Asparagine Hydroxylation of the HIF Transactivation Domain: A Hypoxic Switch

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The hypoxia-inducible factors (HIFs) 1α and 2α are key mammalian transcription factors that exhibit dramatic increases in both protein stability and intrinsic transcriptional potency during low-oxygen stress. This increased stability is due to the absence of proline hydroxylation, which in normoxia promotes binding of HIF to the von Hippel–Lindau (VHL tumor suppressor) ubiquitin ligase. We now show that hypoxic induction of the COOH-terminal transactivation domain (CAD) of HIF occurs through abrogation of hydroxylation of a conserved asparagine in the CAD. Inhibitors of Fe(II)- and 2-oxoglutarate–dependent dioxygenases prevented hydroxylation of the Asn, thus allowing the CAD to interact with the p300 transcription coactivator. Replacement of the conserved Asn by Ala resulted in constitutive p300 interaction and strong transcriptional activity. Full induction of HIF-1α and -2α, therefore, relies on the abrogation of both Pro and Asn hydroxylation, which during normoxia occur at the degradation and COOH-terminal transactivation domains, respectively.

The hypoxia-inducible factor HIF-1α is a ubiquitous bHLH/PAS (basic helix-loop-helix/Per-Arnt-Sim homology) transcription factor that plays a key role in the adaptation of cells to low-oxygen stress. HIF-1α targets the genes encoding erythropoietin, vascular endothelial growth factor, and an array of genes encoding glycolytic enzymes involved in anaerobic energy production (*1*). HIF-1α has a major function in vascularization of the embryo (*2*, *3*), tumor angiogenesis, and the pathophysiology of ischemic disease (*1*). A second, highly related hypoxia-inducible factor, variously termed endothelial PAS protein [EPAS1 (*4*)], HIF-like factor [HLF (*5*)], HIF-related factor [HRF (*6*)], or HIF-2α, is also essential for mouse development (*7*, *8*), although its exact function is not clear. Both HIF-1α and HIF-2α heterodimerize with a general bHLH/PAS partner protein, the aryl hydrocarbon receptor nuclear translocator (Arnt), to form DNA binding transcription factor complexes (*1*, *4*, *5*).

Two separate domains within HIF-1α and HIF-2α respond to hypoxia signaling pathways. The first is the oxygen-dependent degradation domain (ODD), which, at normoxia, is subject to posttranslational modification by an oxygen-dependent prolyl hydroxylase (*9*–*11*). The hydroxylated proline promotes interaction of HIF with the von Hippel–Lindau ubiquitin ligase complex, initiating rapid ubiquitination and subsequent HIF protein destruction via the proteasome. During hypoxia, the prolyl hydroxylase ceases to function, and the HIF proteins escape degradation. A number of prolyl hydroxylases capable of hydroxylating HIF have been cloned (*12*, *13*) and are members of the Fe(II)- and 2-oxoglutarate–dependent dioxygenase superfamily (*10*, *14*). A second hypoxia-sensing region, which functions as a hypoxia-inducible transactivation domain (termed the CAD, COOH-terminal activation domain) (*15*–*19*), lies within the COOH-terminal 100 amino acids of HIF-1α and HIF-2α. The final

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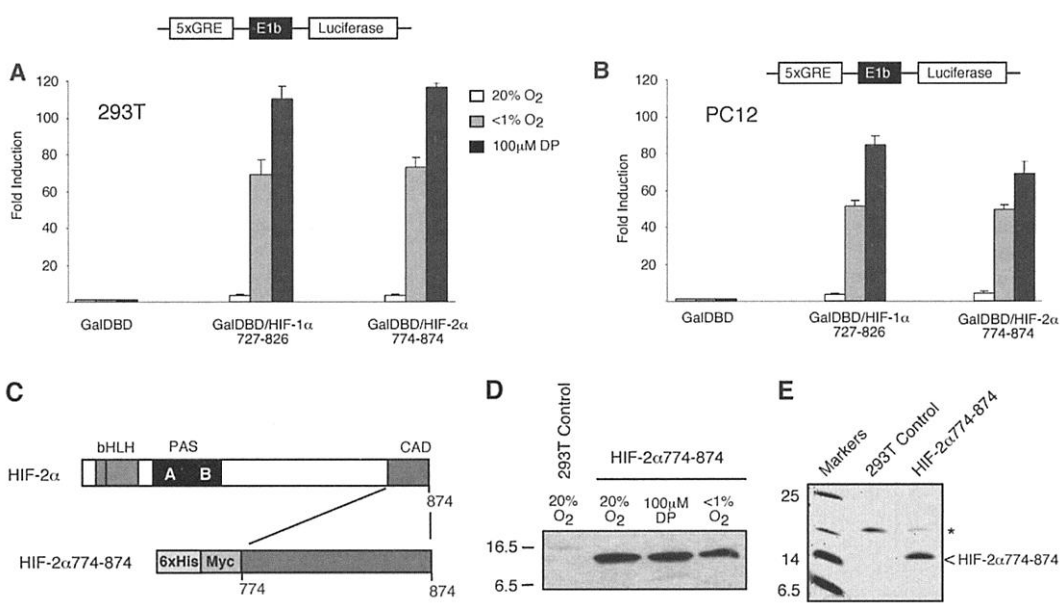

Fig. 1. Activities of the HIF-1α and HIF-2α CAD regions and expression and purification of the HIF-2α CAD from a stably transfected cell line. Expression vectors for either the Gal4 DNA binding domain or the indicated GalDBD/hHIF-1α and /mHIF-2α CAD chimeric proteins (from human and mouse, respectively). They were cotransfected with a Gal4 response element–driven luciferase reporter gene and an internal control renilla luciferase reporter gene. Transfected HEK 293T (**A**) or PC12 (**B**) cells were left untreated or subjected to hypoxia (*30*) or 2,2′-dipyridyl (DP) treatment for 16 hours, then luciferase activities were measured by the dual luciferase assay (Promega). Data are the average of three transfections ± SD. (**C**) Schematic showing domains of HIF-2α and the CAD region fused to a 6xHis tag–*myc* epitope that was used to generate a stable expressing cell line (*31*). (**D**) Protein expression of the HIF-2α CAD is not increased by hypoxia or DP treatment. Stable, transfected HEK 293T cells were incubated at normoxia, or subjected to hypoxia or DP for 3 hours, after which whole-cell extracts (10 μg) were separated by SDS-PAGE and analyzed by immunoblotting with a 9E10 monoclonal antibody against Myc. The 293T control lane contains extract from cells stably transfected with blank expression vector (*31*). (**E**) An SDS-PAGE gel stained with Sypro-Ruby (Bio-Rad) showing an example of HIF-2α CAD purified from stable transfected cells (*32*). Extracts from control 293T cells subjected to the same purification procedure lack the HIF-2α CAD band. The higher molecular weight contaminant (*) that is copurified by the procedure was identified as 60*S* ribosomal protein L27A by mass spectrometry.

COOH-terminal 50 amino acids of HIF-1α and HIF-2α show 60% sequence identity, whereas the preceding 50 amino acids are divergent. Experiments with chimeric proteins containing the CADs fused to the Gal4 DNA-binding domain (Gal4DBD) show that the CADs are silent during normoxia but are potently induced during hypoxia (Fig. 1, A and B) (*15–19*). The Fe(II) chelator 2,2′-dipyridyl (DP) also induces activity of the CADs. As noted by others (*15–19*), hypoxia or hypoxia mimetics do not influence CAD protein levels (Fig. 1D).

To explore the possibility that oxygen-dependent posttranslational modifications might regulate hypoxic activation of the CADs, we used mass spectrometry to analyze CAD domains expressed in normoxic or hypoxic cells. We engineered stable cell lines to express the COOH-terminal 100–amino acid fragment of HIF-2α (Fig. 1, C and D). This fragment was then purified to near homogeneity from normoxic cells (Fig. 1E) and from cells subjected to either hypoxia or DP treatment and subjected to tryptic digestion, followed by matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry (MS). One peptide from the normoxic sample, corresponding to the sequence 846-YDCEVNVPVPGSSTLLQGR-864 (*20*), produced an extra peak with a mass of 16 daltons above the predicted mass (Fig. 2A). The +16 increase indicates the presence of an additional oxygen atom on one of the amino acids. The fragment from DP-treated cells lacked the +16 mass increase, whereas the fragment from cells grown under hypoxic conditions showed nearly complete abrogation of the +16 peak (Fig. 2A). We therefore reasoned that one amino acid in the CAD was being oxidized during normoxia, producing a hydroxylated residue. This scenario is consistent with the fact that both modified and unmodified forms are present in our normoxic sample, which we believe to be a consequence of saturation of the hydroxylating enzyme by the high level of ectopic CAD expression. To identify the modified residue, we analyzed peptides 2090 and 2106 from normoxic cells by tandem mass spectrometry (MS/MS) sequencing, using nano-electrospray ionization in conjunction with quadrupole-quadrupole (Qq)-TOF MS (Fig. 2B) (*21*). Comparison of fragment ions produced from the 2090 and 2106 peptides revealed that it was asparagine (Asn^{851}) that was hydroxylated in peptide 2106 (compare ions y14 with y13 and b6 with b5). In contrast, the conserved Pro^{853} residue, which might be expected to undergo hydroxylation by a member of the oxygen-dependent prolyl hydroxylase family that modifies the ODD, was unchanged (Fig. 2B) (*21*). Corroborative data were obtained using MALDI-TOF post-source decay (PSD) analysis (*22*).

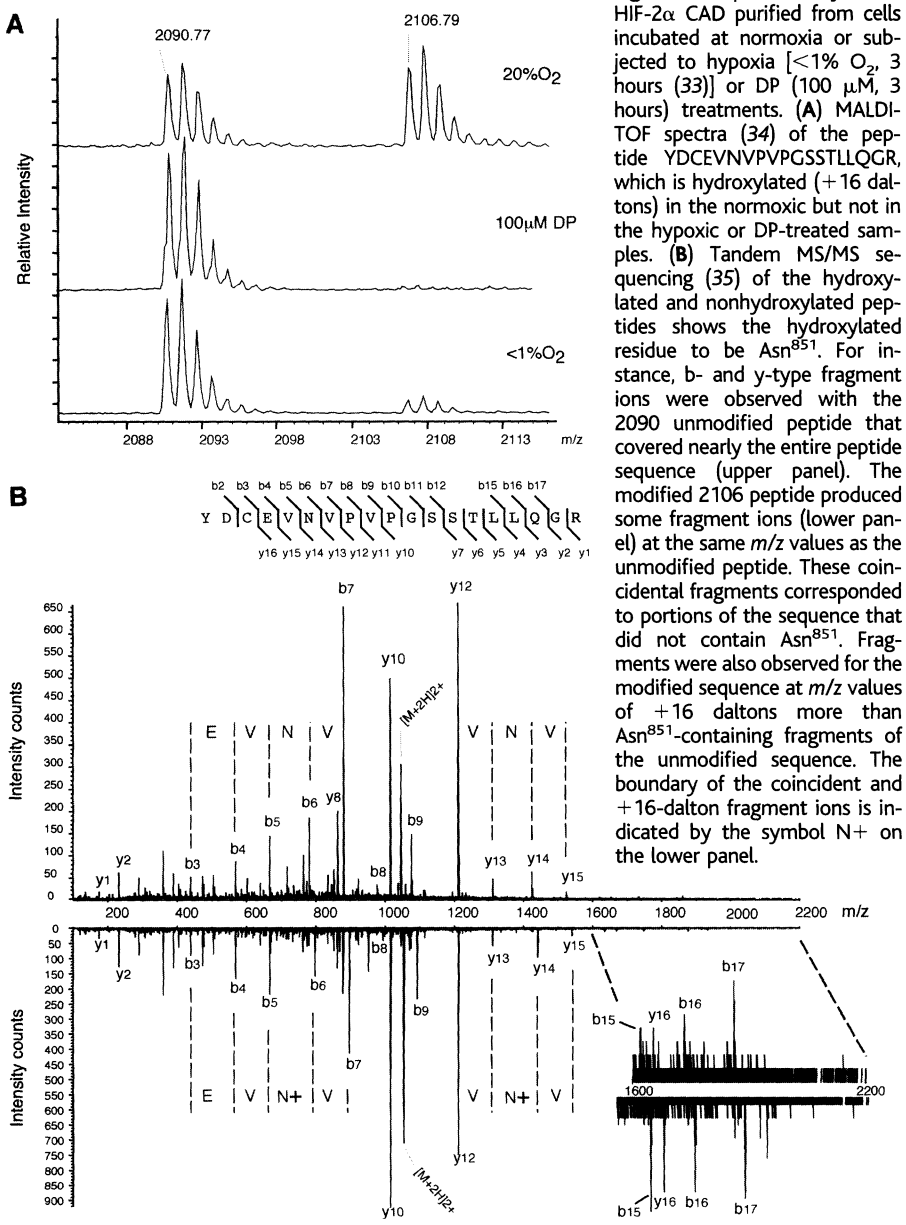

Fig. 2. Mass spectrometry of the HIF-2α CAD purified from cells incubated at normoxia or subjected to hypoxia [<1% O_2, 3 hours (*33*)] or DP (100 μM, 3 hours) treatments. (**A**) MALDI-TOF spectra (*34*) of the peptide YDCEVNVPVPGSSTLLQGR, which is hydroxylated (+16 daltons) in the normoxic but not in the hypoxic or DP-treated samples. (**B**) Tandem MS/MS sequencing (*35*) of the hydroxylated and nonhydroxylated peptides shows the hydroxylated residue to be Asn^{851}. For instance, b- and y-type fragment ions were observed with the 2090 unmodified peptide that covered nearly the entire peptide sequence (upper panel). The modified 2106 peptide produced some fragment ions (lower panel) at the same *m/z* values as the unmodified peptide. These coincidental fragments corresponded to portions of the sequence that did not contain Asn^{851}. Fragments were also observed for the modified sequence at *m/z* values of +16 daltons more than Asn^{851}-containing fragments of the unmodified sequence. The boundary of the coincident and +16-dalton fragment ions is indicated by the symbol N+ on the lower panel.

Asparagine 851 in HIF-2α is highly conserved across species, as is the equivalent Asn^{803} in HIF-1α (Fig. 3A), suggesting that failure to hydroxylate this residue may be the hypoxic switch that activates the CADs. An enzyme that hydroxylates Asp and Asn residues has been described (*23*), and like the HIF prolyl hydroxylases, it is a member of the Fe(II)- and 2-oxoglutarate–dependent superfamily of dioxygenases. If an asparaginyl hydroxylase in this family is responsible for silencing the CADs during normoxia, its activity in cells should be blocked by dimethyloxalylglycine (DMOG), a cell-permeable analog of 2-oxoglutarate that functions as a competitive inhibitor for 2-oxoglutarate–dependent dioxygenases (*10*). Treatment of cells with DMOG resulted in activation of the Gal4DBD/HIF-1α and Gal4DBD/HIF-2α CAD chimeras to an extent similar to that seen during treatment with hypoxia (Fig. 3B), confirming that the HIF CADs are subject to regulation by hydroxylation. Moreover, replacement of the critical Asn residues with Ala (N803A in HIF-1α and N851A in HIF-2α) conferred on the Gal4DBD chimeras full transcriptional activity in normoxia. The activity of these CAD chimeras with single–amino acid substitutions was not increased by treatment with hypoxia, DP, or DMOG (Fig. 3B). These experiments provide strong evidence that hydroxylation of the critical asparagines mediates silencing of the HIF-1α and HIF-2α transactivation domains. A more conservative substitution, replacement of Asn by Gln, conferred on the Gal4DBD/HIF-1α

and Gal4DBD/HIF-2α CAD chimeras weak transcriptional activity (about 4 times as great as that of the Gal4DBD/HIF CAD chimeras at normoxia) that was unaffected by hypoxia, DP, or DMOG (*22*). This implies a substrate-specificity for the hydroxylating enzyme and a size limitation for the amino acid at the Asn position. In contrast, and consistent with the notion that the CADs have a strict structural requirement, replacement of the conserved Pro within this region with Ala (P805A in HIF-1α and P853A in HIF-2α), resulted in complete loss of transcriptional activity under both normoxic and hypoxic conditions (*22*).

The HIF CADs function by recruiting transcriptional coactivator complexes to promoters of target genes. The evidence to date suggests that the central integrating coactivator p300/CBP directly interacts via its CH1 domain with the HIF-1α CAD (*19*, *24*) to induce transcription via recruitment of the accessory coactivators SRC-1, TIF-2, and the redox factor Ref-1 (*17*, *18*). Conversely, FIH-1 interacts with both VHL and the HIF-1α CAD to mediate transcription repression via recruitment of histone deacetylases (*25*). Our data raise the possibility that the Asn hydroxylation status of the CAD controls assembly of coactivator complexes. This model predicts that the CH1 domain of p300/CBP would not interact with CADs in normoxic cells but would interact with CADs in DP- or DMOG-treated cells, whereas the CADs with the Asn replaced by Ala would interact with CH1 regardless of treatment.

To test this hypothesis, we used recombinant glutathione *S*-transferase (GST)–CH1 in GST pull-down experiments with protein extracts from cells transiently transfected with constructs expressing HIF-1α CAD regions. In agreement with our prediction, minimal amounts of the wild-type CAD from untreated cells interacted with the CH1 domain, whereas a substantial portion of the CAD from cells treated with either DP or DMOG bound to the CH1 (Fig. 3C, compare lanes 2, 5, and 8). It is important that the CAD with the N803A substitution showed strong interaction with the CH1 domain from untreated cells, and the level of interaction was not changed by DP or DMOG treatments (compare lane 3 with lanes 6 and 9). We therefore conclude that during normoxia, Asn hydroxylation silences the COOH-terminal transactivation domains of HIF-1α and HIF-2α by preventing their interaction with p300/CBP. During hypoxia or in the presence of inhibitors of 2-oxoglutarate–dependent dioxygenases, the nonhydroxylated CAD is free to bind the CH1 domain of p300/CBP and to assemble transcriptional coactivator complexes. Binding of p300 to the CADs may also occur during experiments where expression levels of HIF proteins are high enough to titrate out Asn hydroxylating enzymes and to override the hydroxylation, thus providing transcriptional activities to the overexpressed proteins.

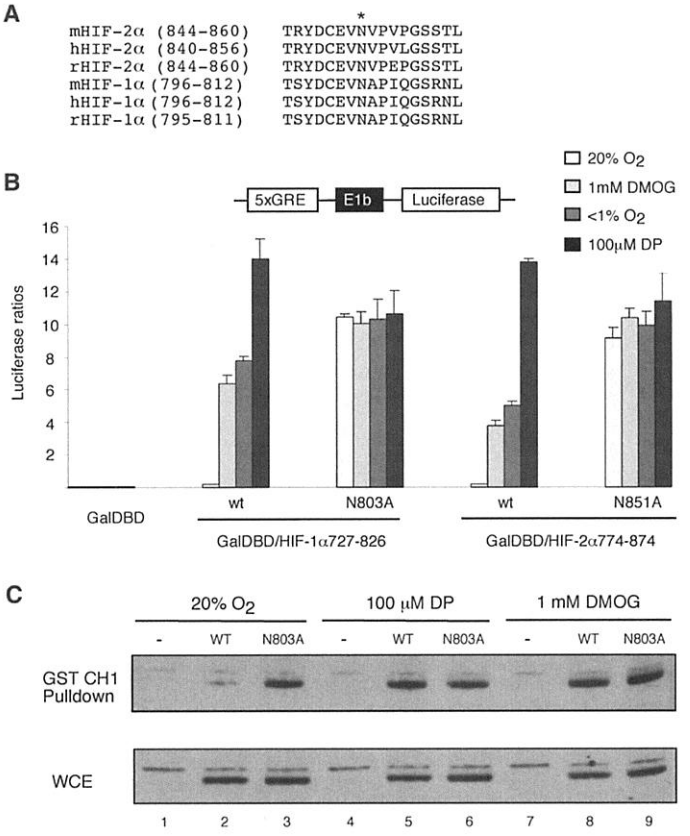

Fig. 3. Substitution of Asn confers strong, constitutive activity to the HIF-1α and HIF-2α CADs. (**A**) Sequence comparisons show conservation of the critical Asn between HIF-1α and HIF-2α across species. (**B**) Reporter gene assays showing constitutive activity of the mutants GalDBD/HIF-1α727–826 (N803A) and GalDBD/HIF-2α774–874 (N851A). HEK 293T cells were transfected according to the protocol of Fig. 1A, and the data represent the average of triplicate transfections ± SD. Expression of wild-type and mutant chimeras was similar during the transient transfection process (*21*). (**C**) The CH1 domain of p300 interacts with forms of the HIF-1α CAD lacking hydroxylation of Asn[803]. HEK 293T cells were cotransfected with expression vectors encoding Gal4DBD/HIF-1α 727 to 826 (wild type, WT) or the corresponding Asn to Ala substitution (N803A) as indicated. The transfected cells were left untreated or exposed to either 100 μM DP or 1 mM DMOG for 3 hours before lysis. Whole-cell extracts (WCE) were mixed with recombinant GST-p300CH1 (amino acids 300 to 528) in a pull-down assay (*36*). Aliquots of WCE (20% of input) and 50% of the GST-CH1 pull-down were separated by SDS-PAGE, and the CAD proteins were detected by immunoblotting with a polyclonal antibody directed against the HIF-1α COOH-terminus. Western blotting of whole-cell extracts shows that the wild-type and N803A CADs are expressed at equivalent levels in untreated and DP- and DMOG-treated cells (bottom panel). No interaction was observed between the GST tag and wild-type or mutant CAD fragments in control pull-down experiments (*22*).

In addition to the CADs, HIF-1α and HIF-2α contain a second transactivation domain that lies within the ODD. To compare the relative contributions of these domains to the overall transcriptional potency of full length HIF-1α and HIF-2α, we analyzed activities of a series of modified proteins with single–amino acid substitutions where either the critical Pro of the ODD or the critical Asn of the CAD, or both Pro and Asn in tandem, were replaced by Ala. Replacement of the Pro in the ODD domains of full-length HIF-1α and HIF-2α (HIF-1αP564A and HIF-2αP530A), provided stable proteins that exhibited low transcriptional activities at normoxia, but high transcriptional activities after treatment with DP (Fig. 4, A and B). Replacement of the critical Asn residues within the CADs had little effect on activities when compared with wild-type proteins, consistent with the proteins remaining unstable at normoxia despite their active CADs. In contrast to the single–amino acid substitutions, the double–amino acid substitutions HIF-1αP564A/N803A and HIF-2αP530A/N851A provided each protein with nearly full activity at normoxia. The higher normoxic potencies of the proteins with double–amino acid substitutions when compared with those of the single–amino acid substitutions in the ODD establish the critical nature of the CAD for transcriptional activation by HIF proteins. They also demonstrate that the mere abrogation of hydroxylation at the critical Pro and Asn residues is adequate for nearly full transcriptional activity of HIF-1α and HIF-2α. Western blotting established that the proteins having a single–amino acid substitution for Pro are expressed at levels similar to those of the proteins with both Pro and Asn replacements (*21*).

The described aspartyl/asparaginyl β-hydroxylase is thought to be anchored in the endoplasmic reticulum and modifies residues within a motif known as the EGF-like domain, which is contained in several proteins with extracellular functions (*23*,

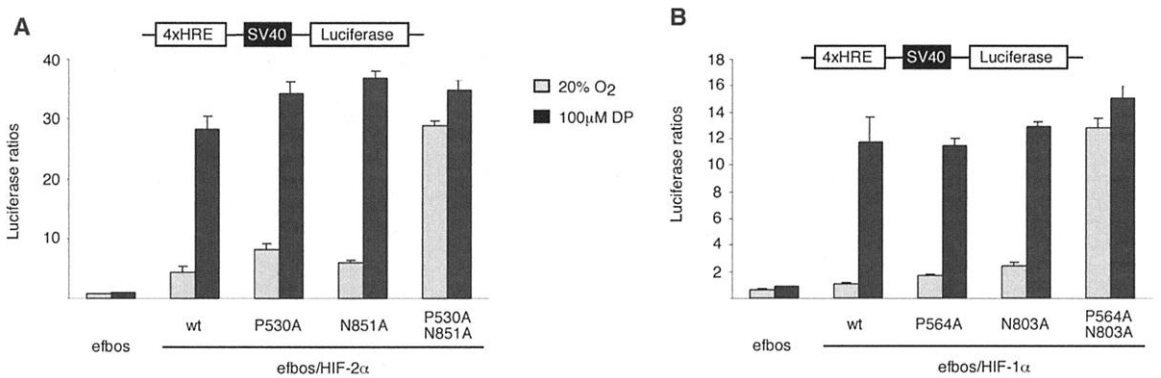

Fig. 4. HIF-1α and HIF-2α containing ODD and CAD single–amino acid substitutions exhibit nearly full transcriptional activities at normoxia. (**A**) Expression vectors containing wild-type HIF-2α, the single–amino acid substitutions P530A or N851A, or the dual substitutions P530A/N851A were cotransfected into Ka13.5 CHO cells with a hypoxia response element (HRE)–driven reporter gene and a renilla luciferase internal control reporter. Ka13.5 cells lack endogenous HIF-1α and HIF-2α and are therefore devoid of any background activity (*37*). Cells were left untreated or subjected to a 16-hour treatment with DP before being assayed with the dual luciferase assay system (Promega). Western blotting shows the Pro mutants are stable enough to be visualized during normoxia, but Asn mutants are not (*21*). (**B**) Similar to (A) with expression vectors containing wild-type HIF-1α, the single single–amino acid substitutions P564A or N803A, or the dual amino acid substitution P564A/N803A. Data are the average of triplicate transfections ± SD. Efbos (*38*) refers to background activity from empty expression vector.

26). As the HIF proteins are not membrane-bound, and consensus residues within the EGF-like domain bear no resemblance to any sequences within HIF-1α and HIF-2α, it is expected that the asparaginyl hydroxylase that modifies the CADs is likely to be a novel cytoplasmic or nuclear member of the Fe(II)- and 2-oxoglutarate–dependent family of dioxygenases.

Our results indicate that there are at least two major steps involved in the hypoxic induction of the HIF proteins: (i) inhibition of oxygen-dependent hydroxylation on Pro residues in the ODD to prevent interaction of HIF with the VHL ubiquitin ligase complex and thus avoid proteasomal destruction, and (ii) inhibition of oxygen-dependent hydroxylation of Asn in the CAD regions to promote interaction with the p300/CBP coactivator and induce transcription. The high constitutive activities of the proteins with both Pro and Asn substitutions confirm that the relevant prolyl and asparaginyl hydroxylases are attractive targets for therapeutic regulation of HIF-1α and HIF-2α.

References and Notes

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20. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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30. For exposure to hypoxia (<1% O_2) cells were placed inside an airtight chamber along with an AnaeroGen sachet (OXOID, Hampshire, UK), according to the manufacturer's instructions.
31. To generate the stable cell line, we subcloned cDNA encoding HisMycHIF-2α into the pEFIRES-puro vector (*27*) and transfected human embryonic kidney HEK 293T cells. Cells were pooled after 2 weeks of selection with 1 μg/ml puromycin. A control cell line was derived from the same selection process after transfection with the empty pEFIRES-puro vector.
32. Cells were lysed with binding buffer [100 mM Na-phosphate (pH 8.0), 8 M urea, 0.1% NP40, 0.15 M $NaCl_2$, 5 mM imidazole with protease and phosphatase inhibitors]. Clarified lysate was incubated with Ni-IDA agarose (Scientifix, Australia) and, after extensive washing, eluted with 100 mM Na-phosphate (pH 8.0), 8 M urea, and 200 mM imidazole. Eluted protein was loaded onto a butyl C4 high-performance liquid chromatography (HPLC) column (Brownlee, PerkinElmer) that had been equilibrated in 0.1% (v/v) trifluoroacetic acid (TFA). HIF-2α774-874 was then eluted with an increasing gradient of 80% (v/v) acetonitrile/0.1% (v/v) TFA.
33. An anaerobic workstation MkIII (Don Whitley Scientific, UK) was used to prepare hypoxic HIF-2α774–874 protein for MS analysis. Cells were washed and lysed inside the anaerobic workstation with buffers that had been deoxygenated overnight. After cell lysis, the purification was carried out at ambient O_2 conditions.
34. The HPLC fractions were reduced and alkylated with iodoacetamide before trypsin digestion. Theoretical products of tryptic digestion were generated by using the Bioanalyst component of Analyst QS software (Applied Biosystems). Digests were analyzed by using a Bruker Reflex MALDI-TOF-MS operated in the positive ion reflector mode, and data were acquired and analyzed with the Bruker XMass suite of software. Post-source decay (PSD) analysis was performed using the Bruker Reflex mass spectrometer (*28*).
35. Peptides were subjected to partial sequence analysis by MS/MS in the positive ion mode with a Sciex QSTAR-Pulsar Qq-TOF-MS under the control of Analyst QS software. Tryptic digests were sprayed from 60% (v/v) aqueous methanol containing 0.1% (v/v) formic acid. Diluted digests (~2 μl) were loaded into drawn capillaries coated with gold or platinum (Protana NanoES capillaries) and fitted onto a Protana NanoES electrospray ion source. Ions were sprayed with a potential of 850 V on the sample capillary. Collisionally activated decomposition of peptides was achieved by selecting doubly charged ions of interest, using Q1 at low resolution and manually varying the energy of collisions with nitrogen gas to achieve optimal spread of fragments across the desired TOF mass ranges (*29*). Identification of proteins was achieved by using MS/MS spectra to search the Mascot database (www.matrixscience.com) with mass error constraints of 0.1 and 0.05 daltons for parent ions and fragments ions, respectively.
36. The GST-p300CH1 domain (amino acids 300 to 528) fusion protein was expressed from pGEX-4T3/p300CH1 and purified from bacterial extract by glutathione agarose. A whole-cell extract (200 μg) from transfected cells was mixed with 2 μg of glutathione bound GST-CH1 in binding buffer [20 mM tris-HCl (pH 8.0), 150 mM NaCl, 0.1% NP40, 1 mM dithiothreitol, 2 μM EDTA, 20 μM $ZnCl_2$ and protease inhibitors] and incubated for 2 hours at 4°C with gentle rotation. The beads were pelleted, then washed three times with binding buffer, and bound proteins were eluted for SDS–polyacrylamide gel electrophoresis (SDS-PAGE) with 1× SDS sample buffer.
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