wild-type Hepa1 and ARNT-mutant Hepa1C4 cells were exposed for 4 hours to hypoxic conditions, and 50 μg each of nuclear extracts (n) and cytoplasmic fractions (c) were separated by SDS-PAGE and analyzed by immunoblotting. Note that the amount of loaded protein was increased twofold compared to Fig. 1, allowing the detection of cytoplasmic HIF-1α. Representative results from a single immunoblot are shown. (B) Hepa1 and Hepa1C4 cells were exposed to hypoxia, and the nuclei extracted with increasing NaCl concentrations. Following incubation with the anti-HIF-1α antibody, the membrane was incubated with an anti-Sp1 control antibody. Ponceau staining of the membranes prior to incubation with antibodies confirmed equal loading and blotting efficiency.
provides evidence against the ‘translocation hypothesis’. Thus, we assume that nuclear ARNT was lost during isolation (but before extraction) of the nuclei rather than being induced by hypoxia. This would suggest a higher affinity of ARNT for the nuclear compartment in hypoxia (i.e. in the presence of HIF-1α) than in normoxia (i.e. in the absence of HIF-1α).

**Nuclear HIF-1α accumulation in an ARNT-mutant hepatoma cell line**

To analyze whether ARNT is necessary for hypoxic induction and nuclear accumulation of HIF-1α, we used an ARNT-mutant Hepa1-derived subline (termed Hepa1C4) that was originally selected for its resistance against 3,4-benzopyrene treatment (Hoffman et al., 1991). We and others previously showed that in Hepa1C4 cells (1) ARNT mRNA levels were decreased, (2) hypoxic response of endogenous as well as reporter gene transcription was impaired and (3) no functional HIF-1 DNA-binding activity was detected (Forsythe et al., 1996; Gassmann et al., 1997; Gradin et al., 1996; Salceda et al., 1996; Wenger et al., 1998; Wood et al., 1996). As shown in Fig. 2A, ARNT protein was also hardly detectable in nuclear extracts from normoxic and hypoxic Hepa1C4 cells. Interestingly, HIF-1α was still induced in nuclear extracts from Hepa1C4 cells, although at lower levels compared to wild-type Hepa1 cells. Reduced HIF-1α levels in nuclear extracts from Hepa1C4 cells were paralleled by increased HIF-1α levels in the cytoplasmic fraction (Fig. 2A). As for ARNT, these findings suggest that the affinity of HIF-1α for the nuclear compartment is higher in the presence of its heterodimerization partner. To directly assess this hypothesis, a titration experiment was performed, using increasing concentrations of NaCl to extract the nuclei. As shown in Fig. 2B, at least 300 mM NaCl was necessary to extract HIF-1α from wild-type cells, whereas in ARNT-mutant cells, HIF-1α had already leaked out of the nuclei in the absence of NaCl. Correspondingly, the HIF-1α content was much lower in cytoplasmic fractions from Hepa1 than from Hepa1C4 cells. In contrast, the unrelated transcription factor Sp-1 was efficiently extracted at 300 mM NaCl in both cell lines. Taken together, these results suggest that ARNT is required to retain HIF-1α in the nucleus but not for HIF-1α nuclear translocation.

**Fig. 3.** Electrophoretic mobility shift assay (EMSA) of nuclear extracts derived from wild-type and ARNT-mutant mouse hepatoma cells. Nuclear fractions were prepared from normoxic or hypoxic wild-type Hepa1 and ARNT-mutant Hepa1C4 cells. Nuclear protein extracts (5 μg) were incubated with a 32P-labelled oligonucleotide probe carrying the HIF-1 binding site derived from the Epo 3' enhancer. The HIF-1 complex is supershifted by the monoclonal anti-HIF-1α antibody mgc3. HIF-1 DNA-binding activity in hypoxic ARNT-mutant Hepa1C4 nuclear extracts is reconstituted following addition of in vitro translated wild-type ARNT protein. Migration positions of hypoxia-inducible (HIF-1), constitutive (ATF-1/CREB-1 family members) and nonspecific factors are indicated.

**Fig. 4.** Co-immunoprecipitation of HIF-1α:ARNT complexes from nuclear extracts of hypoxic hepatoma cells. Nuclear extracts (n, 200 μg) and cytoplasmic fractions (c, 2 mg) were prepared from normoxic and hypoxic wild-type Hepa1 and ARNT-mutant Hepa1C4 cells. Following incubation with either the anti-HIF-1α IgY antibody, followed by a rabbit anti-IgY antibody or the rabbit anti-ARNT antibody, the immune complexes were precipitated by addition of protein A sepharose. The precipitates were analyzed by immunoblotting using the monoclonal anti-ARNT 2B10 antibody or the polyclonal anti-HIF-1α IgY antibody.
Reconstitution of HIF-1 DNA-binding activity in nuclear extracts from Hepa1C4 cells

To prove that nuclear HIF-1α from Hepa1C4 cells is capable of forming a functional complex with ARNT, EMSAs were performed using an Epo 3′ enhancer-derived HIF-1 DNA-binding oligonucleotide (Kvietikova et al., 1995). Hepa1C4 nuclear extracts were complemented with exogenous ARNT protein that was translated in vitro using reticulocyte lysates. As shown in Fig. 3, synthetic ARNT could reconstitute HIF-1 DNA-binding despite a general inhibitory effect of the reticulocyte lysate on DNA-binding activity of HIF-1 and ATF-1/CREB-1 family members, which assemble the constitutive factor (Kvietikova et al., 1995). Supershift experiments using the monoclonal anti-HIF-1α antibody mcg3 confirmed the identity of the HIF-1 band (Fig. 3). Unprogrammed reticulocyte lysate lacking HIF-1α and ARNT cDNAs did not yield any detectable bands (not shown).

Formation of the HIF-1 heterodimer in the nucleus

To assess the site of HIF-1 heterodimer formation, co-immunoprecipitation experiments were performed using the affinity-purified chicken anti-HIF-1α IgY or the rabbit anti-ARNT antibody. Since chicken IgY antibodies have a very low affinity for protein A or G, a rabbit anti-IgY antibody was used as an intermediary for the precipitation with protein A-sepharose beads. The precipitates were analyzed by immunoblotting using either the chicken anti-HIF-1α IgY or the mouse anti-ARNT 2B10 antibody. As shown in Fig. 4 (left), ARNT was co-immunoprecipitated together with HIF-1α from 200 µg hypoxic nuclear extract but not from 2 mg cytoplasmic fraction of Hepa1 cells. On the other hand, HIF-1α was co-immunoprecipitated together with ARNT from 200 µg hypoxic nuclear extract but not from 2 mg cytoplasmic fraction of Hepa1 cells (Fig. 4, right). Since this represents 40-fold more protein than minimally required to detect cytoplasmic HIF-1α in an

![Fig. 5. Indirect immunofluorescence analysis of HIF-1α expression in normoxic and hypoxic human and mouse cell lines. The various cell lines were cultured under normoxic or hypoxic conditions for 4 hours, and prepared for immunofluorescence as described in Materials and methods. The cells were then incubated with the affinity-purified anti-HIF-1α IgY antibody followed by a FITC-conjugated rabbit anti-chicken antibody and analyzed by CLSM (HeLa: b,c; Hep3B: f,g; Hepa1: j,k; Hepa1C4: n,o; VT[2]: r,s). The corresponding transmission images are shown in the outer columns. Normoxic and hypoxic Hepa1 cells were stained with either the anti-HIF-1α IgY (j,k), or with the IgY antibody that had been depleted of specific anti-HIF-1α antibodies by pre-incubation with GST-HIF-1α fusion protein (v,w).](image-url)
immunoblot (see Fig. 2A), these results suggest that HIF-1α:ARNT heterodimer formation occurs mainly in the nucleus. No co-immunoprecipitations were obtained from normoxic Hepa1 or normoxic and hypoxic Hepa1C4 extracts.

Subcellular localization of HIF-1α in wild-type and ARNT-mutant hepatoma cells

Since HIF-1α was prone to leak from the nuclei during biochemical separation in the absence of ARNT (see above), we examined the subcellular localization of HIF-1α by indirect immunofluorescence and confocal laser scanning microscopy (CLSM). Normoxic and hypoxic HeLa, Hep3B, wild-type Hepa1, ARNT-mutant Hepa1C4 as well as ARNT-reconstituted VT(2) (Hoffman et al., 1991) cells were analyzed using the chicken anti-HIF-1α IgY antibody. In normoxically cultured cells, very weak immunofluorescence was detected in the cytoplasm and nuclei of HeLa and Hep3B cells (Fig. 5b,f), but not in the other cell lines (Fig. 5j,n,r). Upon hypoxic exposure, all cell lines, including ARNT-mutant Hepa1C4 cells, responded with a drastic increase in HIF-1α levels in the nucleus but not in the nucleoli (Fig. 5c,g,k,o,s), demonstrating that ARNT is not required for hypoxic accumulation and nuclear translocation of HIF-1α. In contrast to the immunoblot results (Fig. 2A), HIF-1α was not detectable in the cytoplasm of hypoxic Hepa1C4 cells (Fig. 5o), supporting our notion that the presence of HIF-1α in cytoplasmic fractions is mainly due to leakage from the nucleus. In human HeLa and Hep3B cells, the cytoplasmic fluorescence was brighter than that of mouse Hepa1 cells. To ensure that cytoplasmic staining in HeLa and Hep3B cells was not an experimental artifact, HeLa cells were stained using the monoclonal anti-HIF-1α antibody mgc3, and a similar cytoplasmic fluorescence was observed (data not shown). To confirm the specificity of the chicken anti-HIF-1α IgY antibody, it was pre-absorbed with recombinant GST-HIF-1α fusion protein and removed by centrifugation. As demonstrated in Fig. 5w, this treatment completely abolished binding of the antibody to hypoxic Hepa1 cells.

Induction and subcellular localization of ARNT in hypoxia

It has previously been reported that, unlike the dioxin receptor, ARNT levels remain spatially and temporarily constant in the nucleus following treatment with dioxin-analogs (Pollenz, 1996). In contrast, in our immunoblotting experiments (see Figs 1A, 2A), as well as in those of others (see Introduction), ARNT seemed to be regulated by hypoxia. Because these data might be compromised by artifacts occurring during biochemical separation, we performed indirect immunofluorescence experiments using the anti-ARNT monoclonal antibody 2B10. As shown in Fig. 6b,c, the ARNT level in the nucleus of Hepa1 cells increased following exposure to hypoxia. A nonspecific cross-reactivity of the 2B10 antibody gave rise to background signals in Hepa1 and Hepa1C4 cells, which overwhelmed normoxic ARNT signals in Hepa1 cells. Opposite to Hepa1 cells, no hypoxic ARNT induction was observed in HeLa cells (Fig. 6j,k). These results confirm our immunoblot data using total cellular extracts (Fig. 1B), and show that (1) ARNT is a nuclear protein and its disappearance from normoxic nuclear extracts is due to leakage during preparation of the nuclei, and (2) certain cell lines are

Fig. 6. Indirect immunofluorescence analysis of ARNT expression in normoxic and hypoxic mouse and human cell lines. Wild-type Hepa1 (b,c), ARNT-mutant Hepa1C4 (f,g) and HeLa cells (j,k) were incubated with the monoclonal anti-ARNT antibody 2B10 followed by a Cy3-conjugated donkey anti-mouse antibody. The corresponding transmission images are shown in the outer columns.
capable of inducing ARNT protein levels in a hypoxia-dependent manner.

**Subcellular localization of HIF-1α in wild-type and ARNT-deficient embryonic stem cells**

An ARNT point mutation (Gly326→Asp) in Hepa1C4 cells has recently been reported, which leads to increased proteolytic susceptibility and a higher turnover rate (Numayama-Tsuruta et al., 1997). Despite reduced ARNT mRNA and protein levels, as well as lack of HIF-1 DNA-binding and HIF-1-mediated gene activation (Gassmann et al., 1997; Gradin et al., 1996; Wenger et al., 1998), this ARNT mutation did not abrogate its ability to translocate to the nucleus and to form heterodimers with AhR (Numayama-Tsuruta et al., 1997). Thus, residual amounts of ARNT might have been responsible for the nuclear localization of HIF-1α observed in Hepa1C4 cells (Fig. 5). To rule out this possibility, ES cells in which the ARNT gene has been targeted by homologous recombination (Maltepe et al., 1997) were examined. As shown in Fig. 7A, HIF-1α was detected by immunoblotting in nuclear extracts of hypoxic ARNT+/+ as well as ARNT−/− ES cells, albeit at a lower level in the latter cell line. Nuclear ARNT levels were increased in hypoxic ARNT+/+ ES cells, but, as expected, could not be detected in ARNT−/− ES cells. Staining of ARNT+/+ and ARNT−/− ES cells with the anti-HIF-1α IgY antibody and analysis by CLSM revealed a weak homogeneous immunofluorescence in normoxic cells (Fig. 7Bb,f). In hypoxic wild-type ES cells, HIF-1α accumulated in the non-nucleolar part of the nuclei (Fig. 7Bc,d). Nuclear accumulation of HIF-1α was also clearly observed in hypoxic ARNT−/− ES cells. As can be seen in Fig. 7Bg, HIF-1α signals were variable from cell to cell. However, using CLSM, scanning through multiple horizontal sections of the cell aggregates revealed that the HIF-1α was present in the nuclei of a majority of the cells (data not shown). Thus, these data confirmed that whereas hypoxic HIF-1α accumulation and nuclear translocation is ARNT-independent, HIF-1α stability in the nucleus during isolation of the nuclei is lowered in the absence of ARNT.

**Subcellular localization of HIF-1α following proteasome inhibition and thiol reduction**

Salceda and Caro (1997) reported that the proteasome inhibitor lactacystin and the thiol-reducing agent N-(2-mercaptopropionyl)-glycine (NMPG) induced HIF-1α protein in normoxic cells. It is conceivable that additional hypoxia-inducible post-stabilization steps (e.g. phosphorylation) might

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**Fig. 7.** Hypoxic HIF-1α nuclear accumulation in ARNT+/+ and ARNT−/− embryonic stem (ES) cells. (A) Nuclear extracts (n) and cytoplasmic fractions (c) from normoxic and hypoxic ARNT+/+ and ARNT−/− ES cells were analyzed by immunoblotting using the anti-HIF-1α IgY or the rabbit anti-ARNT antibody. (B) Indirect immunofluorescence analysis of normoxic and hypoxic ARNT+/+ (b,c) and ARNT−/− (f,g) ES cells using the anti-HIF-1α IgY antibody. The corresponding phase-contrast images are shown in the outer columns.
be required for nuclear translocation. Therefore, we first assessed HIF-1α protein induction in total cellular lysates derived from HeLa cells. As shown in Fig. 8A, lactacystin and NMPG induced a series of HIF-1α isoforms with different mobilities compared to hypoxically induced HIF-1α, indicating alternative post-translational modifications. However, nuclear accumulation of HIF-1α was observed by immunofluorescence as a result of the treatment with all of these stimuli (Fig. 8B). In addition, we found that the iron chelator desferrioxamine induced protein stability and nuclear accumulation of HIF-1α (data not shown). Thus, hypoxia as well as hypoxia-mimicking agents that interfere with the process of protein modification/degradation lead to an increase in protein levels that is sufficient for nuclear translocation of HIF-1α.

**DISCUSSION**

In the present study, we used ARNT-mutant Hepa1C4 cells (Numayama-Tsuruta et al., 1997) as well as ARNT-deficient ES cells (Maltepe et al., 1997) to demonstrate that HIF-1α protein accumulation and nuclear translocation can occur independently of ARNT. This finding is not compatible with the term ‘nuclear translocator’ in ‘ARNT’. There is a similar discrepancy between biochemical fractionation and immunofluorescence data, as we show in this work for the HIF-1α:ARNT complex, which has also been reported for the AhR:ARNT complex (reviewed in Hankinson, 1995; Schmidt and Bradfield, 1996). In Hepa1, but not in Hepa1C4 cells, the liganded AhR can be purified from the nucleus. The complementing ARNT was hence believed to be responsible for nuclear translocation of AhR. That this model is wrong has been demonstrated by immunofluorescence experiments showing that AhR and ARNT are translocated to the nucleus independently (Hord and Perdew, 1994; Pollenz et al., 1994), and that following ligand binding the AhR translocates to the nucleus where it is rapidly depleted, whereas ARNT remains at constant levels in the nucleus (Pollenz, 1996). Thus, upon homogenization almost all of the ARNT present in a cell is found outside of the nuclei, apparently because it leaks out of the nuclei (Hord and Perdew, 1994).

A plausible explanation for the failure of subcellular fractionation methods to accurately preserve the distribution of AhR and ARNT observed in vivo might be that heterodimer formation in the nucleus renders the complex more stably associated with the nuclear compartment during isolation of the nuclei. Hence monomeric subunits are more prone to leakage. In this work, we found partial loss of both HIF-1 subunits during isolation of the nuclei in the absence of one of the respective heterodimerization partners in Hepa1C4 and ES cells (lacking ARNT) or under normoxic conditions (lacking HIF-1α). Whether this is due to interaction of the heterodimer with the nuclear scaffold and/or specific DNA binding is a matter of further investigations. In this context, it is noteworthy that Poellinger and coworkers reported a conformational change of HIF-1α upon interaction with ARNT, as shown by altered resistance to proteolytic digestion in vitro (Kallio et al., 1997). Even if this effect might be due to steric hindrance, it provides evidence that increased proteolytic stability in vivo during isolation of the nuclei could also contribute to the difference observed in monomeric versus heterodimeric protein levels of the HIF-1α subunits.

Our findings on ARNT-independent HIF-1α induction in Hepa1C4 and ARNT−− ES cells are in striking contrast to a recent publication where the same cell lines were used as HIF-1α-deficient cell culture models (An et al., 1998). The authors claimed that HIF-1α hypoxically stabilizes wild-type...
p53 protein, as substantiated by the lack of hypoxic p53 induction in the ARNT-deficient cell lines. However, since hypoxia normally induces HIF-1α in these cells, the simplified model of increased p53 due to interaction with HIF-1α needs refinement (Wenger et al., 1998).

Recently, nuclear localization signals (NLS) were identified in the amino termini of ArhR, ARNT and HIF-1α as well as a second NLS in the carboxy-terminal part of HIF-1α (Eguchi et al., 1997; Ikuta et al., 1998; Kallio et al., 1998), consistent with their ability to translocate to the nucleus independently. However, these data were based on transient transfection experiments with fusion protein vectors, conditions that lead to unphysiological accumulation of products already in normoxia, probably because of the high copy number of transfected plasmids or because the fusion partners may stabilize the HIF-1α protein (Kallio et al., 1998). In contrast, we showed that endogenous HIF-1α can accumulate and translocate to the nucleus in hypoxic conditions only. Interestingly, a nuclear export signal was also identified in the conserved bHLH region of Arh (Ikuta et al., 1998). Given the rapid degradation of HIF-1α upon reoxygenation (Huang et al., 1996), it will be important to determine whether HIF-1α degradation occurs inside or outside the nucleus, probably involving a nuclear export signal.

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REFERENCES


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