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Research Article

The specific contribution of hypoxia-inducible factor-2α to hypoxic gene expression *in vitro* is limited and modulated by cell type-specific and exogenous factors

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ABSTRACT

Cellular integrity in hypoxia is dependent on molecular adaptations dominated by the heterodimeric transcription factor hypoxia-inducible factor (HIF). The HIF complex contains one of two alternative oxygen-regulated α-subunits considered to play distinct roles in the hypoxia response. Although HIF-2α may be more important in tumour biology and erythropoiesis, the spectrum of individual target genes is still insufficiently characterized. We therefore performed an Affymetrix gene array on Hep3B cells stimulated with a hypoxia-mimetic and transfected with either HIF-1α or HIF-2α siRNA. 271 transcripts were found to be induced HIF-dependently, including most previously identified HIF targets and a number of novel genes. Most were influenced by HIF-1α knock-down, whereas a smaller number were regulated by HIF-2α. Validation of a selection of genes by RNase protection confirmed the hypoxic regulation and HIF-1α- or HIF-2α-dependency in most cases, with the latter showing a lower amplitude. Many HIF-2α targets also responded to HIF-1α knock-down. Interestingly, regulation by HIF-2α was markedly influenced not only by cell type, but also by cell culture conditions, features that were not shared with HIF-1α-regulated genes. Thus, HIF-2α effects are modulated by a number of intrinsic and extrinsic factors which may be most relevant in tumour cells.

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Introduction

Hypoxia, the lack of molecular oxygen, constitutes a life-threatening condition for the vertebrate organism. Oxygen, which is critical for the generation of high energy substrates, is also a limiting factor for survival and proliferation of tumour cells (for review see [1,2]). A pivotal role in the adaptive response of normal and tumour cells to hypoxia is played by the hypoxia-inducible (transcription) factor (HIF), which consists of two subunits, the constitutive HIF-β and the oxygen-responsive HIF-α subunit [3]. At least three alternative HIF-α subunits exist, of which HIF-1α and HIF-2α contribute to the transcriptional response to hypoxia and their oxygen-dependent regulation is at least partially understood. Oxygen regulates

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these proteins in a dual fashion. First, it activates enzymatic hydroxylation of two defined prolyl residues which enables targeting by the von Hippel-Lindau protein (VHL), the recognition component of an E3 ubiquitin ligase complex, and leads to rapid proteasomal destruction. Second, a C-terminal asparagyl residue is hydroxylated, which precludes the recruitment of the transcriptional co-activator CBP/p300 (for review see [4,5]).

Although the expression and hypoxic regulation of HIF-1α and HIF-2α appears to be quite similar in tumour cell lines [6], an increasing body of evidence points to differential biological roles of the two HIF-α subunits. On the one hand, immunohistochemical studies of HIF-1α and HIF-2α in rodent tissues have demonstrated tissue- and cell-specific expression, with very little overlap [7–9]. On the other hand, gene deletion studies of individual HIF-α isoforms in mice have shown either lethality in midgestation or distinct and severely altered phenotypes ([10], for review see [5]). These data suggest that the HIF-α subunits exert at least some non-overlapping functions *in vivo*, probably due to differential expression patterns as well as regulation of specific target genes. In addition, it has also been shown that either HIF-α subunit mediates activation of specific target genes in tissue culture settings [11–15]. By the use of RNA interference (RNAi) we were able to identify erythropoietin (EPO) as a HIF-2α target in Hep3B and Kelly cells [12]. In support of these data, recent gene deletion studies in mice have provided functional evidence for a critical role of HIF-2α in the regulation of EPO [16–19]. However, most other genes investigated so far seem to be responsive only or predominantly to HIF-1α, leading to uncertainty concerning the contribution of HIF-2α to the hypoxic response. Likewise, the significance of the individual HIF-α isoforms in tumours is controversial, although an increasing number of studies reported a decisive role for HIF-2α in renal tumourigenesis [15,20–22].

A number of gene arrays addressing the differential function of HIF-1α vs. HIF-2α have been performed in the past. Most findings were obtained by overexpression of the HIF-α subunits [13,14] or by utilising the HIF-1α-deficient renal carcinoma cell line 786-0, in which HIF-2α has taken over the regulation of at least some HIF-1α targets [23,24], which limits the significance of these studies. Since clonal variability and loss of HIF-α target gene specificity may compromise the findings obtained with HIF-α overexpression, transient downregulation of the endogenous HIF-α genes may give more meaningful results. Therefore, in the present study we chose RNAi to investigate specific HIF-1α and HIF-2α effects in microarray analyses. We utilised Hep3B cells since it is one of the few cell lines that reliably induces EPO under hypoxic conditions, which served as a control for HIF-2α-mediated effects.

By the use of this approach, we identified numerous known and a number of novel HIF targets. Some of these genes are possibly of functional relevance in cancer biology. Further analyses showed that HIF-2α-dependency is cell type-specific and may even be modulated by cell culture conditions, features that are not shared with HIF-1α-regulated genes. The present study therefore contributes a number of potentially important genes to the continuously increasing hypoxic transcriptome, while emphasizing the need for further dissection of the molecular mechanisms of HIF-1α- and HIF-2α-driven gene induction.

Materials and methods

Cells and reagents

Hep3B, HepG2 and HeLa cells were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cell culture reagents were produced by Invitrogen (Karlsruhe, Germany) or PAA Laboratories (Coelbe, Germany). The fetal calf sera (standard, "Gold" and "Clone") were purchased from PAA Laboratories, 2,2′ dipyridyl (DP) from ICN (Costa Mesa, CA, USA) and LPS, IL-1β and TNF-α from Sigma (Taufkirchen, Germany).

Cell culture

Hep3B, HeLa and HepG2 cells, wild-type renal carcinoma cells RCC4 and 786-0 cells were grown in DMEM, 1.0 g glucose/L, 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U penicillin and 100 μg streptomycin /mL, and passaged twice or thrice weekly. VHL-reconstituted RCC4 and 786-0 cells were passaged in the presence of G418 (1 mg/mL).

SiRNAs and transfection procedures

HIF-α siRNAs (HIF-1α sense 5′ GCCACUUCGAAGUAGUGCUdTdT targeting nt 1378–1398 of the human HIF-1α mRNA (acc. no. AF304431.1); HIF-2α sense 5′ GCGACAGCUGGAGUAUGAAdTdT targeting nt 2274–2294 of the human HIF-2α mRNA (acc. no. NM_001430.1) were described before [12] and synthesized by Qiagen (Cologne, Germany) and Eurogentec (Cologne, Germany). SiRNAs against firefly luciferase comprised in the pGL2 and the pGL3 vector, respectively (2luc sense siRNA 5′ CGUACGCGGAAUACUUCGAdTdT, 3luc sense 5′ CUUACGCUGAGUACUUCGAdTdT) were used as controls. Apart from a modest reduction of gene induction by the transfection procedure itself we did not observe differences between these luciferase siRNAs when used as negative controls. Cells were transfected with the siRNAs (final concentration 200 nmol/L) at 50% confluency by the use of Oligofectamine (Invitrogen, Karlsruhe, Germany) in Optimem-1 medium (Invitrogen) according to the manufacturer's protocol. In the second Affymetrix array experiment the established HIF-α siRNA pair was compared with the HIF-1α and HIF-2α siRNAs published by Sowter and colleagues [11] (HIF-1α′ sense 5′ CUGAUGACCAGCAACUUGAdTdT, HIF-2α′ sense 5′ CAGCAUCUUUGAUAGCAGdTdT). To further reduce non-specific side-effects siRNAs were used in this and all subsequent experiments at a concentration of 50 nmol/L.

For knock-down of CITED2 two independent siRNAs were designed (CITED2 A sense 5′ UGGGCGAGCACAUACACUAdTdT, acc. no. NM_006079.3 nt 399–417, and CITED2 B sense 5′ CAGAAGCTCAACAACCAGTATdTdT, nt 595–613).

Immunoblotting

The day after siRNA transfection, cells were stimulated with 2,2′dipyridyl (DP, 100 μmol/L). Immunoblotting for HIF-1α (mouse monoclonal HIF-1α, Transduction Laboratories, Lexington, KY, USA, or rabbit polyclonal antiserum NB100-449,

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