We constructed a dominant negative form of human hypoxia-inducible factor (HIF)-2α, HIF-2αDoN, which inhibited HIF transcriptional activity induced by hypoxia and by HIF-2α. HIF-2αDoN formed a complex with HIF-1α and interacted with DNA containing hypoxia response elements (HREs). Thus, the complex appears to inhibit the binding of HIF-2 to HREs, and HIF-2αDoN might provide a useful therapeutic tool for HIF-2α-related diseases.

Key words: hypoxia-inducible factor-2α; dominant negative form; hypoxia-inducible factor-1α; hypoxia; hypoxia-inducible factor-1β

Mammalian cells upregulate the expression of genes related to energy metabolism, vascularization, and erythropoiesis in response to hypoxia.1) Uprregulation of the expression of such genes largely depends on the expression of hypoxia-inducible factor (HIF).2) HIF binds to consensus hypoxia response elements (HREs) in hypoxia-responsive genes, followed by activation of the transcription of their genes. HIF is composed of α and β subunits. The α-subunit is stably expressed when oxygen concentrations are reduced. The β-subunit (HIF-1β), which is required for DNA binding and the consequent transcriptional response, is constitutively expressed. Two types of α-subunit isoforms, HIF-1α and HIF-2α, share a common modular structure that is composed of a basic helix-loop-helix (bHLH)-containing PER-ARNT-SIM (PAS) (bHLH/PAS) domain, an oxygen-dependent degradation (ODD) domain, an N-terminal transactivation domain (N-TAD), and a C-terminal transactivation domain (C-TAD).3,4) Two nuclear localization signals are located in the N- and C-terminal regions. The bHLH region and the PAS domain are required for DNA binding and dimerization with HIF-1β respectively. The ODD domain includes two critical proline residues, which are hydroxylated under normoxia. Hydroxylated HIF-α undergoes ubiquitination and subsequent proteasome-dependent degradation,5) but HIF-1α and HIF-2α are expressed in distinct temporal and spatial patterns, and do not function redundantly.6,8) Thus the expression of hypoxia-responsive genes depends mainly on the amount and isoform of the α-subunit that is expressed in response to hypoxia.

Solid tumors have unique hypoxic microenvironments, and tumor growth in hypoxia is largely regulated by HIF-mediated gene expression. Therefore, inhibitors of HIF transcriptional activity might be useful as therapeutic tools for hypoxia-related diseases such as cancers.9) Recently, alternatively spliced isoforms of HIF-1α have been reported.3) Two of six HIF-1α isoforms, including wild-type HIF-1α, function as dominant negative isoforms of HIF-1α. On the other hand, no alternatively spliced variants of HIF-2α have been found. HIF-1α and HIF-2α differ in target gene specificity and tumorigenic activity. If dominant negative isoforms of HIF-2α bind to HREs in HIF-2α-responsive genes, such isoforms are expected to be useful as therapeutic tools for HIF-2α-related diseases, including cancers. In the present study, we constructed a dominant negative form of human HIF-2α and determined its function.

Total RNA was extracted from human hepatoma HepG2 cells, and was reverse-transcribed to synthesize cDNAs. Because the human HIF-2α gene is composed of 16 exons, the presence of splicing variants of HIF-2α was monitored by reverse transcription (RT)-PCR analysis between exons 1 and 9 and between exons 9 and 16. The two RT-PCR products covered the full-length open reading frame of HIF-2α mRNA. As shown in Fig. 1A, the RT-PCR products between exons 1 and 9 were detected as a single band by Southern blot analysis, cloned, and sequenced. The nucleotide sequence analysis of cloned DNA was identical to that of the authentic form of HIF-2α mRNA, indicating that there were no splicing variants between exons 1 and 9 of HIF-2α mRNA. On the other hand, Southern blot analysis of the RT-PCR products between exons 9 and 16 detected seven bands. The RT-PCR products corresponding to five of the bands were cloned and sequenced. The biggest PCR fragment corresponded to the authentic form of HIF-2α mRNA, and the second-smallest PCR fragment, which was faintly detected, was identified as a variant lacking exons 12 to 15 of HIF-2α mRNA. In the...
present study, we could not clone the cDNA encoding an alternatively spliced isoform lacking exons 12 to 15 of HIF-2α mRNA (termed HIF-2αDoN). However, as shown in Fig. 1B, the deduced structure of HIF-2αDoN allowed us to predict that HIF-2αDoN functions as a dominant negative form of HIF-2α. Thus, in HIF-2αDoN, the joining of exons 11 and 16 resulted in the generation of a new reading frame for exon 16 (the shaded box in Fig. 1B). Hence the deduced amino acid sequence of HIF-2αDoN was composed of 518 amino acids encoded by exons 1 to 11 and an additional 18 amino acids encoded by exon 16. HIF-2αDoN conserved the bHLH/PAS, the N-terminal nuclear localization signal, and the N-terminal part of N-TAD in the ODD domain. These structural features suggest that HIF-2αDoN can bind to DNA, interact with HIF-1α, and translocate to the nucleus, but cannot induce HIF transcriptional activity.

To determine whether HIF-2αDoN inhibits endogenous HIF transcriptional activity, a cDNA encoding HIF-2αDoN was constructed. HEK293 cells were transiently transfected with pH2αDoN and pHRE-Luc, followed by exposure to hypoxia. pH2αDoN is an expression vector encoding N-terminal HA- and C-terminal Myc-tagged HIF-2αDoN, and pHRE-Luc is a reporter vector that has three tandem repeats of an HRE of the human erythropoietin 3′-flanking region in pGL3-promoter vector. HRE of erythropoietin has a higher affinity for HIF-2α than HIF-1α. HIF-2αDoN suppressed hypoxia-induced HIF transcriptional activity in a dose-dependent manner (Fig. 2A). In contrast, HIF-2αDoN had no inhibitory effect of HIF-2αDoN on HIF-1α- or HIF-2α-induced HIF transcriptional activity, HIF-1αDM and HIF-2αDoN cDNAs encoding mutant HIF-1α and HIF-2α, which are stably expressed even in normoxia, were synthesized by site-directed mutagenesis. Plasmids expressing HIF-1αDM and HIF-2αDM with the N-terminal HA- and C-terminal Myc-tags (pHL1DM and pH2αDM respectively) were constructed and transfected into HEK293 cells. Recombinant HIF-1αDM and HIF-2αDM activated HIF transcriptional activity in normoxia, and HIF-2αDoN inhibited endogenous HIF-1α- or HIF-2α-activated HIF transcriptional activity.

![Fig. 1. Schematic Representation of HIF-2αDoN.](image)

![Fig. 2. Inhibitory Effects of HIF-2αDoN on HIF Activity.](image)
precipitation analysis with anti-HA antibody was co-transfected with pHRE-Luc, and plasmid immunoprecipitated neither HIF-1α nor HIF-2α, to HREs. In contrast, two HIF-1α dominant negative isoforms, HIF-1αZ15) and HIF-1α516,16) interact with HIF-1α in the cytosol and appear to block the nuclear translocation of HIF-1β. Thus the mechanisms by which HIF-2αDoN and HIF-1α variants inhibit HIF-2αDoN is expected to be useful as a therapeutic tool for cancers (e.g., breast cancer and renal cancer) that preferentially express HIF-2α.

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