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Bacterially produced human HIF-1α is competent for heterodimerization and specific DNA-binding

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Abstract

Hypoxia-inducible factor 1α (HIF-1α) is the regulatory subunit of HIF-1, the transcriptional activator and key mediator of the cellular response to hypoxia. Regulation of HIF-1α occurs at multiple levels and involves several different post-translational modifications. In order to examine the importance of these modifications for the basic function of HIF-1α, we have produced in bacteria recombinant full-length human HIF-1α using different expression systems. We show that this unmodified form of HIF-1α is able to form a stable heterodimer with the second subunit of HIF-1 (HIF-1β or ARNT) when both proteins are co-expressed in *Escherichia coli*. Furthermore, this bacterially reconstituted heterodimer exhibits specific DNA-binding activity. These data indicate that post-translational modification of HIF-1α is not essential for its interaction with ARNT and DNA, and provide an in vitro system for the characterization of the molecular properties of HIF-1α.

Keywords: Hypoxia-inducible factor 1; HIF-1α; ARNT; Bacterial expression; Heterodimerization; DNA-binding

Upon hypoxia (reduced oxygen availability), cells respond by increasing the expression of genes encoding proteins that facilitate oxygen delivery (e.g., erythropoietin, VEGF) or enhance anaerobic production of energy (e.g., glycolytic enzymes) thus maintaining energy homeostasis [1]. A crucial component in the induction of hypoxia-regulated genes is the transcription factor hypoxia-inducible factor 1 (HIF-1), which activates transcription by binding to a specific *cis*-acting regulatory sequence referred to as hypoxia response element (HRE), a hallmark of hypoxia-sensitive target genes [2]. The HIF-1 protein complex consists of a heterodimer composed of HIF-1α and HIF-1β subunits, both members of the basic-helix–loop–helix–PAS protein family of transcription factors [3,4]. HIF-1α is unique to HIF-1 while HIF-1β is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT) that also dimerizes with the aryl hydrocarbon receptor. Although HIF-1β is constitutively expressed, the activity of HIF-1α is regulated at multiple levels [5].

Under normoxic conditions, HIF-1α is maintained at a low level through proteasomal degradation. HIF-1α is targeted for proteolysis through its interaction with the von Hippel-Lindau (VHL) tumor suppressor protein and subsequent polyubiquitination [6,7]. VHL can only bind to HIF-1α when one or possibly two critical proline residues in the ODD (oxygen dependent degradation) domain of HIF-1α are hydroxylated [8–10]. The proline hydroxylases that modify HIF-1α (members of the EGLN family, termed PHDs or HPHs) are Fe^{2+} dependent and require oxygen [11]. According to these findings, it has been suggested that hypoxia inhibits the hydroxylation of HIF-1α thus allowing its stabilization

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[12,13]. Expression and stability of HIF-1α under normoxic conditions can also be regulated by major signal transduction pathways induced by growth factors and cytokines, including those involving PI3K and ERK/MAPK [5,14]. Once stabilized, HIF-1α translocates inside the nucleus, dimerizes with ARNT, binds to DNA, and activates the transcription of its target genes. This final step is also thought to be regulated by hypoxia via an asparagine hydroxylase, which, when active, modifies the C-terminal transactivation domain (CTAD) of HIF-1α and inhibits its association with the transcriptional co-activator CBP/p300 [10,15].

In addition to hydroxylation, HIF-1α is also subjected to various other post-translational modifications including acetylation, SUMOylation, phosphorylation, and S-nitrosation [16]. Acetylation has been suggested to negatively regulate HIF-1α, facilitating its normoxic destabilization [17]. On the opposite, SUMOylation [18], phosphorylation [19], and S-nitrosation [20] are thought to increase the stability and/or transcriptional activity of HIF-1. All the aforementioned modifications are known to occur in the carboxy-terminal half of HIF-1α, where the ODD and two transactivation domains (NTAD and CTAD) are located [16]. The studies that have analyzed the function of these domains and their modifications have often been performed using recombinant or synthetic fragments of HIF-1α and their fusions to reporter proteins. It is, therefore, not exactly known how these domains or their modification status may affect, in the context of the full-length protein, the function of the amino-terminal half of HIF-1α that contains the heterodimerization and DNA-binding bHLH-PAS domain.

To start addressing this issue, we have attempted to produce the full-length form of human HIF-1α in bacteria, which lack the eukaryotic modification enzymes acting normally on HIF-1α, and test this unmodified form of HIF-1α for functionality in vitro. To achieve this, the cDNA of HIF-1α was cloned in a number of different *E. coli* expression vectors and conditions were established that led to production and purification of soluble tagged full-length HIF-1α. We show that recombinant HIF-1α can interact with recombinant ARNT (HIF-1β) and form stable heterodimers, when both proteins are co-expressed in *E. coli* cells. Furthermore, this bacterially reconstituted HIF-1 is able to bind to DNA, recognizing specifically the HRE sequence.

Materials and methods

Plasmid constructions. The cDNA of full-length HIF-1α was amplified by PCR from plasmid pcDNA3-HIF-1 [21], using as primers: forward: TTTTTGGATCCCGATTCACC*ATG*GAGGGC; reverse: TTTTTGGATCC*TCA*GTTAACTTGATCCAAAGC (*Bam*HI sites underlined, start and stop codons of HIF-1α ORF in italics, respectively). The PCR product was cloned into the pCR2.1-TOPO-TA vector (Invitrogen), sequenced and subsequently subcloned as a *Bam*HI fragment into the bacterial expression vectors pQE-80L, pQE-60 (Qiagen), zzpQE-60, pGEX-4T-1 (Amersham), and pHisGFP. The zzpQE-60 vector is a pQE60 derivative coding for an N-terminal ProtA-tag in addition to the C-terminal His-tag [22]. The pHisGFP vector permits expression of the cloned protein with an N-terminal His-tagged GFP-fusion and was a generous gift by Dr. D. Görlich. The pARNT/GEX4T-3 plasmid [23] was used to subclone the ARNT cDNA as a *Bam*HI fragment into the *E. coli* expression vector pET24d-GST-Tev, a pET24d (Novagen) derivative [24].

Protein expression and purification. Plasmids encoding for HIF-1α-His (pQE60-HIF-1α) and ProtA-HIF-1α-His (zzpQE60-HIF-1α) were transformed into the BLRpREP4 strain of *E. coli*. Those encoding for His-HIF-1α (pQE80-HIF-1α), HisGFP-HIF-1α (pHisGFP-HIF-1α), and GST-HIF-1α (pGEX-HIF-1α) were transformed into the BL21(RIL) strain of *E. coli* and the one encoding for GST-ARNT (pET-GST-ARNT) was transformed into the BL21(DE3)RIL strain of *E. coli.* Co-expression of HisGFP-HIF-1α and GST-ARNT was also performed in the latter strain. Expression of the recombinant proteins was induced by addition of 1 mM IPTG in the culture medium.

Bacterial cells expressing individual His-tagged proteins or the HIF-1 complex (HisGFP-HIF1α/GST-ARNT) were lysed by sonication in buffer A containing 50 mM Tris–Cl, pH 7.4, 200 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 5% glycerol, 1 mM PMSF, EDTA-free protease inhibitor cocktail (Roche), and 0.1% Triton X-100. The soluble extracts were applied onto a Ni–NTA–agarose (Qiagen) affinity column and bound proteins were eluted with 250 mM imidazole (Fluka) in buffer A containing 0.02% Triton X-100. His-GFP-HIF1α and the HIF-1 complex were further purified using a HiPrep 16/60 Sephacryl S-200 HR column (Amersham) equilibrated in EMSA buffer (see below) and run at a flow rate of 0.6 ml/min. The column was calibrated using commercially available gel filtration standards (Bio-Rad). The column fractions containing the recombinant proteins were concentrated by ultrafiltration and stored at −80 °C.

Bacterial cells expressing GST-fusion proteins were lysed by sonication in buffer B containing 20 mM Tris–Cl, pH 7.6, 137 mM NaCl, 1 mM PMSF, EDTA-free protease inhibitor cocktail, 0.1% Tween 20, and 5 mM DTT. The soluble extracts were applied onto a glutathione–Sepharose 4B (Amersham) column and bound proteins were eluted with 10 mM reduced glutathione (Sigma) in buffer B without Tween 20. GST-ARNT, expressed in *E. coli* from plasmid pET-GST-ARNT, was purified as previously described [23]. The GST-tagged proteins were dialyzed against EMSA buffer and stored at −80 °C.

SDS–PAGE and Western analysis. Protein samples from all expression and purification steps were resolved by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and analyzed by Coomassie blue staining or Western blotting using an anti-HIF-1α mouse monoclonal antibody (1:500; BD Transduction Laboratories), anti–ARNT (1:500; BD Transduction Laboratories) mouse monoclonal antibody or an anti-GST goat antibody (1:1000; Amersham). Membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) or mouse anti-goat IgG (Jackson Immunoresearch) followed by ECL detection (Amersham) or by detection with color reaction using chloronaphthol and H_2O_2 as substrates.

Electrophoretic mobility shift assay. The W18 oligonucleotide (sense strand; 5′-GCCCTACGTGCTGTCTCA-3′) containing the hypoxia response motif (underlined) from the human erythropoietin enhancer [25] and its mutant form M18 (5′-GCCCTAAAAGCTGTCTCA-3′) were end-labeled using the T4 polynucleotide kinase (MBI Fermentas) and [γ-^{32}P]ATP (Amersham), purified by the Qiaquick (Qiagen) nucleotide removal kit, and annealed with 5-fold excess of their corresponding unlabeled complementary antisense strand oligonucleotides. Binding reactions for the gel shift assays were set up in a volume of 15 μl. Single purified proteins (250 ng) or the reconstituted HIF-1 complex (500 ng) were incubated for 30 min at RT, in EMSA buffer