Hypoxia-induced activation of HIF-1: role of HIF-1α-Hsp90 interaction

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Abstract The protein chaperone heat shock protein 90 (Hsp90) is a major regulator of different transcription factors such as MyoD, a basic helix loop helix (bHLH) protein, and the bHLH-Per-aryl hydrocarbon nuclear translocator (ARNT)-Sim (PAS) factors Sim and aryl hydrocarbon receptor (Ahr). The transcription factor hypoxia-inducible factor-1α (HIF-1α), involved in the response to hypoxia, also belongs to the bHLH-PAS family. This work was aimed to investigate the putative role of Hsp90 in HIF-1 activation by hypoxia. Using a EGFP-HIF-1α fusion protein, co-immunoprecipitation experiments evidenced that the chimeric protein expressed in COS-7 cells interacts with Hsp90 in normoxia but not in hypoxia. We also demonstrated that Hsp90 interacts with the bHLH-PAS domain of HIF-1α. Moreover, Hsp90 is not co-translocated with HIF-1α into the nucleus. At last, we showed that Hsp90 activity is essential for HIF-1 activation in hypoxia since it is inhibited in the presence of geldanamycin. These results indicate that Hsp90 is a major regulator in HIF-1α activation.

Key words: Hypoxia; Hypoxia-inducible factor; Heat shock protein 90

1. Introduction

In response to a reduced oxygen level, activation of hypoxia-inducible factor-1 (HIF-1) regulates the transcription of several genes such as erythropoietin, vascular endothelial growth factor, inducible nitric oxide synthase, ... [1]. All these genes are involved in the adaptative response of the cell and tissue to the hypoxic conditions. HIF-1 is a heterodimeric complex composed of two subunits belonging to the Per-aryl hydrocarbon nuclear translocator (ARNT)-Sim (PAS)-basic helix loop helix (bHLH) family, HIF-1α and ARNT [2,3]. The mechanism of hypoxia-dependent function and activation of the HIF-1α-ARNT complex is currently poorly understood. The HIF-1α protein level is specifically upregulated under hypoxic conditions, through a redox-dependent proteolytic stabilization to prevent HIF-1α rapid degradation by the proteasome pathway [4,5]. HIF-1α then translocates into the nucleus where it dimerizes with ARNT [6] to form the active complex HIF-1.

ARNT is a common factor already known as Ah receptor nuclear translocator [7], which functions in association with Ah receptor as an activator of the transcriptional response of cells to dioxin and other xenobiotics [7]. In the absence of ligand, aryl hydrocarbon receptor (Ahr) exists in an inducible cytoplasmic form associated with the chaperone protein heat shock protein 90 (Hsp90) [8]. Upon ligand binding, Hsp90 dissociates concomitantly, unmasking the dimerization and DNA binding activities of Ahr [9]. Hsp90 chaperone is among the most abundant proteins in the cytosol of eukaryotic cells [10,11]. As chaperone, Hsp90 prevents the aggregation of unfolded proteins generated by heat shock [12], oxidative [13] or ischemic stresses [14]. In addition, Hsp90 is a major partner of different transcription factors. Among these factors are zinc finger proteins such as glucocorticoid receptor [15], bHLH proteins such as MyoD [16] and bHLH-PAS proteins such as Sim and Ahr [6].

Although HIF-1α activity does not seem to be upregulated by ligand binding such as Ahr, there are striking similarities in functional properties between these two factors. Most notably is the dimerization with the common subunit ARNT. In addition, Gradin et al. demonstrated that HIF-1α is able to bind Hsp90 in vitro [17], but it is not known whether Hsp90 modulates HIF-1α activity as it does for Ahr.

This work was aimed to elucidate the role of this chaperone in the regulation of HIF-1α translocation and of HIF-1 transcriptional activity. We observed that Hsp90 is associated with HIF-1α in normoxia and is necessary to obtain an activable form of HIF-1 by hypoxia in COS-7 and human microvascular endothelial (HMEC-1) cells.

2. Materials and methods

2.1. Cell culture

HMEC-1 [19] were grown in MCDB 131 (Gibco, Paisley, UK) containing 15% fetal calf serum and 10 ng/ml EGF, 1 μg/ml hydrocortisone, 10 mM glutamine, 50 U/ml penicillin G, 50 ng/ml amphotericin B. COS-7 cells were grown in DMEM (Gibco, Paisley, UK) containing 10% fetal calf serum, 50 U/ml penicillin G, 50 ng/ml amphotericin B and 20 mM HEPES (pH 7.4). Chemical hypoxia were performed in the presence of 150 μM $CoCl_2$ [20]. Normoxia and hypoxia incubations were performed in CO_2-independent medium (Gibco, Paisley, UK) supplemented with 2% fetal calf serum, 10 mM glutamine, 50 U/ml penicillin G, 50 ng/ml amphotericin B (Gibco) with respectively 21 and 1% O_2.

2.2. Transient transfection and reporter gene assays

To assay the activity of HIF-1, we used the pGL-3SV40HRE vector which contains three copies of the EPO hypoxia responsive element (HRE) downstream from the luciferase gene [21], for which the expression is driven by a heterologous SV40 promoter. HMEC-1/COS-7 transfection was performed in a 96 well plate at 80–90% confluency with DOTAP transfection reagent (Boehringer Mannheim), 600 ng of PGL-SV40. This vector was co-transfected with 200 ng of the control vector pRL-SV40 (Promega, Madison, WI, USA). Twenty-four hours post-transfection, luciferase activity is measured. Luciferase activity was quantitated in a luminometer using the Dual-Luciferase-Reporter

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Abbreviations: HIF-1, hypoxia-inducible factor-1; Hsp90, heat shock protein 90; Ahr, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon nuclear translocator; bHLH, basic helix loop helix; PAS, Per-ARNT-Sim; HRE, hypoxia responsive element; HMEC-1, human microvascular endothelial cells

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System (Promega, Madison, WI, USA). The EGFP-HIF-1α and EGFP-CHIF-1α fusion proteins were obtained by cloning respectively the complete HIF-1α coding sequence (2480 bp) and the sequence between position 1578 and 2480 in the pEGFP-C1 (Clontech, Palo Alto, CA, USA). The resulting plasmids were called pEGFP-HIF-1α and pEGFP-CHIF-1α. The EGFP-HIF-1α and EGFP-CHIF-1α fusion proteins were transiently expressed in COS-7 cells grown in 25 cm^2 flasks for immunoprecipitation experiments and on glass coverslips placed in a six well plate for immunofluorescence experiments. The cells were transfected with DOTAP and 2.5 μg of plasmid. The cells were incubated during 4 h with the transfection solution. Then, the cell medium was replaced with fresh DMEM containing 10% serum.

2.3. Immunoprecipitation and Western blot analysis

Total cell extracts were prepared from COS-7 cells grown in 25 cm^2 flasks and transfected with the pEGFP-HIF-1α, pEGFP-CHIF-1α or pEGFP. Twenty-four hours post-transfection, the cells were incubated for 2 h and 30 min in normoxia or in hypoxia. Then, the cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed using the extraction buffer (Tris 40 mM pH 7.5, KCl 300 mM, EDTA 2 mM, Triton X-100 1%, protease and phosphatase inhibitors). The lysate was collected in micro-centrifuge tubes and incubated on ice for 30 min. The lysate was centrifugated 15 min at 15 000 rpm, 4°C, and the supernatant was collected. An aliquot of 50 μl of this supernatant was boiled and kept frozen (supernatant 1). Hsp90 antibodies (H9010 antibodies provided by Prof. D. Toft, Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN, USA) and 40 μl of Sepharose-protA beads (Pharmacia, Uppsala, Sweden) were added and incubated with the supernatant overnight at 4°C. Then, the beads were collected with the Hsp90 antibodies and the interacting complex by centrifugation for 5 min at 15 000 rpm, 4°C. The supernatant was boiled and kept frozen (supernatant 2). The pellet was washed three times with 800 μl of cold extraction buffer and collected by centrifugation for 5 min at 15 000 rpm, 4°C. At last, 40 μl of loading buffer was added to the beads and the mix was boiled before being frozen. The different extracts (supernatant 1, 2 and immunoprecipitated fraction) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to a PVDF membrane. After blocking in phosphate saline buffer containing 0.2% Tween and 5% dried milk, the blot was probed with mouse anti-EGFP antibodies (Clontech, Palo Alto, CA, USA) or with mouse anti-Hsp90 antibodies (both at 1/2500 dilution). Anti-mouse horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia, Uppsala, Sweden) were used for detection by chemiluminescence.

2.4. In vitro transcription-translation

The bHLH-PAS domain (amino acid positions 1–330) [18] coding sequence of HIF-1α was cloned into the pET-3a expression vector (Novagen, Madison, WI, USA). Two μg of this new plasmid was incubated with wheat germ extract, [^{35}S]methionine and T7 polymerase according to the procedure described in the TNT (Promega, Madison, WI, USA) protocol. In order to know whether the bHLH-PAS domain of HIF-1α interacts with Hsp90, the radiolabelled transcription-translation product was incubated for 4 h with total extracts of COS-7 cells. These samples were prepared using the extraction buffer described in Section 2.3. Hsp90 antibodies were added with Sepharose-protA beads and the mix was incubated overnight. The supernatant was then discarded and the beads and the interacting proteins were washed five times as described in the immunoprecipitation procedure. The extracts were loaded on a 14% SDS-PAGE gel. Then, the gel was dried and the immunoprecipitated product was detected by autoradiography.

2.5. Northern blot analysis

Total RNA was isolated from hypoxic and normoxic COS-7 cells by the guanidium thiocyanate method. Three μg of total RNA from each sample of COS-7 cells was loaded on a 1.5% agarose formaldehyde gel and transferred to a Hybond-N^+ nylon membrane (Amersham). The probe used is 207 bp long and hybridizes on the third exon of the human Hsp90. As internal control, an ubiquitin probe was used (Clontech, Palo Alto, CA, USA). Both probes were labelled by random priming (Amersham Pharmacia, Uppsala, Sweden) using [γ-^{32}P]dCTP. Hybridization was performed at 42°C in a 50% formamide medium.

2.6. Immunofluorescence

COS-7 cells grown on glass coverslip were transiently transfected with the pEGFP-HIF-1α or the pEGFP-CHIF-1α vectors. Twenty-four hours post-transfection, the medium was replaced by fresh CO_2-independent medium and the cells were incubated for 2 h and 30 min in normoxia or in hypoxia. Then, the cells were washed with PBS and fixed 10 min with PBS containing 4% paraformaldehyde. The fixed cells were washed three times with PBS and permeabilized with a solution of PBS+Triton X-100 1%. After three washing steps with PBS+BSA 3%, the cells were incubated for 2 h with the anti-Hsp90 antibodies in PBS+BSA 3%. Then, the cells were washed three times with PBS+BSA 3% and the secondary anti-mouse TRITC-conjugated antibodies were added to the cells. After 1 h of incubation, the cells were washed three times with PBS+BSA before fixation in mowiol. Observations were performed using a confocal microscope (Leica, Hamburg, Germany).

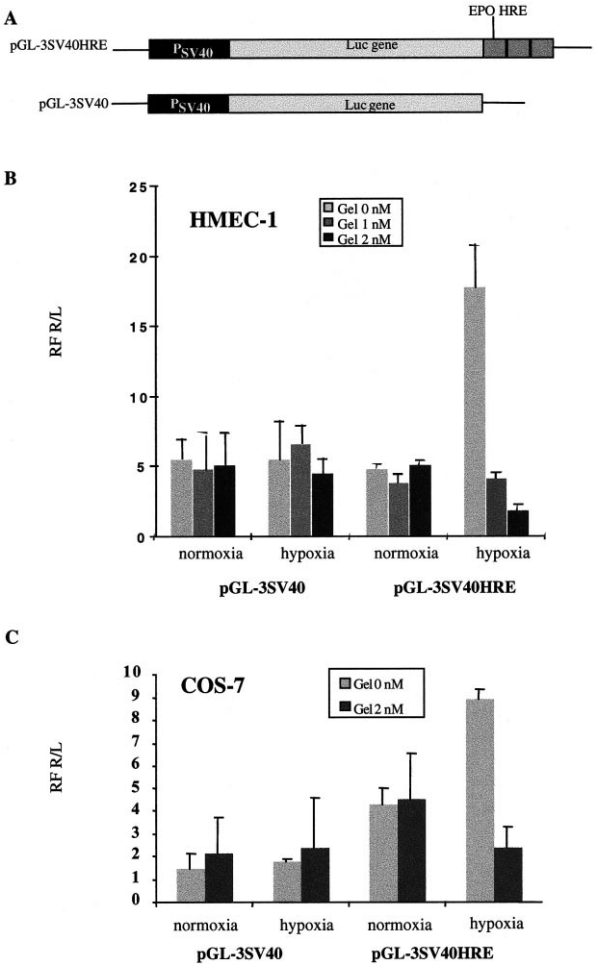

Fig. 1. Hsp 90-dependent activation of HIF-1. (A) Schematic representation of the reporter plasmids used. The pGL-3SV40 is a luciferase reporter plasmid, the Luc gene expression is dependent on a heterologous SV40 promoter. The pGL-3SV40HRE reporter construct expresses a luciferase reporter gene under the dependence of both the SV40 promoter and three HREs. HMEC-1 cells (B) and COS-7 cells (C) were co-transfected either with the pGL-3SV40 or pGL-3SV40HRE together with the pRL-SV40 control vector. Five hours after transfection, the cells were incubated for 20 h either in the absence or in the presence of 150 μM $CoCl_2$ inducing a chemical hypoxia. Then, the cells were lysed for luciferase assays. Data represent the ratio between test firefly luciferase activity and renilla luciferase activity (RF/R). Results are presented as means ± S.D. for triplicates.

3. Results

3.1. Hsp90 is necessary for HIF-1 activity

Several works were aimed to understand the role of Hsp90 in the regulation of transcription factor activity (glucocorticoid receptor, Ahr). The glucocorticoid receptor is activated in the presence of dexamethasone, but this activation is inhibited when cells are treated with geldanamycin, a Hsp90 specific inhibitor [22,23]. In yeast expressing a regulatable level of Hsp90, the Ahr signaling pathway is disrupted when the expression of Hsp90 falls to 5% of its normal level [24]. All these data suggest that Hsp90 is a major component in the regulation of the activity of transcription factors such as steroid receptors and bHLH-PAS factors [22,25]. In order to investigate whether Hsp90 is involved in HIF-1 activation under hypoxic conditions, we used a luciferase reporter system pGL-3SV40HRE (Fig. 1A) where the Luc gene expression is regulated by three HREs [21]. It has previously been shown that the luciferase activity is affected by Hsp90 only in vitro [10]. The reporter system was transfected into endothelial cells (HMEC-1) and into COS-7 cells. The cells were then incubated during 24 h in the absence or in the presence of 150 μM $CoCl_2$, a HIF-1 activator [20], with or without geldanamycin. This chemical hypoxia was able to increase the luciferase activity by 4-fold in HMEC-1 (Fig. 1B) and by 2-fold in COS-7 cells (Fig. 1C). Interestingly, a very strong inhibition of HIF-1 activation was observed in both $CoCl_2$-treated HMEC-1 (Fig. 1B) and COS-7 (Fig. 1C) cells in the presence of geldanamycin. No inhibition of luciferase expression was detected in normoxic cells treated with geldanamycin and transfected with the pGL-3SV40HRE. The luciferase expression remained constant under the different conditions in cells transfected with the pGL-3SV40 control plasmid (Fig. 1B,C). These results suggest that Hsp90 activity is needed for the hypoxia-induced activation of HIF-1.

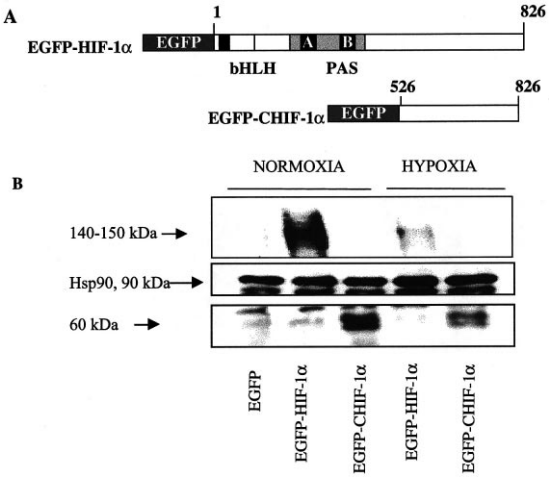

Fig. 2. EGFP-HIF-1α chimeric protein but not EGFP-CHIF-1α protein is co-immunoprecipitated with Hsp90. (A) Schematic representation of the chimeric proteins. (B) COS-7 cells were transfected with the pEGFP-HIF-1α or pEGFP-CHIF-1α. Twenty-four hours post-transfection, the cells were incubated in either hypoxia (1% O_2) or normoxia (21% O_2) and the total protein extracts were prepared. Co-immunoprecipitation was performed using Hsp90 specific antibodies. (a) Western blot analysis was performed using anti-EGFP antibodies. (b) The amount of Hsp90 immunoprecipitated in each sample was checked by Western blot using antibodies against Hsp90. (c) Western blot analysis performed on the co-immunoprecipitation supernatant using anti-EGFP antibodies.

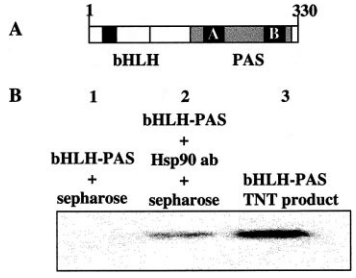

Fig. 3. The bHLH-PAS domain of HIF-1α is co-immunoprecipitated with Hsp90. (A) Schematic representation of the HIF-1α bHLH-PAS domain. (B) The in vitro transcription-translation product (lane 3) was incubated with COS-7 cell protein extracts. Sepharose-protA beads alone (lane 1) or Sepharose-protA beads+Hsp90 antibodies (lane 2) were added to the samples and incubated overnight. After five washing steps, the co-immunoprecipitated proteins were resolved on a SDS-PAGE gel and detected by autoradiography.

3.2. HIF-1α is associated with Hsp90 in normoxia

ARNT is a nuclear factor that does not interact with Hsp90 [26], so that we hypothesized that Hsp90 interacts with HIF-1α. In order to confirm the interaction between HIF-1α and Hsp90, we used the chimeric EGFP-HIF-1α and EGFP-CHIF-1α proteins which are respectively fusion proteins between EGFP and the complete HIF-1α sequence and between EGFP and the carboxy-terminal domain of HIF-1α. The fusion between the EGFP and HIF-1α or its carboxy-terminal domain produces a more stable protein than HIF-1α alone [18], which is degraded in normoxia [4]. This system allowed us to overexpress HIF-1α chimeric proteins in COS-7 cells. COS-7 cells were transfected with plasmids encoding EGFP, EGFP-HIF-1α or EGFP-CHIF-1α (Fig. 2A). Twenty-four hours later, the cells were incubated in normoxia or in hypoxia for 2 h and 30 min. The cells were then lysed and incubated in the presence of Hsp90 antibodies in order to co-immunoprecipitate Hsp90 and Hsp90-interacting proteins. The co-immunoprecipitated proteins were resolved on a polyacrylamide gel and the EGFP chimeric proteins were detected by Western blotting using anti-EGFP antibodies. As shown in Fig. 2B, EGFP-HIF-1α was co-immunoprecipitated with Hsp90 in normoxia. In hypoxia, a very small amount of EGFP-HIF-1α interacting with Hsp90 was detected (Fig. 2B). EGFP alone did not interact with Hsp90 (not shown). No EGFP-CHIF-1α proteins were immunoprecipitated nor in normoxia neither in hypoxia (not shown). A Western blotting analysis using Hsp90 antibodies was performed on the same blot, showing that a similar amount of Hsp90 was immunoprecipitated in each sample (Fig. 2C). Interestingly, the EGFP-CHIF-1α was recovered into the supernatant of immunoprecipitation (Fig. 2D). These results indicate that HIF-1α interacts with Hsp90 in normoxia and that the interacting domain is not within the 300 last amino acids.

3.3. Hsp90 interacts with the bHLH-PAS domain of HIF-1α in vitro

It has been shown that Hsp90 is able to interact with two different domains of Ahr, the first being in the bHLH domain [9] and the second in the PAS B domain [9]. Results from Fig.

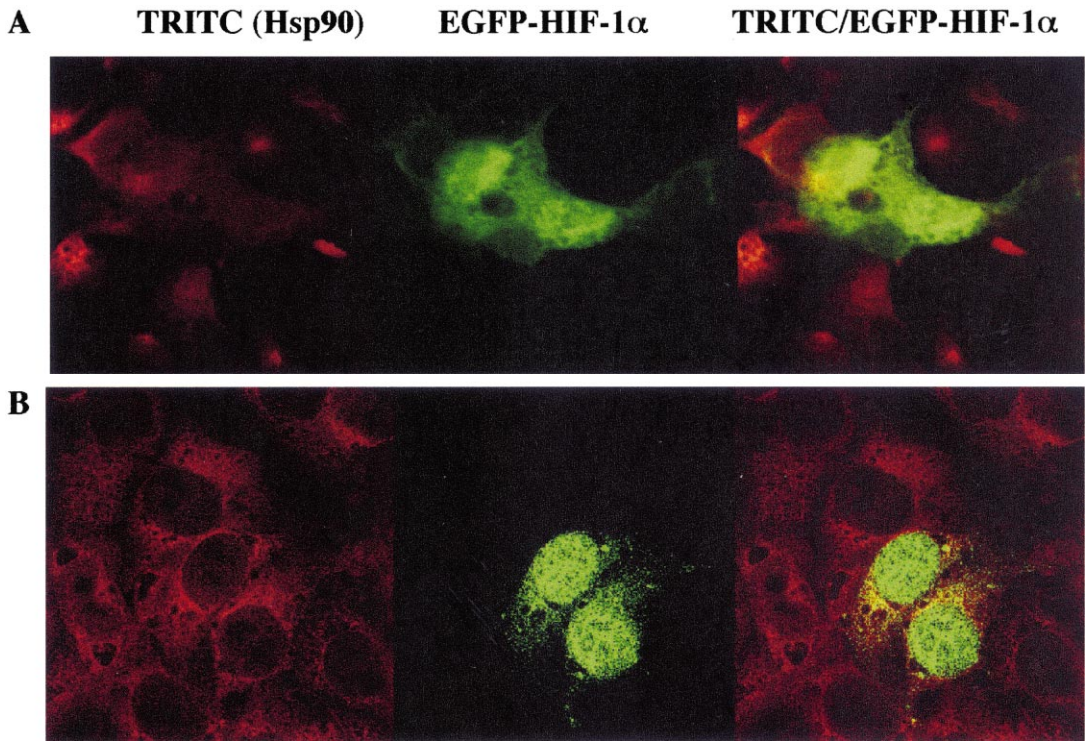

Fig. 4. Hsp90 is not co-translocated with EGFP-HIF-1α into the nucleus upon hypoxia. COS-7 cells were transfected with the pEGFP-HIF-1α vector. Twenty-four hours post-transfection, the cells were incubated in either normoxia (21% O_2) or hypoxia (1% O_2) for 2 h and 30 min. After fixation and permeabilization, the cells were labelled with Hsp90 antibodies and TRITC anti-mouse-conjugated antibodies. The respective cellular localization of Hsp90 and fusion proteins was detected in confocal microscopy. Localization of the EGFP-HIF-1α protein and of Hsp90 in normoxia (A) and hypoxia (B). Magnification: 1000×

2 indicate that HIF-1α interacts with Hsp90 and that this interaction does not involve the carboxy-terminal end of HIF-1α. We then wanted to know whether Hsp90 interacts with the bHLH-PAS domain of HIF-1α. The HIF-1α bHLH-PAS domain [3] was cloned into the pET-3a expression vector. In vitro transcription-translation was performed in wheat germ lysate in order to produce radiolabelled HIF-1α bHLH-PAS domain (Fig. 3A). It is important to notice that in other experiments using this system, no binding of wheat Hsp90 was obtained with the bHLH-PAS domain of Ahr, avoiding false positive results [9]. Then, the radiolabelled HIF-1α bHLH-PAS domain was incubated in the presence of COS-7 cell extract and a co-immunoprecipitation experiment using anti-Hsp90 antibodies was performed. As shown in Fig. 3B, the bHLH-PAS domain of HIF-1α was co-immunoprecipitated by the anti-Hsp90 antibodies. When the anti-Hsp90 is not added, the bHLH-PAS domain was not recovered.

3.4. Hsp90 is not translocated into the nucleus with EGFP-HIF-1α

One hypothesis concerning the nuclear translocation of the bHLH-PAS factor Ahr is that the binding of one molecule of dioxin onto the Ahr protein leads to its dissociation from Hsp90 allowing for its translocation to the nucleus [27]. The interacting element of Ahr with Hsp90 is the bHLH-PAS domain [9]. In comparison, the oxygen-dependent mechanism of activation of HIF-1α is not known. Indeed, there are no data regarding a putative ligand for HIF-1α. This ligand does not seem to be a heme but phosphorylation [28] has been reported. Phosphorylation could provoke the dissociation of HIF-1α and Hsp90 under hypoxic conditions. We have indeed shown in Fig. 2 that the interaction of EGFP-HIF-1α and Hsp90 is weaker in hypoxia. In order to investigate whether Hsp90 is involved in HIF-1α translocation into the nucleus during hypoxia, we performed immunofluorescence experiments. Hypoxic or normoxic COS-7 cells expressing EGFP-HIF-1α or EGFP-CHIF-1α were fixed, permeabilized and

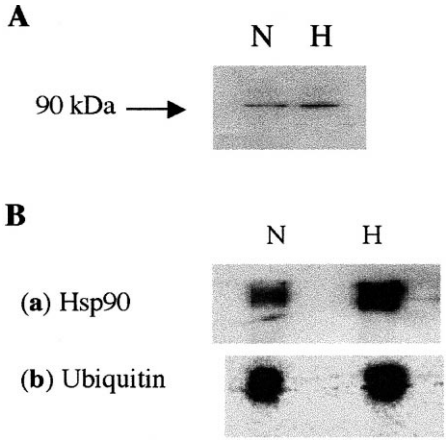

Fig. 5. Hypoxic regulation of Hsp90 expression. COS-7 cells were exposed to either normoxia (21% O_2) or hypoxia (1% O_2) during 2 h and 30 min. (A) Western blot analysis of Hsp90 in normoxia (N) and in hypoxia (H). (B) Northern blot analysis of Hsp90 (a) and ubiquitin (b) mRNA in normoxia (N) and hypoxia (H).

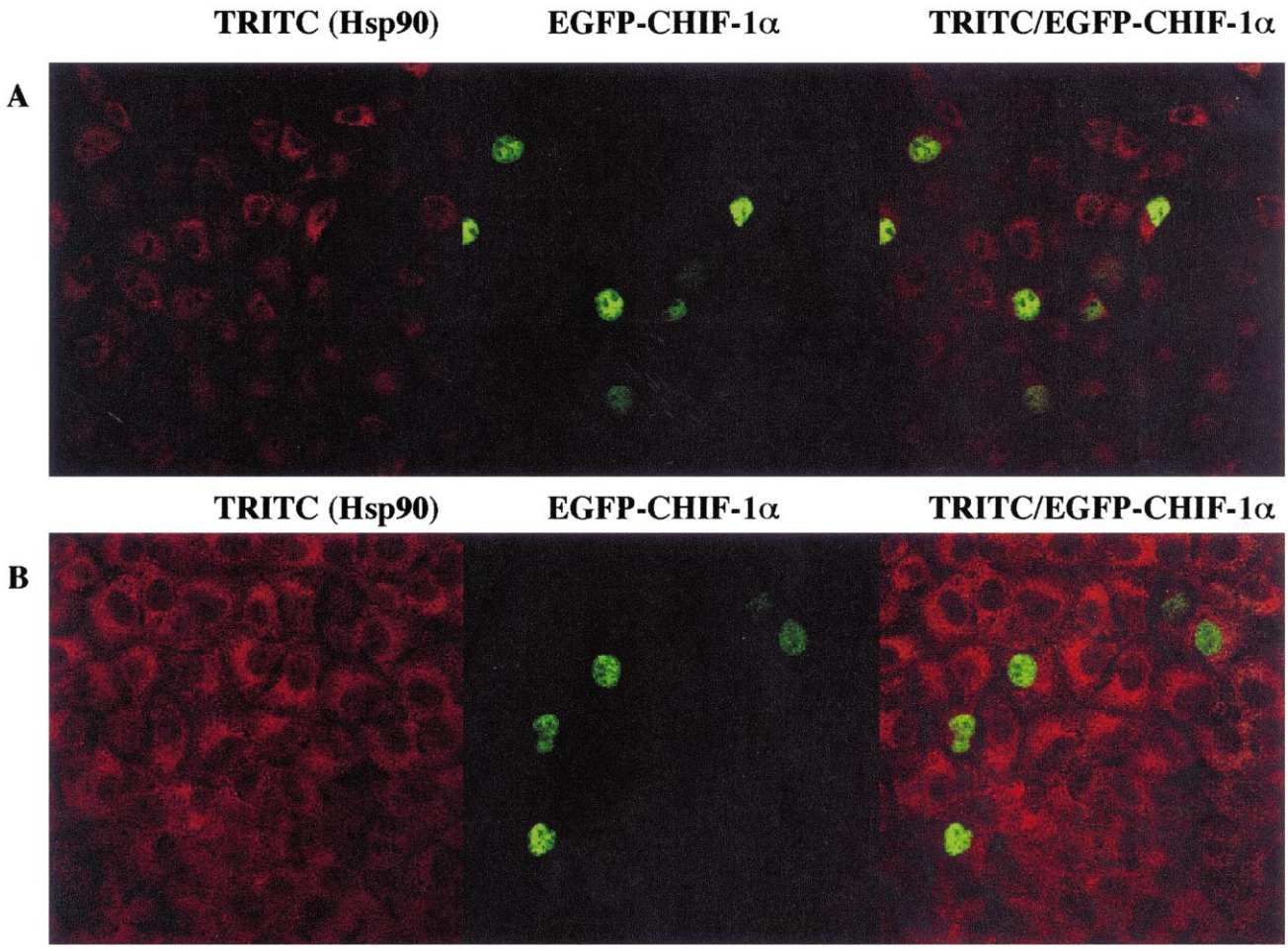

Fig. 6. Hsp90 is not co-translocated with EGFP-CHIF-1α into the nucleus. COS-7 cells were transfected with the pEGFP-CHIF-1α vector. Twenty-four hours post-transfection, the cells were incubated in either normoxia (21% O_2) or hypoxia (1% O_2) for 2 h and 30 min. After fixation and permeabilization, the cells were labelled with Hsp90 antibodies and TRITC anti-mouse-conjugated antibodies. The respective cellular localization of Hsp90 and fusion proteins was detected in confocal microscopy. Localization of the EGFP-CHIF-1α protein and of Hsp90 in normoxia (A) and hypoxia (B). Magnification: 400×.

labelled with anti-Hsp90 antibodies detected by TRITC anti-mouse Ig. Putative co-localization of Hsp90 and EGFP-HIF-1α/EGFP-CHIF-1α was studied using confocal microscopy. First, we detected an increase in cytosolic Hsp90 during hypoxia (Fig. 4A,B). This increase was confirmed by Northern and Western blotting (Fig. 5A,B). As shown in Fig. 4A, in normoxia, the EGFP-HIF-1α protein was present through the whole cell and Hsp90 is mainly peri-nuclear but also in the cytoplasm. In hypoxia (Fig. 4B), EGFP-HIF-1α was translocated into the nucleus, but this did not provoke any increase in the Hsp90 present within the nucleus in comparison to non-transfected cells. Regarding the EGFP-CHIF-1α (Fig. 6), this chimeric protein was nuclear in normoxia (A) and in hypoxia (B) as already shown by Kallio et al. [18]. These results indicate that Hsp90 does not seem to translocate into the nucleus with HIF-1α in hypoxia.

4. Discussion

Studies of interactions between Hsp90 and transcription factors such as steroid hormone receptors [15], MyoD [16], HSF1 [29], Sim and Ahr [6] allowed us to elucidate the role of this chaperone molecule in the complex regulatory mechanisms leading to the activation of these factors. The folding of the bHLH and PAS domains of the dioxin receptor Ahr is dependent on Hsp90 [9]. Moreover, its ability to bind its ligand [30], to dimerize with ARNT [30] and to bind DNA is also Hsp90-dependent [30].

In this work, we investigated the role of Hsp90 in HIF-1 activation by hypoxia. However, the mechanism leading to its activation upon hypoxia is far from being completely understood. The results shown here evidence that geldanamycin inhibits the activation of HIF-1 in hypoxia. Geldanamycin is able to inhibit the ATP binding activity of Hsp90, leading to the formation of a low affinity Hsp90 complex for substrates [15,31]. The fact that geldanamycin inhibited HIF-1 activation indicates that Hsp90 activity is needed for this activation. Since it is known that ARNT does not interact with Hsp90 [26], we sought whether HIF-1α is a partner for Hsp90. By co-immunoprecipitation studies using anti-Hsp90 antibodies and total extracts from cells expressing the stabilized EGFP-HIF-1α and EGFP-CHIF-1α chimeric proteins, we showed that EGFP-HIF-1α interacts with Hsp90 in normoxia but to a much lesser extent in hypoxia. The truncated protein EGFP-CHIF-1α expressing the C-terminal end of HIF-1α and located within the nucleus does not interact with

Hsp90. These results indicate that HIF-1α actually interacts with Hsp90 and that this interaction is not mediated by the C-terminal domain of the protein. Moreover, HIF-1α seems to dissociate from Hsp90 in hypoxia. Interestingly, both processes are similar to what is observed for Ahr [27]. First, in Ahr-Hsp90 complex, Hsp90 proteins are bound to the bHLH-PAS domain [9]. As the C-terminal end of HIF-1α did not immunoprecipitate with Hsp90, we supposed that the interaction between Hsp90 and HIF-1α is also located within the bHLH-PAS domain. This hypothesis was confirmed by an in vitro experiment showing that the HIF-1α bHLH-PAS domain was co-immunoprecipitated by anti-Hsp90 antibodies.

Secondly, with respect to Ahr, Hsp90 dissociates when the ligand, dioxin, interacts with its receptor, enabling nucleus translocation and dimerization with ARNT [27]. Results from Fig. 2 suggest that Hsp90 is released from HIF-1α under hypoxic conditions, i.e. when HIF-1α is activated by hypoxia, the exact mechanism leading to this activation still being unclear. One can thus hypothesize that Hsp90 could play a similar role for HIF-1α as for Ahr, holding HIF-1α in a repressed state and/or chaperoning its folding and the 'activable' conformation of HIF-1α under normoxic conditions [8,9,30].

Another interesting feature of Hsp90 is its putative role in regulating nuclear translocation [23]. Results from Fig. 6 show that the chimeric protein EGFP-CHIF-1α is constitutively translocated into the nucleus. This translocation is due to the presence of an unmasked RKRK motif in the C-terminal domain of HIF-1α [18]. This was already observed by Kallio et al. [18]. They suggested that either the complete HIF-1α protein folds upon itself so that this NLS is masked in normoxic conditions and that HIF-1α is located within the cytosol or that Hsp90 interacting with HIF-1α could mask this NLS. Using the EGFP-HIF-1α chimeric protein, we were not able to detect by confocal microscopy any co-translocation into the nucleus of Hsp90 with HIF-1α during hypoxia. This result confirms that in hypoxia, the binding of Hsp90 to HIF-1α is unstable as observed in the co-immunoprecipitation studies. However, the role of Hsp90 in the translocation mechanism of HIF-1α into the nucleus upon hypoxia remains unclear and it could be possible that nuclear Hsp90 will also be involved in HIF-1α regulation as for Ahr [31].

It must be noted that an increase in the Hsp90 mRNA and protein levels was observed under hypoxic conditions. This increase could be due to cell injuries resulting from hypoxia [32]. Other Hsps have already been shown to be induced by ischemic conditions [14].

In conclusion, we showed that HIF-1α interacts with Hsp90 in vivo in normoxia and that the interaction involves the bHLH-PAS domain of HIF-1α, as for Ahr. In addition, the presence of an active Hsp90 is necessary for HIF-1 activation in hypoxia. However, there is no evidence for the involvement of Hsp90 in the translocation of HIF-1α. In the future, it will be interesting to investigate precisely the levels at which the chaperone is acting: folding, regulation of proteasomal degradation and/or nuclear import regulation.

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