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Hypoxia-inducible factor (HIF)-3α is subject to extensive alternative splicing in human tissues and cancer cells and is regulated by HIF-1 but not HIF-2

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ABSTRACT

The hypoxia-inducible transcription factors (HIFs) play a central role in the response of cells to hypoxia. HIFs are αβ dimers, the human α subunit having three isoforms. HIF-3α is unique among the HIF-α isoforms in that its gene is subject to extensive alternative splicing. Database analyses have predicted the generation of six HIF-3α splice variants that utilize three alternative transcription initiation sites. None of these variants is likely to act as an efficient transcription factor, but some of them have been reported to inhibit HIF-1 and HIF-2 functions. We analyzed here for the first time in detail whether these six variants are indeed generated in various human tissues and cell lines. We identified four novel variants, named here HIF-3α7 to HIF-3α10, whereas we obtained no evidence for the predicted HIF-3α3 and HIF-3α5. Distinct differences in the expression patterns of the variants were found between human tissues, the levels being particularly low in many cancer cell lines. Hypoxia upregulated transcription from all three alternative HIF-3α promoters. siRNA experiments showed that this induction is mediated specifically by HIF-1 and not by HIF-2. The tissue-specific differences in the expression patterns and levels of the HIF-3α variants can be expected to modulate the hypoxia response of various tissues and cell types to different extents during development and in pathological situations. A further level of regulation is brought about by the fact that the levels of the HIF-3α transcripts themselves are regulated by hypoxia and by changes in HIF-1 levels.

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1. Introduction

The hypoxia-inducible transcription factors (HIFs) play a central role in the response of cells to hypoxia by regulating the expression of genes involved in functions such as hematopoiesis, angiogenesis, iron transport, glucose utilization, extracellular matrix synthesis, cell proliferation, survival and apoptosis and tumor progression (Kaelin and Ratcliffe, 2008; Myllyharju, 2008; Semenza, 2009). The HIFs are αβ dimers, the human α subunit having three isoforms (Kaelin and Ratcliffe, 2008; Myllyharju, 2008; Semenza, 2009). Hydroxylation of at least one of two critical prolines, Pro402 and Pro564, in the oxygen-dependent degradation domain (ODDD) of HIF-1α mediates its interaction with the von Hippel-Lindau E3 ubiquitin ligase complex, which targets it for rapid proteasomal degradation in normoxia (Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001). This hydroxylation is catalyzed in humans by three HIF prolyl 4-hydroxylases (HIF-P4Hs) having a preference for Pro564 (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2002; Hirsilä et al., 2003; Chan et al., 2005; Koivunen et al., 2006). A further hydroxylase, factor inhibiting HIF (FIH), acts on an asparagine in the C-terminal transactivation domain (C-TAD) of HIF-1α and HIF-2α, which prevents binding of the coactivator p300 and thus full activity of HIF-1 and HIF-2 (Hewitson et al., 2002; Lando et al., 2002a,b). Hypoxia inhibits both the HIF-P4Hs and FIH, leading to stabilization of HIF-1α and HIF-2α and their full transcriptional activity.

The HIF-1α and HIF-2α isoforms have similar domain structures, with an N-terminal basic helix-loop-helix (bHLH) domain followed by two Per-ARNT-Sim (PAS) domains, the ODDD, and N-terminal and C-terminal TADs, the former partially overlapping with the ODDD (Patel and Simon, 2008). Although HIF-1 and HIF-2 have many common target genes they also show specificity in that HIF-1 appears to act more effectively on genes for glycolytic enzymes, for instance, and HIF-2 on the gene for erythropoietin (Patel and Simon, 2008). The first mouse and human HIF-3α isoforms to be characterized were 662 and 667-amino-acid polypeptides, respectively, which lack the C-TAD (Gu et al., 1998; Hara et al., 2001). They dimerize with HIF-β and induce expression of a hypoxia response element (HRE) reporter when overexpressed together with HIF-β, and may thus act as weak transcription factors. On the other hand, they also suppress HIF-1 and HIF-2-mediated gene expres-

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sion when HIF-β is limited, probably by competing for recruitment of the common partner (Gu et al., 1998; Hara et al., 2001). The HIF-3α ODDD differs from those of HIF-1α and HIF-2α in that it contains only a single hydroxylatable proline corresponding to Pro564 in HIF-1α. The HIF-3α ODDD is regulated in an oxygen-dependent manner and peptides representing its single hydroxylation site are efficiently hydroxylated by HIF-P4Hs (Hirsilä et al., 2003; Maynard et al., 2003; Koivunen et al., 2006).

The HIF-3α mRNA is subject to alternative splicing. The 307-residue mouse inhibitory PAS domain protein (IPAS) differs from HIF-3α in that the first 8 amino acids are coded by the IPAS-specific exon 1a instead of exon 1b, and it lacks the ODDD and N- and C-TADs (Makino et al., 2001; 2002). IPAS forms a complex with HIF-1α but not with HIF-β, and the IPAS-HIF-1α complex is not able to recognize or bind to HRE, so that IPAS functions as a dominant negative regulator of HIF-1 (Makino et al., 2001). Another mouse variant, NEPAS, a 664-amino acid polypeptide that differs from HIF-3α only in that exon 1b is replaced by the IPAS exon 1a, is expressed mainly during embryonic and neonatal stages (Yamashita et al., 2008). NEPAS also dimerizes with HIF-β and suppresses HIF-1 and HIF-2 activity (Yamashita et al., 2008). Mice with a targeted disruption of the *Nepas*/*Hif-3α* locus are viable but have abnormal heart development and lung remodeling (Yamashita et al., 2008).

Database analyses have suggested that six splice variants are generated from the human HIF-3α locus, which consists of 17 exons (Fig. 1A) (GenBank accession no. AC007193) (Maynard et al., 2003). These variants utilize three alternative transcription start sites in exons 1a, 1b and 1c, and exons 13 and 14 have two and three alternative forms, respectively (Fig. 1A and B). The first variant to be characterized, HIF-3α1 (Hara et al., 2001), starts from exon 1c and ends at exon 16, the encoded polypeptide containing a leucine zipper (LZIP) at its C terminus. The predicted HIF-3α2 starts from exon 1a, ends at exon 13a, and codes for a 632-residue polypeptide, while HIF-3α3 starts at exon 1b, ends at exon 17, lacks exons 15 and 16, and codes for a 648-residue protein. HIF-3α3 differs from HIF-3α1 and HIF-3α2 in that it lacks the N-terminal bHLH domain. Three short variants lacking the ODDD and N-TAD domains have also been predicted (Maynard et al., 2003) (Fig. 1B). The HIF-3α4 transcript starts from exon 1a and ends within intron 8, intron 7 not being spliced, and codes for a 363-residue polypeptide that is most similar to, but not identical with, the mouse IPAS. The HIF-3α5 and HIF-3α6 both start from exon 1b and lack exon 3. HIF-3α5 also lacks exons 12 and 13 and ends at exon 15, while HIF-3α6 is similar to HIF-3α4 from exon 4 onwards, and the encoded polypeptides consist of 450 and 237 residues, respectively. The cloned human HIF-3α4 forms a complex with both HIF-1α and HIF-β, and inhibits transcriptional activity of HIF-1 in a dominant negative manner (Maynard et al., 2005).

The HIF-1α and HIF-2α mRNAs are not induced by hypoxia, while contrasting data have been reported on the oxygen-dependent regulation of HIF-3α transcription. Hypoxia activates expression of the mouse and rat HIF-3α mRNAs, the activation of the former being driven by HIF-2 but not by HIF-1 (Heidbreder et al., 2003; Hatanaka et al., 2009). The mouse IPAS mRNA is likewise upregulated in hypoxic tissues, the mechanism involving at least partly hypoxia-dependent alternative splicing of the HIF-3α locus and the presence of an HRE element that can be regulated by both HIF-1 and HIF-2 (Makino et al., 2002, 2007). Human HIF-3α1 has been shown to be induced by hypoxia (Li et al., 2006), while human HIF-3α4 mRNA has been reported to be downregulated in hypoxia (Maynard et al., 2005).

HIF-3α and its variants may have major effects on the function of HIF-1 and HIF-2, and thus on the hypoxia response. Detailed information on the characteristics and regulation of the HIF-3α variants will thus be of considerable biological relevance. We set out for the first time to analyze in detail whether the predicted HIF-3α variants are indeed generated in human cell lines and tissues. We identified four novel variants, named here HIF-3α7 to HIF-3α10, but obtained no evidence for the existence of the predicted HIF-3α3 and HIF-3α5. Distinct differences in the expression patterns of the variants were found between the human tissues and cancer cell lines studied. We also show that hypoxia upregulates transcription from all three alternative HIF-3α transcription initiation sites, indicating that all human HIF-3α variants are induced by hypoxia. siRNA experiments suggest that this induction is mediated specifically by HIF-1 and not by HIF-2.

2. Materials and methods

2.1. Cloning of full-length human HIF-3α variants

Full-length human HIF-3α variants were amplified by PCR from human placenta or fetus Marathon-Ready cDNAs (Clontech) according to the manufacturer's protocol, using Phusion polymerase (Finnzymes) and two PCR rounds. A 1/50 aliquot of the reaction amplified in the first round was used as a template in the second PCR round. Sequences of the oligos used in the cloning are given in Table 1. The full-length cDNAs were cloned into pMOSBlue or pJET1 blunt vectors using the pMOSBlue blunt-ended cloning (Amersham Biosciences) and GeneJET™ PCR cloning (Fermentas) kits, respectively. The sequences were analysed using an automated DNA sequencer (ABI Prism 377, Applied Biosystems). The full-length cDNAs were amplified from these plasmids using primers with artificial NotI or XhoI restriction sites in the 5′ end and a HindIII site in the 3′ end to facilitate cloning into a NotI/XhoI-HindIII digested pcDNA3.1(−)Zeo (Invitrogen) expression vector.

2.2. PCR analysis of HIF-3α mRNA expression

Expression of the HIF-3α variant mRNAs was studied by PCR analysis of the human multiple tissue cDNA (MTC™) panel I, the human fetal multiple tissue cDNA panel and the human cancer cell line MTC™ Panel (Clontech) according to the manufacturer's protocol, using Phusion polymerase (Finnzymes). 1/50 aliquots of the PCR reactions amplified in the first round were used as a template for the second round. Specific oligo pairs were designed for each variant, the sequences of which are given in Table 1. Amplification of the full-length cDNAs was necessary in order to distinguish variants 1, 8 and 9 (Fig. 1B). PCR products were analysed on a 1% agarose gel and their identity was verified by DNA sequencing. HIF-1α oligos were used to show the presence of HIF-1α cDNA in the samples.

2.3. In vitro transcription and translation

HIF-3α variant cDNAs were transcribed and translated in vitro using pcDNA plasmids containing the full-length cDNAs and the TnT® Quick Coupled Transcription/Translation kit (Promega) with ^{35}S-methionine. ^{35}S-labeled proteins were analyzed by 8% SDS-PAGE followed by fluorography with exposure of the dried gel to an X-OMAT film at −80 °C for 6 h.

2.4. Cell culture

Hep3B hepatoma cells were cultured in Eagle's minimum essential medium (Sigma) with 0.1 mM non-essential amino acids (Sigma) and 1.0 mM sodium pyruvate (Sigma), embryonic kidney HEK293 cells in Dulbecco's minimum essential medium (Biochrom AG) with 0.375% sodium bicarbonate (Sigma), neuroblastoma Kelly cells in 90% RPMI 1640 (Sigma) and ChoK1 cells in Dulbecco's minimum essential medium (Biochrom AG) with 0.375% sodium bicarbonate (Sigma), 0.1 mM non-essential amino acids

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