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Nuclear translocation of hypoxia-inducible factors (HIFs): Involvement of the classical importin α/β pathway

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Abstract

Hypoxia-inducible factors are the key elements in the essential process of oxygen homeostasis of vertebrate cells. Stabilisation and subsequent nuclear localisation of HIF-α subunits results in the activation of target genes such as *vegf*, *epo* and *glut1*. The passage of transcription factors e.g. HIF-1α into the nucleus through the nuclear pore complex is regulated by nuclear transport receptors. Therefore nucleocytoplasmic shuttling can regulate transcriptional activity by facilitating the cellular traffic of transcription factors between both compartments. Here, we report on the identification of specific interactions of hypoxia-inducible factors with nuclear transport receptors importin α/β. HIF-1α, -1β, and HIF-2α are binding to importin α1, α3, α5, and α7. The direct interaction of HIF-1α to α importins is dependent on a functional nuclear localisation signal within the C-terminal region of the protein. In contrast, the supposed N-terminal NLS is not effective. Our findings provide new insight into the mechanism of the regulation of nuclear transport of hypoxia-inducible factors.

Keywords: Hypoxia-inducible factor; HIF; Importin; Nuclear transport; NLS; Oxygen homeostasis

1. Introduction

Hypoxia, a local or general reduction of oxygen tension below a tissue-specific threshold, can be observed during acute and chronic vascular or pulmonary diseases, cancer and in distinct healthy tissues (e.g. testis). For this reason all organisms feature mechanisms to sustain oxygen homeostasis. In vertebrate cells key elements in this essential process are the hypoxia-inducible factors (HIFs) (reviewed by [1,2]). These proteins are heterodimer complexes consisting of an α and a β subunit. In addition to ubiquitous HIF-1α, the protein family contains HIF-2α, HIF-3α and a number of different splice variants [3–6]; reviewed by [7]. Both, the α and β subunits are basic-helix–loop–helix Per/Arnt/Sim (bHLH-PAS) transcription factors [8] which form a heterocomplex that recruits a number of co-activators inside the nucleus and thus facilitates transactivation of the expression of a greater number of target genes implicated in oxygen homeostasis (reviewed by [9,10]).

HIF-1β (ARNT) is constitutively expressed, whereas HIF-1α is negatively regulated in normoxia [8]. The interrelation with oxygen is provided by enzymatic hydroxylation of conserved prolyl residues [11–15]. Turnover of the α subunit is mediated by the ubiquitin-proteasome pathway via specific binding of the "von Hippel-Lindau" protein (pVHL) [12–15]. Important structural elements of the primary structure of HIF-1α are the oxygen-dependent-degradation-domain (ODDD), the bHLH-domain, the PAS domains (A and B) and the predicted nuclear localisation

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signals (NLS) (Fig. 1). The bHLH/PAS domains induce the dimerisation of the α and β subunits [16]. The process of nuclear translocation of HIF subunits consists of stabilisation and subsequent nuclear import. The nuclear translocation of HIF-1α was discussed to be hypoxia-inducible [17], whereas others reported that hypoxia is not a necessary factor for nuclear localisation of HIF-1α and HIF-2α [18]. The HIF-α subunits are regulated by protein degradation and only available for protein import after stabilisation. While HIF-α stabilisation was the subject of intensive research in recent years, less is known about the details of NLS-dependent nuclear translocation of HIFs and the transport receptors involved in this process. Here, we addressed the question of the mechanism of nuclear transport of HIF-α using *in vitro* and *in vivo* assays.

Import of large proteins (>40–60 kDa) into the nuclear compartment depends on the presence of targeting sequences in the molecule which are usually recognised by a specific nuclear transport receptor in the cytoplasm. While many proteins are imported constitutively, others, like some transcription factors are only imported in response to a specific stimulus. In these cases nuclear transport may constitute a distinct level of regulation of gene expression (reviewed by [19]). In many proteins the targeting information is encoded by at least one copy of the so-called "classical" nuclear localisation sequence (NLS) [20]. These NLS may either contain one or two cluster of basic amino acids (monopartite or bipartite NLS, respectively) (reviewed by [21]). "Classical" NLS usually do not bind to their nuclear transport receptor importin β directly, but rather bind to α importins, which in turn are bound by importin β via their importin β binding domain (IBB). Thus α importins act as cytoplasmic adaptor molecules for NLS-bearing proteins (reviewed by [22,23]). Importin αs bind the NLS via a domain of highly structured armadillo (ARM) repeats containing two binding sites [24]. Monopartite NLSs can bind both of these sites, however, it is suggested that they mainly bind to the N-terminal major binding site of importin α, formed by ARM 2, 3, 4 [24]. Bipartite NLS are recognised by N-terminal ARM repeats in combination with the minor binding site shaped by ARM 7, 8, 9 in the C-terminal part of the molecule [25]. To date, six importin α isoforms have been identified which show different affinity to particular substrates [26–28]. The binding specificity of a substrate to a particular α importin depends on the NLS sequence [29,30] but also on the protein domains surrounding the NLS [31].

The transport pathway of HIFs into the nucleus is so far not fully understood. Clearly, heterodimerisation of HIF-α and HIF-β is not a prerequisite for the translocation process in the nuclear compartment [32]. Moreover, it has been shown, that import may be facilitated by a sequence with similarities to a classical monopartite-type NLS (a series of basic amino acids) in the C-terminal part of HIF-1α [17]. Others provide evidence that the C-terminal NLS of HIF-2α and HIF-1α is a novel variant of bipartite-type NLS [33]. Additionally, a bipartite NLS (two series of basic amino acids interrupted by a linker sequence) is predicted for the N-terminus of the protein. It was hypothesised that the N-terminal NLS might be masked by parts of the PAS-B domain [17]. However, the nature of the nuclear transport receptors involved remained elusive.

In this study, we report results of experiments aimed at characterising the specific interactions between the hypoxia-inducible factors and the importins known to interact directly with classical NLS. Protein–protein binding assays show for the first time that HIF-α (-1, -2) and HIF-1β (ARNT) bind directly to different importin α subtypes, suggesting that several importin α are likely to transcolate HIF isoforms into the nucleus. Further we report the structural elements of HIF-1α and importin αs involved in their direct interaction, identifying the C-terminal NLS of HIF-1α and armadillo repeat 3 of importin α3 as necessary for direct protein–protein binding. We found no evidence that the PAS-B domain is involved in importin α dependent nuclear translocation as had been suggested previously [17,34].

2. Materials and methods

2.1. Plasmids and DNA manipulation

For isolation and manipulation of DNA and *E. coli* transformation, we used standard procedures as described by Ausubel et al. [35]. Vector constructs encoding C-terminally GST-tagged importin α1 and α3 genes have been described formerly [27]. Importin α5, α7 and importin β were generated by replacing C-terminal His-tags of precursor-constructs by GST-tags [31]. Plasmids encoding full-length nucleoplasmin (nucleoplasmin/pQE70) have been reported by Gorlich et al. [36]. Plasmid constructs for the expression of GST-tagged importin α ARM mutants have been described by Melen and co-workers [37]. Plasmids encoding full-length cDNA of HIF-1α, HIF-2α, HIF-1β and HIF-1α ΔPAS-B were cloned into the pcDNA3.1 vector and have been described already [38].

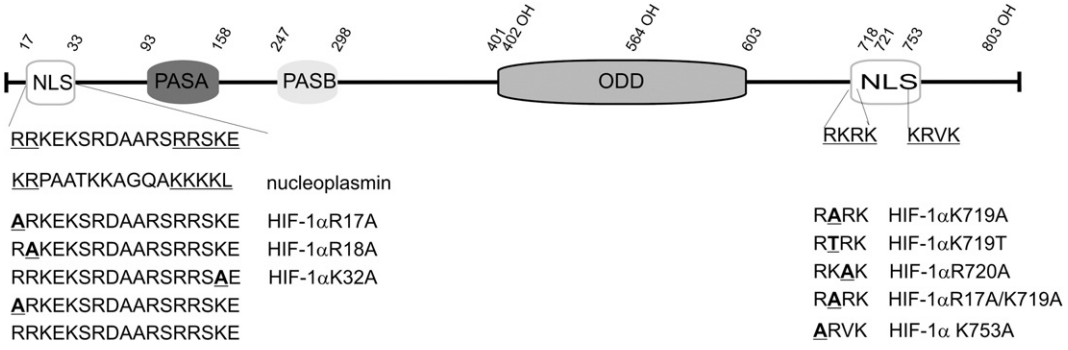


Fig. 1. Schematic representation of the HIF-1α domain structure. ODD: oxygen-dependent-degradation-domain, PAS: Per/ARNT/Sim domains A and B, NLS: N- and C-terminal nuclear localisation signals. The nucleoplasmin bipartite NLS is shown in comparison to the proposed N-terminal HIF-1α NLS. Amino acid residues which were mutated in the predicted NLSs are shown in bold.

For site-directed mutagenesis of the N-terminal and C-terminal NLS motifs we used the QuikChange Mutagenesis® kit and followed the manufacturer's instructions (Stratagene, Amsterdam, Netherlands). The correct exchange of coding triplets following mutagenesis was monitored by sequencing of the resulting plasmids. The corresponding primer sequences are available on request.

2.2. Protein expression and purification

Recombinant human importins α1, α3, α5, α7 and β were expressed as GST-fusion proteins and purified as described [27,31], importin β was also expressed as His-tagged protein. Briefly, transformed *E. coli* BL21/pRep4 were grown with vigorous shaking at 37 °C in LB medium. At mid-log phase

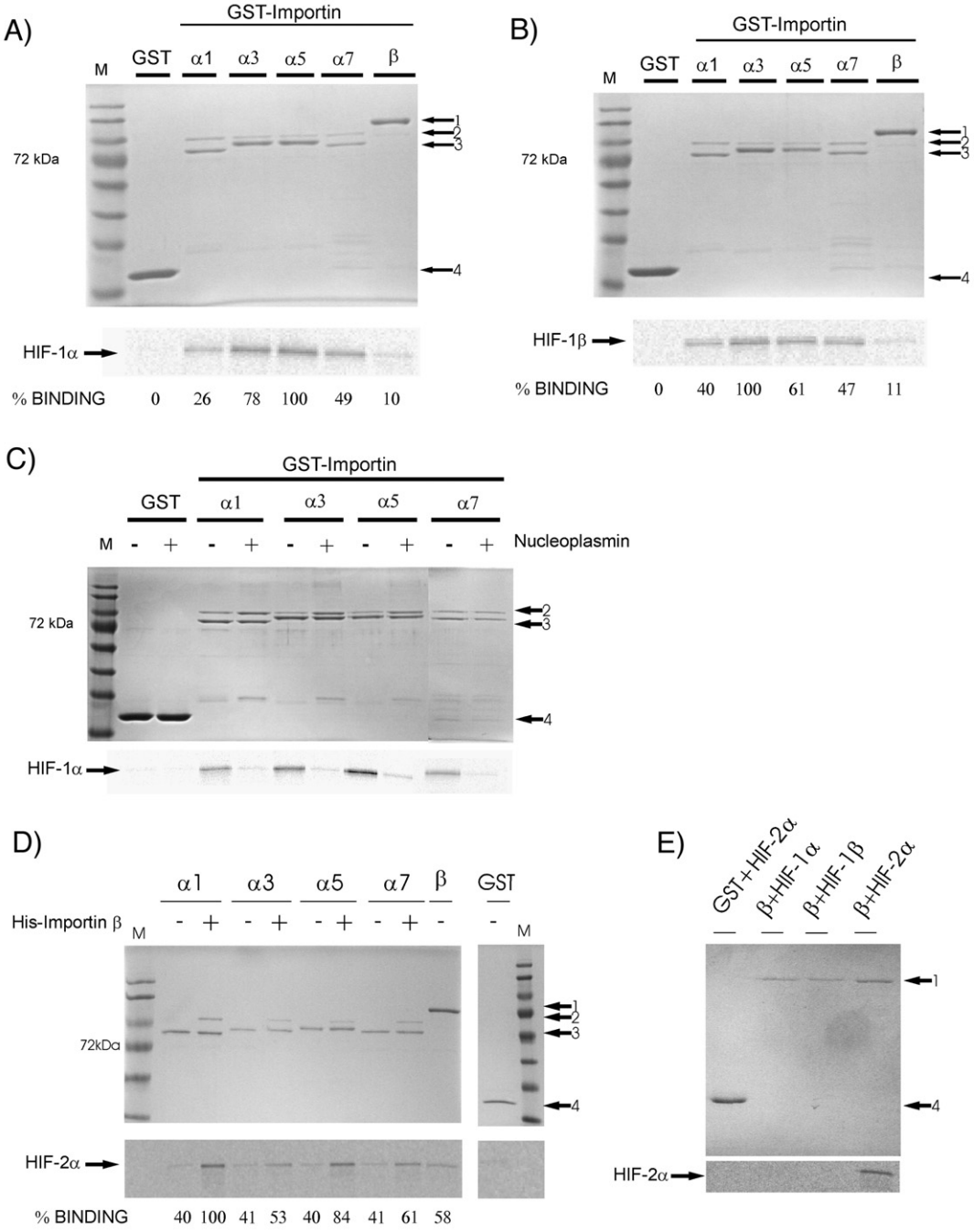


Fig. 2. Various importins interact specifically with HIF isoforms. Protein–protein interaction of *in vitro* transcribed/translated HIF-1α, HIF-1β and HIF-2α with various purified GST-importin isoforms. GST or GST-importins were immobilised on glutathione-Sepharose and incubated with *in vitro* transcribed/translated HIF-1α A), C), HIF-1β B) or HIF-2α D), E). Protein–protein interaction was analysed by SDS-PAGE, subsequent Coomassie staining of GST or GST-importins (α1, α3, α5, α7, β) and autoradiography of the [^{35}S]-methionine labelled HIF isoforms. A protein ladder was used as a marker (M). 1: importin β, 2: His-tagged importin β, 3: various importin αs, 4: GST (negative control). %Binding = densitometry data in relation to the strongest protein–protein interaction measured in the assigned assay. A) HIF-1α is binding to various importin α B) HIF-1β is binding to various importin α. C) HIF-1α competition with nucleoplasmin for importin binding. Protein–protein interaction of *in vitro* transcribed/translated HIF-1α with GST-importins (α1, α3, α5, α7) was tested under the influence of nucleoplasmin as a competing second substrate. D) HIF-2α is binding to various importin α and importin β. E) Importin β binding to HIF-1α, HIF-1β and HIF-2α. The HIF isoforms were tested for importin β binding in parallel.

of growth, overexpression was induced by 0.6 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 4 h at 25 °C. The cells were collected and disintegrated in a French Press. The lysate was clarified (1 h, 22,000 rpm, SS 34 rotor) and incubated for 2–3 h at 4 °C with glutathione-Sepharose (Amersham Biosciences, München, Germany). The sepharose was washed 3 times and the protein was eluted by incubation with elution buffer (50 mm Tris–HCl, pH 8.0, 10 mM reduced glutathione). Protein samples were dialysed against dialysis buffer (50 mM HEPES-KOH, pH 7.5, 200 mM NaCl, 5% glycerol). If required the protein was concentrated in centrifugal filter devices (Millipore, Schwalbach, Germany). His-tagged importin β was separated from the protein extract by metal affinity chromatography using BD TALON metal affinity resin (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. The protein concentrations were measured using the RC DC protein assay (Bio-Rad, München, Germany).

2.3. GST-importin pull-down assay

GST or GST-importins were allowed to bind to glutathione-Sepharose 4B. GST-importin pull-down assays were carried out with purified GST as a negative control. In a typical experiment 100 μl beads were pre-equilibrated in IP-buffer

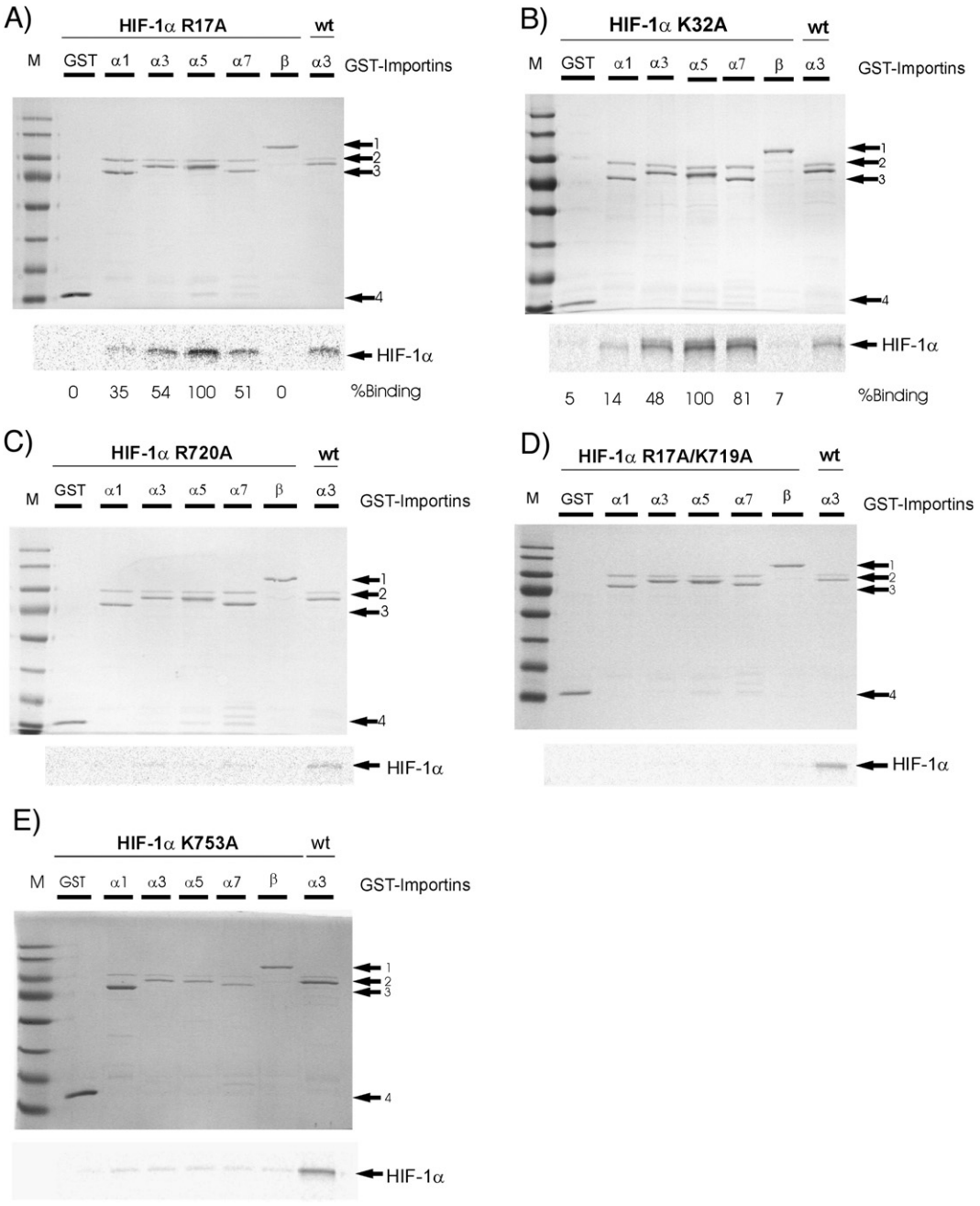
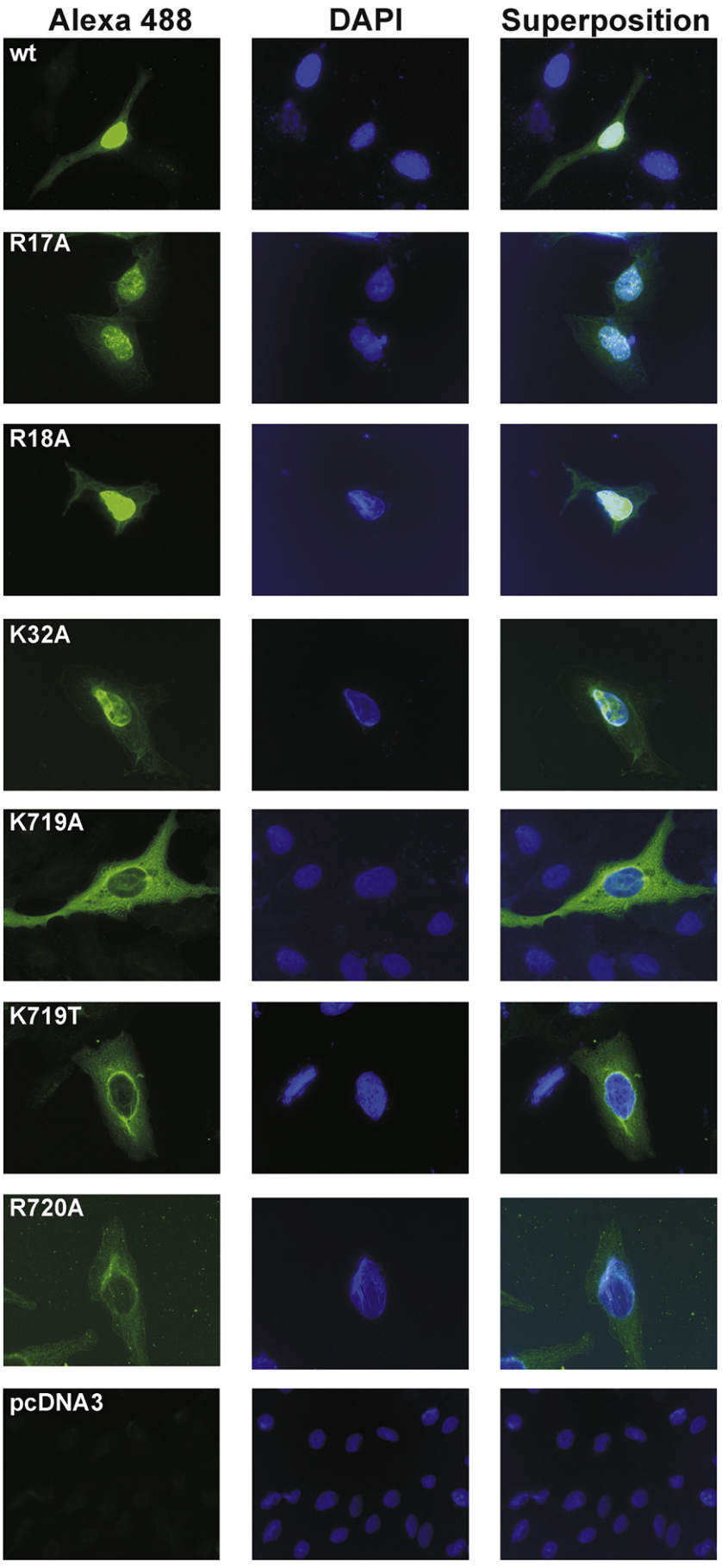


Fig. 3. The C-terminal NLS of HIF-1α is crucial for importin binding. Protein–protein interaction of *in vitro* transcribed/translated NLS-mutant HIF-1α with GST-importins (α1, α3, α5, α7 and β). GST or GST-importins were immobilised on glutathione-Sepharose and incubated with *in vitro* transcribed/translated HIF-1α with various point mutations A) R17A, B) K32A, C) R720A, D) R17A/K719A E) K753A. Protein–protein interaction was analysed by SDS-PAGE, subsequent Coomassie staining of GST or GST-importins (α1, α3, α5, α7, β), and autoradiography of the [^{35}S]-methionine labelled HIF-1α. A protein ladder was used as a marker (M). 1: GST-Importin β, 2: His-Importin β, 3: various importin αs 4: GST (negative control).


Alexa 488
DAPI
Superposition
wt
R17A
R18A
K32A
K719A
K719T
R720A
pcDNA3

(20 mM Hepes pH 7.5, 100 mM KOAC, 0.5 mM EGTA, 5 mM MgOAc, 250 mM sucrose, 4 °C), mixed with 15 μg GST-fusion proteins and His-tagged importin β and incubated at 4 °C for 1 h. HIF-1α, HIF-1α mutants, HIF-2α or HIF-1β were transcribed and translated *in vitro* in the presence of ^{35}S-methionine (TNT Coupled Reticulocyte Lysate System, Promega, USA) according to the manufacturer's protocol. After incubation, 10 μl of the reaction batch were allowed to bind to the immobilised fusion proteins. In competition experiments purified nucleoplasmin and importins were added in a 1:1 ratio. ^{35}S-labelled methionine was obtained from Hartmann Analytic (Braunschweig, Germany). After washing three times with IP-buffer, the sepharose beads were dissolved in 60 μl Laemmli buffer [39]. Proteins were separated by SDS-PAGE (10%) and visualised by Coomassie Brilliant Blue staining [39]. To detect the [^{35}S]-labelled proteins, the dried gels were autoradiographed (16 h–24 h). The binding efficiency was quantified by analysing densitometry data of the gels (PCBAS 2.09 g, Raytest Isotopenmessgeräte GmbH).

2.4. Cell culture, DNA transfection and hypoxia

Cells were maintained in a saturated humidified atmosphere at 37 °C, 5% CO_2 and 95% air. Hela and U2OS cells were maintained in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum (Invitrogen, Karlsruhe, Germany). Transient transfections were performed with TransFectin™ using standard protocols of the manufacturer (Bio-Rad, München, Germany).

2.5. Immunofluorescence analysis

U2OS cells were grown on coverslips and transiently transfected with full-length wild-type or NLS-mutant constructs of HIF-1α expression plasmids. Following washing steps with PBS the cells were treated with methanol/acetone (1:1) for fixation (−20 °C, 5 min), washed twice with PBS and treated with 0.5% Triton-X/PBS for 5 min. After blocking in 5% skim milk, the cells were incubated with mouse anti-HIF-1α antibodies (BD Transduction Laboratories, Erembodegem, Belgium). Cells were washed with PBS and incubated with secondary antibodies (Alexa 488 labelled anti-mouse IgG (Molecular Probes, Karlsruhe, Germany) or Cy3 labelled anti-mouse IgG (Zymed, San Francisco, CA, USA)). Nuclear counterstaining was accomplished by DAPI staining in a 1.5 μg/ml solution (5 min/RT). Finally coverslips were mounted on glass slides and visualised by fluorescence microscopy (Axioplan 2000, Carl Zeiss Vison GmbH, Germany).

3. Results

3.1. Hypoxia-inducible factors interact with various importin α isoforms

We set out to investigate whether distinct importin α isoforms, in the presence or absence of importin β, can bind to HIF-1α, -2α and -1β and thus might be the driving force of HIF nuclear import. The proposed interaction of the NLS-containing hypoxia-inducible factors with various importins was investigated by using an *in vitro* glutathione *S*-transferase pull-down assay (Fig. 2). Recombinant purified GST-importin fusion proteins were expressed in *E. coli*, purified to almost homogeneity and immobilised on sepharose. Radioactively labelled *in vitro* transcribed and translated HIF-α and β subunits (1α, 1β, and 2α) were added and allowed to bind to GST-importins α1, α3, α5, and α7 in the presence of His-tagged importin β or to GST-importin β alone. Fig. 2 (A and B) displays representative results for HIF-1α and HIF-1β (ARNT) pull-down assays. Both proteins efficiently bound to importins α1, α3, α5 and α7, whereas binding to importin β was very weak and interaction with GST alone was not detectable, thus showing the existence of the predicted importin α dependent interaction. Densitometry data indicates that importin α3 and importin α5 were binding with the strongest affinity (Fig. 2A and B). To further confirm the specificity of the HIF-1α/importin complex formation, we performed nucleoplasmin competition experiments. Nucleoplasmin can bind to various importin αs [31]. When purified nucleoplasmin was added in concentrations equimolar to importins, binding of importin α to HIF-1α was reduced rigorously due to the competing second substrate (Fig. 2C).

To examine whether binding properties of HIF-2α are divergent to HIF-1α, we used the same assay and examined *in vitro* transcribed and translated HIF-2α (Fig. 2D). Binding features of this isoform were different from HIF-1α. HIF-2α bound to all importin α subtypes tested, but in addition it also bound to importin β alone (Fig. 2D). However, interaction of HIF-2α to importin β was weaker than its binding efficiency to most of the various importin α/β complexes. To further confirm this observation, importin β binding to the three different HIF isoforms were tested in parallel experiments (Fig. 2E). Only HIF-2α bound to importin β. In contrast to HIF-1α and HIF-1β was HIF-2α binding importin α3 with the lowest affinity. This interaction is approximately on the same level with importin β. When His-tagged importin β was removed from the assay, binding of HIF-2α to importin αs was markedly reduced (Fig. 2D).

3.2. Characterisation of essential residues of the HIF-1α C- and N-terminal nuclear localisation signals for importin binding and nuclear translocation

To investigate which of the proposed NLS of HIF-1α are necessary for importin binding, we examined the molecular interactions of various HIF-1α mutants with different importin αs and importin β by pull-down assays and immunofluorescence experiments. Point mutations were introduced into full-length HIF-1α in the *in silico* predicted putative N-terminal bipartite NLS (R17A, R18A or K32A) by a PCR based procedure. Since the C-terminal NLS has been discussed to be either monopartite or bipartite [17,18], mutations at residues K719A, K719T, R720A and K753A were generated. Additionally, a double mutant of both NLSs (R17A/K719A) was created. HIF-1α mutants R17A, K32A, and R18A showed binding to all importin α subtypes (Fig. 3A, B and data not shown). In contrast, no binding of NLS-C mutants of both parts of the putative bipartite C-terminal NLS (R720A, K753A) and of the double-NLS-mutant could be detected (Fig. 3C, D, E).

To analyse the functional relevance of the different HIF-1α NLSs proposed for nuclear translocation in a full-length protein context *in vivo*, HIF-1α immunofluorescence studies were

Fig. 4. Subcellular localisation of HIF-1α (wild-type, R17A, R18A, K32A, K719A, K719T, R720A (×650 magnification) pcDNA3 (negative control (×200 magnification)) by immunofluorescence. U2OS cells were fixed and subjected to immunofluorescence staining with HIF-1α antibodies and Alexa 488 labelled secondary antibodies. Nuclear counterstaining was accomplished by DAPI staining.

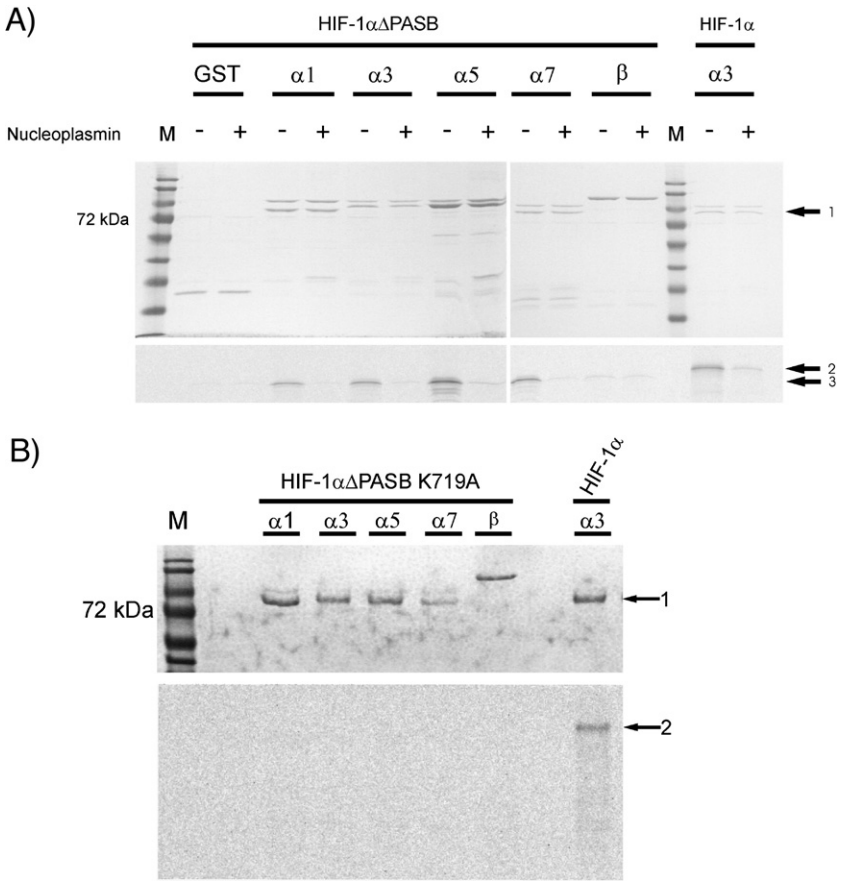


Fig. 5. Influence of HIF-1α PAS-B domain on the interaction with importins. Protein–protein interaction of *in vitro* transcribed/translated HIF-1α ΔPAS-B with GST-importins (α1, α3, α5, α7 and β). GST or GST-importins were immobilised on glutathione-Sepharose and incubated with *in vitro* transcribed/translated HIF-1α. A) Pull-down assay with HIF-1α ΔPAS-B with and without nucleoplasmin as a competing substrate. HIF-1α to importin α3 binding (positive control). 1: various importin αs, 2: HIF-1α wild-type 3: HIF-1α ΔPAS-B. Proteins with lower molecular weight in the SDS-PAGE represents degraded GST-importin fusion proteins. B) Pull-down assay with HIF-1α ΔPAS-B K719A. HIF-1α to importin α3 binding (positive control). Binding of HIF-1α ΔPAS-B K719A to importins was not detected. 1: various importin αs, 2: HIF-1α.

performed. For this purpose U2OS cells were transiently transfected with HIF-1α wt or HIF-1α mutants (R17A, R18A, K32A, K719A, K719T, and R720A). Wild-type HIF-1α was in particular detected in the nucleus of these cells (Fig. 4). Similarly mutants R17A, R18A, and K32A with alanine substitutions in either of both parts of the predicted N-terminal bipartite NLS localised in the nucleoplasma, showing no differences to HIF-1α wild-type behaviour. On the other hand, NLS-C mutants K719A, K719T, or R720A exhibited stringent cytoplasmic accumulation and did not translocate into the nucleus. These results are consistent with our observations from *in vitro* binding assays.

3.3. The PAS-B domain is not involved in HIF-1α/importin α interaction

It has been supposed, that the N-terminal NLS might be masked by an inhibitory or regulative motif which is present in the PAS domain of HIF-1α [17]. Therefore, we analysed the function of the HIF-1α PAS-B domain for the HIF-1α/importin α interaction. First, we determined whether HIF-1α PAS-B deletions can bind to various importins by GST pull-down assays. *In vitro* transcribed and translated HIF-1α ΔPAS-B variants (wt or K719A) were added to protein–protein binding assays (Fig. 5A). The results clearly demonstrated that HIF-1α ΔPAS-B protein was able to bind to importin α1, α3, α5, and α7 in a HIF-1α full-length manner. Like wild-type HIF-1α, the HIF-1α PAS-B deletion did not directly interact with importin β. Specificity was shown by incubating GST alone and by nucleoplasmin competition experiments (Fig. 5A). To exclude interaction of HIF-1α ΔPAS-B to importin via the C-terminal NLS of HIF-1α, we used a HIF-1α ΔPAS-B K719A mutant in the protein–protein binding assay (Fig. 5B). This C-NLS mutation completely prevented binding, showing that the deletion of the PAS-B domain (aa 222 to aa 390) is not sufficient to unmask the putative N-terminal NLS and to enhance its capability for importin-dependent nuclear translocation of HIF-1α. Next we performed immunofluorescence experiments to verify the relevance of the specified results in living cells. Fig. 6 highlights the intracellular localisation of HIF-1α ΔPAS-B and HIF-1α ΔPAS-B K719A protein. The PAS-B deletion alone did not cause any change in the intracellular distribution of HIF-1α, whereas the additional C-terminal NLS mutation

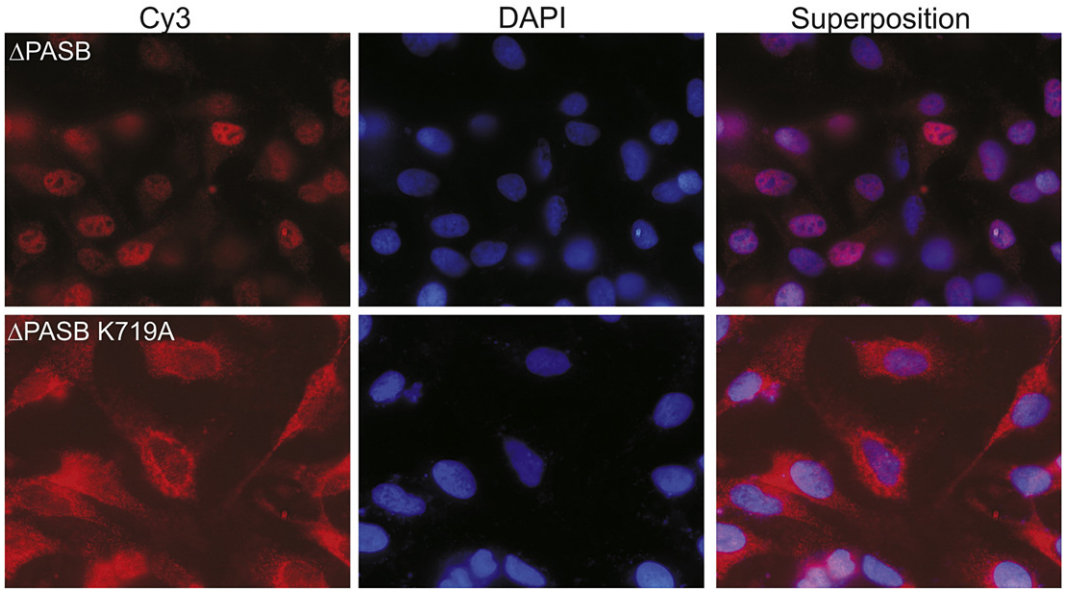


Fig. 6. Subcellular localization of HIF-1α ΔPAS-B and NLS-mutant HIF-1α ΔPAS-B. U2OS cells were fixed and subjected to immunofluorescence staining with HIF-1α antibodies and Cy3-labelled secondary antibodies. Nuclear counterstaining was accomplished by DAPI staining showing the location of the nuclei for orientation.

blocked nuclear import of the protein, according to our observations seen with full-length HIF-1α C-terminal NLS-mutants.

3.4. Identification of the importin α3 NLS-binding site for HIF-1α

Since for monopartite NLS one of the two NLS-binding pockets formed by α importins could be sufficient for binding, we set out to determine the HIF-1α binding sites of importin α.

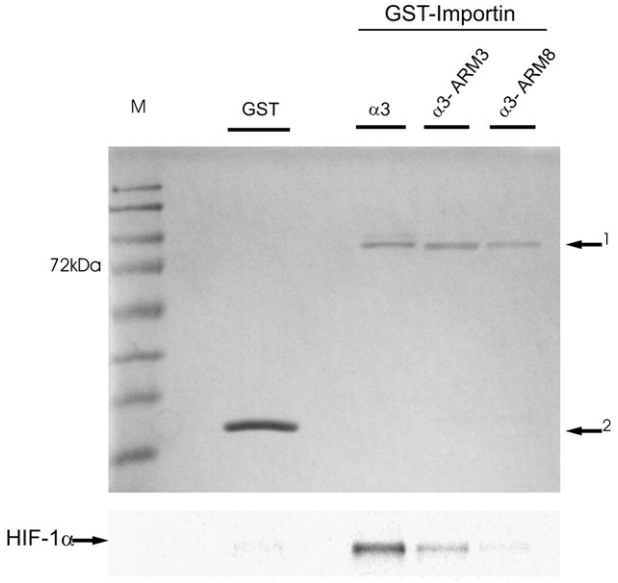


Fig. 7. Importin α3 binding to HIF-1α via the minor NLS-binding site shaped by armadillo repeats 7, 8 and 9. Protein–protein interaction of *in vitro* transcribed/translated HIF-1α GST-importin α3, α3-ARM 3 and α3-ARM 8. GST or GST-importin were immobilised on glutathione-Sepharose and incubated with *in vitro* transcribed/translated HIF-1α. 1: importin α3, 2: GST, M: protein ladder.

We used GST-importin α3 vector constructs with point mutations in ARM repeats 3 and 8, respectively, in the protein–protein pull-down assay. Importin α3 ARM 3 features mutations W191A and N195A, whereas importin α3 ARM 8 is mutated at sites W390A and N394A. As shown in Fig. 7, an amino acid exchange in ARM 8 almost completely prevented the HIF-1α importin α3 interaction. In contrast, the ARM 3 repeat mutant showed reduced but still significant binding activity to HIF-1α.

4. Discussion

The family of HIF transcription factors must be translocated into the nucleus to exert its function of activating specific genes within the nuclear compartment, which is the molecular basis for cellular oxygen homeostasis. Besides the regulation of HIF-1α stability by prolyl-hydroxylation, the regulated nuclear import of HIF subunits may constitute another level of activity control. It has been shown that the classical nuclear import pathway is down-regulated via the nuclear accumulation of importin α and the down-regulation of importin β in response to various stresses (e.g. oxidative stress and heat shock stress) [40,41]. The nuclear accumulation of HIF-1α was reported to be induced by hypoxia [17]. To test whether or not an oxygen-dependent importin level might influence HIF nuclear transport we determined the importin α/β expression in normoxic and hypoxic Hela and U2OS cells. We found no differences in the level of importin α/β protein expression levels (data not shown).

Our results showing direct binding of HIF-1α, HIF-2α, and HIF-1β to α/β importins and the reported coincidence between the loss of this binding and the loss of import capability in living cells suggests strongly, that nuclear import of HIF proteins is triggered by importin α/β complexes. This raises the question, whether a distinct set of importin αs is involved in this process. Our *in vitro* pull-down assays showed for the first time that all

tested isoforms of the three importin α subfamilies (α1, α3, α5, and α7) are able to bind to HIF subunits pointing out the importance of the HIF system for living cells and the necessity of redundancy for this specific transport process. In this case, loss of one functional importin α *in vivo* would not cause loss of cellular response to hypoxia. On the other hand, import of transcription factors which are transported by only one or two importin α isoforms [37] should be more sensitive to a lack of distinct α importins.

The observed binding intensities between the various importin proteins are only slightly different. However, these differences may be of physiological relevance *in vivo* as suggested by reports, which propose different nuclear translocation efficiencies of the various importin α isoforms [28,42–43] and other studies showing that certain proteins are regulated by specific importin α subfamilies [28,44]. The *in vitro* methods employed in these studies and in our own experiments can only unravel substantial differences in the affinity, but may fail to detect subtle ones. This means, that one cannot exclude, that under appropriate *in vivo* conditions a substrate like HIF may be imported by just one type of importin α. However, this also suggests, that in case the naturally used importin α is not available for HIF it can more easily use other isoforms present in the cell.

In contrast to HIF-1α, HIF-2α binds directly to importin β *in vitro*. The efficiency of this interaction is higher than binding of HIF-2α to importin αs solely. These results indicate that importin β alone might be necessary and sufficient for the import of HIF-2α. As the absolute amount of importin β protein in human cells can exceed the amount of protein of importin α isoforms [43], the direct interaction with HIF-2α might bypass the need for the classical nuclear import of this substrate in cases where the transport capacity of importin αs is limited.

The HIF-β subunit is constitutive and therefore a permanent target for nuclear translocation by the transport system. Direct interaction of HIF-1β with various importin α subunits (Fig. 2B) demonstrates that nuclear translocation of HIF-β is addicted to importin α. On the other hand it supports the hypothesis that HIF-α and β subunits are independently imported from each other. This is in accordance with earlier reports showing that HIF-1α is accumulating in the nucleus of HIF-1β deficient embryonic stem cells [45].

The proof that HIF-αs and HIF-1β form complexes with importin nuclear transport factors was a missing step in our understanding of the transport of HIFs into the nucleus. We followed the question of the localisation of the HIF-1α nuclear localisation signal, because previous studies investigating the functional HIF-1α NLS were controversial.

Involvement of the C-terminal NLS of HIF-1α had been suggested mainly on the basis of the cytoplasmic retention of mutant HIF-1α GFP fusion proteins [17,33]. In contrast to these previous studies we used untagged full-length HIF constructs for both, *in vitro* binding as well as *in vivo* intracellular localisation studies for two reasons: First, earlier reports demonstrated that the GFP-part of the fusion protein stabilised HIF-1α nuclear localisation in normoxia, an effect which may have an influence on the observed subcellular localisation of the protein [17–18,33]. Secondly, the large GFP-part of the fusion protein may affect the passage through the nuclear pore and the ability to directly bind to interacting proteins.

Our data demonstrate that point mutations in the C-terminal NLS, but not mutations in the proposed N-terminal NLS disturb the ability of HIF-1α to bind to various importin αs and block its import into the nucleus in living cells. The alteration of both predicted NLSs showed that no residual binding capacity to importins is left if the specific motif is mutated. In accordance with our data is the finding that an isoform lacking the C-terminal NLS, namely HIF-1α^{516}, is solely located in the cytoplasm [46]. However, in contrast to our findings Lee et al. observed an isoform (HIF-1α^{417}) with a C-terminal truncation, also missing the C-terminal NLS in the nuclear fraction of cell extracts [47]. Likewise, a HIF-1α isoform, HIF-1αZ (aa 1–557), was found to be located both in the cytoplasmic and the nuclear compartment, although the C-terminal NLS is deleted [48]. These reports pointed to the existence of an alternative import signal. Interestingly, a GFP-HIF-1α N-terminal NLS fusion protein (HIF-1α^{1-74}) was shown to be transported into the nucleus [17]. On the other hand, the elongation of this fusion protein up to amino acid 330 inhibited the hypoxia induced nuclear accumulation. It was therefore suggested that the PAS-B domain might mask a N-terminal NLS of HIF-1α. However, one should bear in mind that the GFP-HIF-1α^{1-74} construct is small enough to let the fusion protein pass freely through the nuclear pore complex instead of a regulated passage. Interestingly, the PAS-B domain has been shown to be crucial for binding of HSP90 to HIF-1α [38,49,50]. Based on these data, the HIF-1α PAS-B domain has been implicated in influencing nuclear localisation of HIF-1α possibly by masking/unmasking a NLS in a ligand, e.g. HSP90-dependent manner under certain conditions, as demonstrated for the Aryl Hydrocarbon Receptor (Ahr) [17,51–53].

HSP-90 binding to HIF-1α was mapped to the PAS-B domain of HIF-1α [38]. In our hands, the deletion of the PAS-B domain did not alter the strict dependence of both, binding to importin α/β and nuclear localization of HIF-1α on the integrity of the C-terminal NLS. Moreover, addition of HSP-90 in binding assays had virtually no effect on HIF-1α/importin α interaction (data not shown). This is in accordance with data of Minet et al. who precluded any co-translocation of HIF-1α and HSP-90 by confocal microscopy [53]. Thus, our data fit best with a model were HIF-1α has only one functional nuclear import signal in the C-terminus of the protein. Further work has to be done to answer the question why HIF isoforms without a C-terminal NLS can translocate to the nucleus.

The NLS of the cargo protein is only one half of the interacting structure, the other part can be constituted by one or both NLS-binding pockets of importin α. To determine the structural elements of the importin α/HIF-1α interaction we used mutant importin α molecules with point mutations in the binding domain of armadillo repeats 3 and 8, respectively. Binding of HIF-1α depends on the functionality of both binding sites, since importin α3 with an altered ARM 8 shows almost no binding to HIF-1α in our assay and the interaction of an ARM 3 mutant is clearly reduced. Monopartite NLS is said to bind to ARM repeats 2–4 which is the major binding site whereas the C-terminus of

bipartite NLS is interacting additionally with the minor binding site at ARM 7–9 [24,54]. The strong dependency of HIF-1α binding to importin α on an intact minor binding pocket therefore substantiates the hypothesis that the C-terminal NLS of HIF-1α is a bipartite NLS, as discussed above. The HIF-1α mutant K753A possesses an amino acid exchange in the C-terminal part of the predicted bipartite NLS and the abolished binding of this mutant to importins also supports the hypothesis of a bipartite C-terminal NLS. This behaviour of HIF-1α is reminiscent to data obtained for STATs, using a similar assay system [27]. It has been suggested that STAT proteins have an unusual bipartite NLS with a long spacer between a monopartite-like N-terminal cluster of basic amino acids and the C-terminal basic region [55]. Interestingly, the surrounding of the monopartite NLS of HIF-1α constitutes of basic amino acids in a distance similar to that found in STAT 1 and 2, suggesting that the C-terminal NLS of HIF-1α is in fact also an unusual bipartite one.

In summary, this study extends the current knowledge of the NLS-dependent nuclear translocation of hypoxia-inducible factors. HIFs are able to bind the "classical" importins and these complexes are likely to govern the specificity and the rate of the nuclear translocation process.

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