Identification of Residues Critical for Regulation of Protein Stability and the Transactivation Function of the Hypoxia-inducible Factor-1α by the von Hippel-Lindau Tumor Suppressor Gene Product

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Under normoxic conditions the hypoxia-inducible factor-1α (HIF-1α) protein is targeted for degradation by the von Hippel-Lindau (pVHL) tumor suppressor protein acting as an E3 ubiquitin ligase. Binding of pVHL to HIF-1α is dependent on hydroxylation of specific proline residues by O2-dependent prolyl 4-hydroxylases. Upon exposure to hypoxia the hydroxylase activity is inhibited, resulting in stabilization of HIF-1α protein levels and activation of transcription of target genes. One of the two critical proline residues, Pro563 in mouse HIF-1α, is located within a bifunctional domain, the N-terminal transactivation domain (N-TAD), which mediates both pVHL-dependent degradation at normoxia and transcriptional activation at hypoxia. Here we have identified two N-TAD residues, Tyr564 and Ile565, which, in addition to Pro563, were critical for pVHL-mediated degradation at normoxia. We have also identified D568A/D570A, F571A, and L573A as mutations of the N-TAD that abrogated binding to pVHL both in vitro and in vivo, and constitutively stabilized N-TAD against degradation. Moreover, the mutations Y564G, L556A/L558A, and F571A/L573A drastically reduced the transactivation function of either the isolated N-TAD or full-length HIF-1α in hypoxic cells. Interestingly, the F563A mutant exhibited a constitutively active and potent transactivation function that was enhanced by functional interaction with the transcriptional coactivator protein CBP.

In conclusion, we have identified by mutation analysis several residues that are critical for either one or both of the interdigitated and conditionally regulated degradation and transactivation functions of the N-TAD of HIF-1α.

Mammalian organisms are able to adapt to low oxygen levels by activating a network of genes encoding erythropoietin, vascular endothelial growth factor, and glycolytic enzymes (1). Hypoxia-dependent activation of transcription is mediated by the heterodimeric complex of the hypoxia-inducible factor-1α (HIF-1α) with the structurally related partner factor Arnt. In contrast to Arnt, HIF-1α protein expression is regulated in response to alterations in cellular oxygen levels. At normoxia, HIF-1α is degraded by the ubiquitin-proteosomal pathway (2–5), whereas upon hypoxic treatment the protein is stabilized, translocates to the nucleus, and activates transcription of target genes (6). HIF-1α contains two distinct transactivation domains that mediate hypoxia-dependent activation of transcription, the N-TAD (residues 531–584 and 532–585 in mHIF-1α and hHIF-1α, respectively) and the C-TAD (residues 772–822 of mouse HIF-1α corresponding to residues 776–826 in hHIF-1α) (7–11). The transactivation function mediated by the N- and C-TAD motifs has been shown to be enhanced by cofactors such as CBP, SRC-1, and Ref-1 (9, 10). However, the mechanism of regulation of these two TADs is quite distinct because N-TAD protein stability is strictly regulated by oxygen levels, whereas the C-TAD is constitutively stable (9, 12). C-TAD has been recently shown to be hydroxylated at Asn503 (of hHIF-1α) by a Fe(II)-2-oxoglutarate-dependent dioxygenase (13–15), and this modification has been proposed to inhibit the binding to the cysteine/histidine-rich domain 1 of CBP at normoxia. HIF-1α is targeted for normoxia-dependent ubiquitylation by the von Hippel-Lindau tumor suppressor gene product (pVHL) (16–20). The binding of pVHL to HIF-1α is regulated by hydroxylation of Pro402 and Pro464 which is mediated by members of the Fe(II)-2-oxoglutarate-dependent dioxygenase family of enzymes (21–26). The VHL tumor suppressor gene was first identified as the gene responsible for a rare inherited autosomal dominant cancer syndrome characterized by the development of clear cell renal carcinoma, hemangioblastoma, and pheochromocytoma (27). In addition, the VHL gene is also inactivated in sporadic clear cell renal carcinoma. VHL-negative neoplasms are characterized by being hypervascularized and by expressing constituatively hypoxia-inducible mRNAs such as vascular endothelial growth factor (28, 29). pVHL has been shown to harbor an E3 ubiquitin ligase activity in vitro (30, 31) and shows structural similarity to the SKP1-CUL-1-F-
box E3 ubiquitin ligase complex (32). HIF-1α and its paralogues HIF-2α and HIF-3α are so far the only known substrates recognized by the pVHL E3 ubiquitin ligase complex (19). The β domain of pVHL has been shown to interact with the N-TAD of HIF-1α, resulting in ubiquitylation and prolyl-oxidase-dependent degradation of HIF-1α at normoxia (17–20, 33). The crystal structure of a N-TAD peptide with the pVHL-ElonginB-ElonginC (pVHL-BC) complex has been solved by two independent groups (34, 35) that observed that the hydroxyproline is buried in a hydrophobic pocket of pVHL and has a central role in the complex formation. We have identified previously a central PYI motif in the N-TAD of pVHL and conditional degradation of the N-TAD. In the present work we have identified further residues within the N-TAD that are important for binding of pVHL. Amino acid mutations that inhibit interaction with pVHL, and subsequent degradation at normoxia include P563A, V564G, I565G, D568A/D569A/ D570A, F571A, and L573A. Moreover, reporter gene assays demonstrated that the P563A mutation generates a more potent and constitutively active transactivation domain, as compared with the wild type N-TAD. We have also shown that mutations that decrease N-TAD transactivation activity affect the activation of transcription mediated by the full-length HIF-1α.

MATERIALS AND METHODS

Plasmid Constructs—pFLAG-GAL4/mHIF-1α (531–584) and pFLAG-GAL4/mHIF-1α (546–574) were constructed by using PCR fragments generated with primer pairs carrying EcoRI and BamHI ends. The PCR fragments were inserted in-frame into EcoRI-BamHI-restricted pFLAG-GAL4 (36). pSP2-FLAG-GAL4/mHIF-1α (531–584) and pSP2-FLAG-GAL4/mHIF-1α (546–574) were generated by inserting SacI-SmaI fragments from the corresponding pFLAG-GAL4 construct into SacI-EcoRI-digested pSP272 (Promega). pRc/RSV-CBP-HA (expressing full-length mouse CBP) was obtained from R. H. Goodman (Vollum Institute, Portland, OR). pTS1/HRE-luc, GAL4-driven luciferase reporter gene plasmid, pCMX-VHL, pFLAG-mHIF1α, pFLAG-GAL4, and pFLAG-GAL4-mHIF1α (772–822) have been described previously (10, 17, 36). The inserts generated by PCR were completely sequenced using the Dynamic sequencing kit (Amersham Biosciences).

Amino acid mutations were introduced using the QuickChange site-directed mutagenesis kit (Stratagene) according to the instructions of the manufacturer, and positive mutants were screened by sequencing.

Cell Culture and Transient Transfection Experiments—Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F-12 medium in a 1:1 ratio containing 10% fetal calf serum, 50 IU/ml penicillin, and 50 mg/ml streptomycin sulfate. Medium and other products for cell culture were purchased from Invitrogen. HEK 293 cells were transfected using LipofectAMINE (Invitrogen) following the instructions of the manufacturer. Cells were cultured 24 h before transfection in 6-well plates, and cells incubated after transfection were cultured at normoxia (21% O2) or hypoxia (1% O2) for 16 h. We and others (17, 19, 23) have observed previously that pVHL to the N-TAD was increased also in the cells treated with MG132 under hypoxic conditions, resulting in ubiquitylation and proteosome-dependent degradation of HIF-1α, maintained wild type levels of pVHL binding activity, whereas P566E/M567E (lane 10) did not show any in vitro interaction with pVHL. Three additional N-TAD mutations, M560A/L561A (lane 5), D568A/D569A/D570A (lane 11), and F571A/L573A (lane 12) also completely abrogated binding of the N-TAD to pVHL.

pVHL Interacts with N-TAD under Conditions of Reoxygenation—We and others (17, 19, 23) have observed previously that pVHL is able to interact with hHIF-1α in assays using whole cell extracts from cells expressing both proteins (hereafter referred as in vivo interaction) under conditions where prolyl-oxidase-mediated degradation has been inhibited. This interaction is inhibited when cells are exposed to hypoxia or hypoxia-mimicking agents (21–23). In the present report we have examined if binding of pVHL to the N-TAD can be observed without the use of proteasome inhibitors. To this end we used experimental conditions where overexpression of N-TAD will saturate the endogenous degradation machinery (17) (Fig. 1C). HIF-1α and VHL expression plasmids, and the cells were exposed to normoxia (21% O2) or hypoxia (1% O2) for different times, as detailed in the figure legends. For reporter gene assays the cells were harvested 36 h after transfection, and the luciferase activity was determined. Total protein concentration was analyzed in whole cell extracts using the Bradford method (Bio-Rad).

Immunoprecipitation Assays—In vitro immunoprecipitation assays were performed with proteins translated in rabbit reticulocyte lysate (Promega) either in the presence or absence of [35S]methionine. Translated proteins were incubated for 1 h at room temperature in lysis buffer in a volume of 100 μl (17) and added to 20 μl of protein G-Sepharose (Amersham Biosciences) preincubated with anti-FLAG-M2 (Sigma) antibodies. After a further incubation for 1.5 h at room temperature under rotation, the Sepharose pellet was washed three times with lysis buffer, and the precipitated proteins were analyzed by SDS-PAGE followed by autoradiography.

In in vitro immunoprecipitation assays transfected cells were used to prepare whole cell extracts by sonication of cells for 3 s (performed twice) in lysis buffer (17). Whole cell extracts (800 μg to 1 mg of total protein) were incubated at 4 °C for 16 h with anti-FLAG-M2 antibodies bound to protein G-Sepharose as described previously (36). The Sepharose pellet was washed three times with TBS buffer (150 mM NaCl and 50 mM Tris-HCl, pH 4.0), and precipitated proteins were eluted from Sepharose by incubation under rotation with 0.5 mg/ml FLAG peptide (Sigma) in TBS for 1.5 h at room temperature.

Immunoblotting Assays—Immunoprecipitated proteins or 50 μg of whole cell extract protein were separated by SDS-PAGE and blotted onto nitrocellulose filters. Blocking was performed in TBS with 5% non-fat milk and was followed by incubation for 1 h at room temperature with anti-FLAG or anti-VHL (Pharmingen) antibodies diluted 1:500 and 1:250, respectively, in TBS with 5% non-fat milk, respectively. After several washes with TBS containing 0.5% Tween 20, the filters were incubated with 1:1000 dilutions of anti-mouse IgG-horseradish peroxidase conjugate (Amersham Biosciences) in TBS with 1% non-fat milk and washed several times with TBS containing 0.5% Tween 20. Proteins were visualized using enhanced chemiluminescence (Amersham Biosciences).

RESULTS

In Vitro Binding of pVHL to the N-TAD Degradation Box Depends on the Integrity of the PYI Motif and on Hydrophobic and Acidic Residues Surrounding the PYI Sequence—We have previously identified the N-TAD as an interaction interface with pVHL and have identified the PYI motif (residues 563–565 in mHIF-1α) as an essential motif for this interaction (17). In the present study we have generated point mutants of each single residue of the PYI motif and constructed double and triple mutants of the residues located N- or C-terminally of this motif (schematically represented in Fig. 1A). Interaction of N-TAD (FLAG-GAL4/mHIF-1α (531–584)) and mutants with pVHL was investigated using proteins in vitro translated in rabbit reticulocyte lysate. In these assays pVHL was [35S]methionine-labeled and precipitated by the FLAG-GAL4/mHIF-1α fusion proteins. As shown in Fig. 1B two of the N-TAD mutants, L556A/L558A (lane 4) and Q572A/R574A (lane 13), maintained wild type levels of pVHL binding activity, whereas P566E/M567E (lane 10) demonstrated reduced but detectable levels of pVHL binding activity. In contrast, all the point mutants of the PYI motif, P463A (lane 7), Y564G (lane 8), and I565G (lane 9) did not show any in vitro interaction with pVHL. Three additional N-TAD mutations, M560A/L561A (lane 5), D568A/D569A/D570A (lane 11), and F571A/L573A (lane 12) also completely abrogated binding of the N-TAD to pVHL.
Identification of mHIF-1α N-TAD residues critical for pVHL interaction in vitro and in vivo. A, schematic representation of the functional architecture of mHIF-1α and generated N-TAD mutants. The asterisks indicate mutated residues. B, in vitro interaction of N-TAD mutants with pVHL. pSP72-FLAG-GAL4-(531–584) (N-TAD) and mutants thereof were expressed using in vitro translation in rabbit reticulocyte lysate. Equal concentrations of in vitro translated proteins were incubated with in vitro translated 35S-labeled pVHL. Immunoprecipitation assays were performed using anti-FLAG antibodies (α-FLAG-IP) and analyzed by SDS-PAGE and autoradiography. C, analysis of the binding of pVHL to the N-TAD at normoxia (N) or under conditions of reoxygenation (R). HEK 293 cells were transfected with 800 ng of pFLAG-GAL4/mHIF-1α-(531–584) and 200 ng of pCMX-VHL. After transfection the cells were allowed to grow for 24 h and were exposed to normoxia or hypoxia for 12 h before reoxygenation. Cells exposed to hypoxia were harvested after different times of reoxygenation (1 min, R1; 10 min, R10; 60 min, R60). In indicated cases the cells were treated with 1 mM MG132 (MG) during the last 12 h of incubation. Immunoprecipitation assays were performed using the anti-FLAG antibodies (α-FLAG-IP), and precipitated proteins were detected by immunoblotting with anti-FLAG or anti-VHL antibodies (α-FLAG and α-VHL). Input material was analyzed by SDS-PAGE of 50 μg of whole cell extract protein and immunoblotting. D, in vitro interaction of wild type or mutant N-TAD proteins with pVHL. HEK 293 cells were transfected with pVHL expression plasmid, and pFLAG-GAL4/mHIF-1α-(531–584) containing the wild type sequence (N-TAD) or the indicated mutations, pFLAG-GAL4/mHIF-1α-(546–574), pFLAG-GAL4/mHIF-1α-(772–822), pFLAG-GAL4/mHIF-1α-(546–574). Transfections and immunoprecipitations were performed as described in the legend of C. The cells were exposed to normoxia (N) or hypoxia for 12 h and 10 min of reoxygenation (R) before harvesting. LL-A, L566A/L568A; ML-A, M559A/L570A; PYI-D, P563D/Y564D/I565G; QR-A, Q572A/R574A.

Identification of Critical Residues for in Vivo Interaction between N-TAD and pVHL. Binding of pVHL Depends on the PYI Motif and the Integrity of Residues Asp568–Asp569–Asp570 and Phe571–Leu573. In order to know if the mutants that disrupt the interaction between the N-TAD and pVHL in vitro are also critical for the binding in vivo, we performed immunoprecipitation assays using transfected cells expressing FLAG-GAL4-(531–584) mutants and pVHL. Abrogation of the interaction between the N-TAD and pVHL was observed in the case of the mutant P563D/Y564D/I565G (lanes 5 and 6) and the corresponding point mutants P563A (lanes 23 and 24) and Y564G (lanes 13 and 14) (Fig. 1D). Notably, the hydrophobicity of Tyr564 plays an essential role in the binding to pVHL. Mutation of tyrosine to glycine completely disrupted binding of pVHL (Fig. 1D). On the other hand, phosphorylation of Tyr564 does not seem to be relevant for pVHL interaction because the tyrosine residue could be replaced by phenylalanine without affecting the pVHL binding activity (data not shown). Mutation of residue Ile568 drastically reduced the interaction with pVHL but did not completely inhibit binding activity (lanes 15 and 16). Moreover, the two HIF-1α N-TAD mutants, D568A/D569A/D570A and F571A/L573A, which did not interact with pVHL, also failed to bind pVHL in vivo (Fig. 1D, lanes 9–12). In contrast to the in vitro results (Fig. 1B), mutation of residues Met566 and Leu567 did not inhibit pVHL binding in vivo (Fig. 1D, lanes 3 and 4), possibly because of the absence in reticulocyte lysate of appropriate modifying enzymes (i.e. prolyl 4-hydroxylases) required for binding of pVHL to these mutants. Interestingly, the mutant P566E/M567E demonstrated significantly stronger pVHL binding activity than the wild type N-TAD (Fig. 1D, lanes 7 and 8). Interaction of pVHL was also observed with a shorter protein fragment of the N-TAD spanning residues 546–574 (lanes 17 and 18), whereas no binding was observed with the C-TAD (Fig. 1D, lanes 19 and 20) that has been shown previously to be a constitutively stable but hypoxia-regulated transactivation domain of HIF-1α (9).

N-TAD Mutants That Fail to Bind pVHL Are Resistant to pVHL-mediated Protein Degradation. To evaluate the effect of different point mutations introduced into the N-TAD on the ability of pVHL to mediate protein degradation at normoxia (21% O2), we transiently expressed in HEK 293 cells pFLAG-
Fig. 2. Normoxia-dependent degradation of wild type or mutant forms of N-TAD by pVHL. A and B, degradation of mHIF-1α N-TAD mutants under normoxic conditions in the presence of increasing levels of pVHL. HEK 293 cells were transfected with pFLAG-GAL4/mHIF-1α-(531–584) encoding either the wild type (N-TAD) or point-mutated forms of the N-TAD motif, pFLAG-GAL4/mHIF-1α-(546–574) or pFLAG-GAL4/mHIF-1α-(772–822) (C-TAD) at concentrations (750 ng) allowing detection of the protein at normoxia. In indicated cases the cells were also transfected with increasing concentrations of pCMX-VHL (+, 500 ng; ++, 1000 ng). Following transfection the cells were incubated for 36 h before harvest. Whole cell extract proteins (25 μg) were analyzed by SDS-PAGE and immunoblotting using anti-FLAG or anti-VHL antibodies. 

Identification of Residues Important for pVHL Binding Located at the C Terminus of the PYI Motif—The mutants D568A/D569A/D570A and F571A/L573A failed to interact with pVHL in vitro and in vivo and were resistant to pVHL-mediated degradation at normoxia (Fig. 1, B and C, and Fig. 2A). Because these are triple and double mutants, we also generated single amino acid point mutants of all these residues in a shorter protein fragment of the NTAD spanning residues 546–574 (pFLAG-GAL4-mHIF-1α-(546–574)). The mutants D568A, D569A, and Q572A were able to interact with pVHL in an in vitro binding assay (Fig. 3A, lanes 5, 6, and 9, respectively). In the case of the mutant D568A (lane 5) we consistently observed weaker binding to pVHL. In contrast to these results, mutation of residues Asp570, Phe571, and Leu572 completely abrogate pVHL-N-TAD interaction (Fig. 3A, lanes 7, 8, and 10, respectively). In the in vivo pVHL-mediated degradation assay, the shorter fragment of the N-TAD spanning residues 546–574 and the mutant D570A were degraded by pVHL in a dose-dependent manner. The D570A was more resistant to pVHL-mediated degradation than the wild type. These results indicate the presence in HBEK 293 cells of enzymes (notably one or several prolyl 4-hydroxylases) that facilitate binding of pVHL in vitro. The other two mutants F571A and L573A, which failed to bind pVHL in vitro, were resistant to pVHL-mediated degradation.
even at the highest doses of pVHL tested. These data demonstrated that, in addition to the PYI motif, residues Phe at residue 571 and Leu at residue 573 were critical for the physical interaction with pVHL and pVHL-mediated degradation of the N-TAD.

The P563A Mutant of N-TAD Functions as a Constitutively Active Transactivation Domain—The HIF-1α N-TAD motif has been shown previously to function as a bifunctional domain both constituting a hypoxia-regulated degradation box as well as a hypoxia-dependent transactivation domain that can be potentiated by coactivators such as p300/CBP (7–10). We therefore investigated the effect of the different mutations within the N-TAD on the ability of this domain to mediate the hypoxia-dependent transactivation response. To this end we performed transient transfection experiments in HEK 293 cells using a GAL4-driven luciferase reporter gene and pFLAG-GAL4-mHIF-1α-(546–574) and mutants as described in Fig. 1. B, pVHL-dependent degradation at normoxia of mutants of the residue 546–574 fragment of the N-TAD. Transfections were performed with pFLAG-GAL4-mHIF-1α-(546–574) and mutants as described in Fig. 2, A and B.

The P563D Mutant of N-TAD Functions as a Constitutively Active Transactivation Domain—The HIF-1α N-TAD motif has been shown previously to function as a bifunctional domain both constituting a hypoxia-regulated degradation box as well as a hypoxia-dependent transactivation domain that can be potentiated by coactivators such as p300/CBP (7–10). We therefore investigated the effect of the different mutations within the N-TAD on the ability of this domain to mediate the hypoxia-dependent transactivation response. To this end we performed transient transfection experiments in HEK 293 cells using a GAL4-driven luciferase reporter gene and pFLAG-GAL4-mHIF-1α-(546–574) or different mutants of this motif. At normoxia the wild type N-TAD construct mediated about 2.4-fold activation of transcription when compared with the activity of the GAL4 DNA binding domain alone. However, in cells treated for 24 h with hypoxia 9-fold activation of transcription was observed over the values produced by the GAL4 DNA binding domain at normoxia, thus resulting in transactivation levels similar to those produced by the wild type HIF-1α N-TAD motif (2.7- and 9.2-fold activation over background values at normoxia and hypoxia, respectively). Also P566E/M567E mutant transactivates as efficiently as the wild type N-TAD (results not shown). The short protein fragment of the N-TAD spanning residues 546–576 was able to mediate a 2.6-fold hypoxia-dependent transactivation response, indicating a reduced ability to transactivate although it maintained all properties of conditionally regulated protein degradation observed with the larger N-TAD fragment. The transactivation results obtained with the Y564G and Y564F mutants are in excellent agreement with the pVHL binding experiments (Fig. 2B). Mutation of Tyr to phenylalanine affected neither pVHL binding nor the transactivation function of the N-TAD, suggesting that phosphorylation of the Tyr residue may not be required for interaction with pVHL or the ability to transactivate. On the other hand, hydrophobicity of this residue plays an important role in regulation of N-TAD function because the substitution of the tyrosine by glycine abrogated pVHL binding (Fig. 1) and generated a weak, con-

![Fig. 3. Identification of critical residues for pVHL interaction located in the C-terminal region of the N-TAD. A, in vitro binding of pVHL to residues 546–574 of wild type or point-mutated forms of N-TAD. Immunoprecipitations (IP) were performed with in vitro translated pSP72-FLAG-GAL4/mHIF-1α-(546–574) and mutants as described in Fig. 1. B, pVHL-dependent degradation at normoxia of mutants of the residue 546–574 fragment of the N-TAD. Transfections were performed with pFLAG-GAL4/mHIF-1α-(546–574) and mutants as described in Fig. 2, A and B.](Image 90x587 to 274x738)

![Fig. 4. Analysis of the transactivation function of N-TAD mutant proteins. A, mutation of residue Pro to alanine generates a constitutively active and potent transactivation domain. HEK 293 cells were transfected with 500 ng of a GAL4-responsive reporter gene plasmid. 20 ng of wild type N-TAD (pFLAG-GAL4/mHIF-1α-(531–584)), or mutant expression plasmids and carrier DNA pFLAG-CMV-2 to keep a constant DNA concentration of 1 μg. The cells were cultured for 12 h after transfection and exposed to 24 h of normoxia or hypoxia. Data are presented as luciferase activity relative to cells transfected with pFLAG-GAL4 alone at normoxia. Values represent the mean ± S.E. of three independent experiments performed in duplicate. LL-A, L556A/L558A; ML-A, M559A/L570A; PYI-D, P563D/Y564D/I565D; FL-A, F571A/L573A. B, CBP potentiates transactivation activity mediated by the N-TAD or the P563A mutant. HEK 293 cells were transfected as described in A in the absence or presence of 400 ng of CBP expression plasmid.](Image 362x392 to 518x737)
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Contritively active transactivation domain (Fig. 4). Interestingly, mutation of Pro563 to alanine also generated a constitutively active transactivation domain that was much more potent than the wild type N-TAD. N-TAD P563A was able to mediate 28- and 25-fold activation responses over the background values at normoxia or hypoxia, respectively (Fig. 4A). Although all the three mutants of the PYI motif abrogated pVHL-mediated degradation of the N-TAD, only the mutation of the proline to alanine generated a more potent, constitutively active transactivation function. We therefore conclude that the P563A mutation not only inhibited binding of pVHL and subsequent degradation of the N-TAD but also conferred a conformational change onto the N-TAD that improved the transactivation potency of this domain.

Activation of Transcription Mediated by P563A Is Enhanced by Coexpression of CBP and Correlates with the Ability of P563A to Bind CBP—The mutation of Pro563 to alanine generated a constitutive transactivation domain that was significantly more potent in activation of transcription than the wild type N-TAD in hypoxia. In addition, in contrast to the wild type N-TAD fragment, this mutant showed constitutive functional activity. We therefore investigated if the transactivation mediated by the N-TAD P563A mutant could still be potentiated by coexpression of CBP in a manner similar to that of the wild type N-TAD. As shown in Fig. 4B, transient cotransfection of CBP moderately (about 1.7-fold) potentiated transactivation mediated by the N-TAD both at normoxia and hypoxia. CBP enhanced to a similar degree transcription activation mediated by the P563A mutant of N-TAD, indicating that CBP is able to interact functionally with both the N-TAD and P563A mutant.

Mutations That Negatively Affect Activation of Transcription Mediated by the N-TAD Decrease Transactivation Mediated by Full-length HIF-1α—In addition to the N-TAD, HIF-1α contains a second hypoxia-responsive transactivation domain, the C-TAD. In our previous experiments (Fig. 4A), we identified mutations that affected the activation of transcription mediated by the N-TAD. We therefore examined if these mutations interfere with the transactivation function of the full-length HIF-1α. We inserted several of the relevant N-TAD mutations into the context of the full-length mHIF-1α (pFLAG-mHIF-1α). These mutants were tested in transactivation assays by transfected HEK 293 cells together with an HRE-driven luciferase reporter gene. We also monitored the expression levels of the different mHIF-1α mutants (Fig. 5B) using the same DNA preparations tested in the luciferase reporter assays. Activation of transactivation mediated by wild type HIF-1α produced 8.8- and 27.4-fold activation at normoxia and hypoxia, respectively, over the value of the expression of pFLAG at normoxia (Fig. 5A). Three of the tested mutants, mHIF-1α(P563A), mHIF-1α(I565G), and mHIF-1α(P402A/P563A), generated a transactivation response similar to that of the wild type protein, whereas the other mutants showed a significant reduction of the transactivation function both at normoxia and hypoxia (Fig. 5A). The mutant mHIF-1α(P563A) transactivated as efficiently as the wild type protein but was expressed at lower levels. It is possible that this mutation alone increased the transactivation efficiency of mHIF-1α. Mutation of both Pro562-Pro563 generated a mutant that showed elevated levels of expression and transactivated 2-fold more potently at normoxia than the wild type mHIF-1α but nevertheless was still responsive to hypoxia (2-fold hypoxia-dependent activation response). The results obtained with mHIF-1α(P563A) and mHIF-1α(P402A/P563A) are in contrast with a previous study using RCC4 cells stably transfected with pVHL (24) where mutation of Pro563 in hHIF-1α increased transactivation in normoxia and protein expression in non-treated cells. In our study the mutant mHIF-1α(P563A) is as well degraded as the wild type protein. The double mutant P402A/P564G in hHIF-1α is presented as a constitutive transactivator in this report (24), whereas in the present study this protein presents a higher transactivation in normoxia but is still responsive to hypoxia.

The three mutants that showed reduced transactivation activity are expressed at levels similar to the wild type protein. Mutation of residue Tyr564 to glycine generates a much weaker transactivation and degradation functions (Fig. 5A). The mutant mHIF-1α(P563A), HIF-1α(LL-A), HIF-1α(L556A/L558A), HIF-1α(PP-A), HIF-1α(P402A/P563A), and HIF-1α(P571A/L573A) demonstrate a transactivation capacity that was reduced at hypoxia to 40 and 50%, respectively, of the wild type levels. Given the fact that these mutants show wild type, hypoxia-regulated expression levels, these latter results show that some of the mutations that decreased the transcription potential of the N-TAD in GAL4 fusion protein experiments also decrease the total transactivation activity of full-length mHIF-1α.

DISCUSSION

In the present study we have characterized by mutation analysis the bifunctional N-TAD which mediates both transactivation and conditionally regulated protein degradation. We have identified P563A, Y564G, I565G, D568A/D569A/D570A, F571A, and L573A as mutations that interfere with the in vivo
HIF-1α Transactivation and Degradation Functions

binding of pVHL and pVHL-mediated degradation of the N-TAD at normoxia. Furthermore, we have identified the residues Met566-Leu568 and Asp569 as critical for in vitro binding of pVHL using proteins expressed in rabbit reticulocyte lysate. Binding of pVHL to the N-TAD has been shown to be dependent on the hydroxylation status of Pro564 (21–23). This modification is oxygen-dependent and is mediated by a number of recently identified prolyl 4-hydroxylases (25, 26). Two independently elucidated crystal structures of the pVHL-BC complex with a peptide spanning the PYI core of the N-TAD (34, 35) have recently demonstrated that hydroxyproline 563 of the N-TAD is the residue that establishes more contacts with pVHL in a site that is a hotspot for tumorigenic mutations. As expected (21–23), mutation of Pro564 to alanine completely disrupted binding of pVHL both in vitro and in vivo. In addition, our results demonstrated that the P563A mutation generated a constitutively active and more potent transactivation domain as compared with the wild type N-TAD. Thus, in contrast to the C-TAD (13), there is no requirement of any additional hypoxic signal in order for the constitutively stabilized N-TAD to transactivate. All the other N-TAD mutations that abrogate pVHL binding negatively affected the transactivation function mediated by the N-TAD demonstrating overlapping structural requirements for both pVHL binding and functional interaction with the transcription machinery, and establishing that only the P563A mutation conferred a conformation favorable for the transactivation function.

We have demonstrated previously (36) that inactivation of the C-TAD in the full-length HIF-1α by deletion or point mutation does not completely abrogate functional interaction with CBP, as assessed in gene reporter assays or assays monitoring nuclear colocalization of HIF-1α and CBP in accumulation foci. Furthermore, N-TAD-mediated activation of transcription has been shown to be potentiated by coactivators such as CBP/p300 (9, 10). In good agreement with this observation, our results showed that the transcription mediated by the constitutively active P563A mutant could be further enhanced by CBP indicating that this coactivator participates in this transcripational complex.

In our studies we have identified two mutants L556A/L558A and M560A/L561A that impaired transactivation mediated by N-TAD without affecting pVHL binding in vivo. The residue Leu695 in hHIF-1α (corresponding to Leu558 in mHIF-1α) has previously been mutated alone (37) or in the context of a double mutant together with a mutation of Asp555 (23) and shown not to affect the binding of pVHL. Moreover, the mutations L556A/L558A in the N-TAD did not affect pVHL binding in vitro or in vivo. However, in contrast to mutants such as Y564F and P566E/M567E that bind pVHL and transactivate as well as the wild type N-TAD, the L556A/L558A mutations impaired the transactivation activity of the N-TAD and significantly reduced the transactivation potency of full-length HIF-1α. Residue Leu695 in hHIF-1α (Leu561 in mHIF-1α) was also previously mutated in several reports with apparently contradictory results (22, 23, 37). In our assays mutation of M560A/L561A resulted in abrogation of the binding of pVHL in vitro but not in vivo. The use of rabbit reticulocyte lysate or cell extracts could explain the different results described in the previous reports (22, 23, 37). In the co-crystals of the N-TAD peptide and pVHL-BC complex, both Met661 and Leu662 have direct contacts with pVHL residues (34, 35). However, our results demonstrated that even when the two residues are mutated pVHL can bind and degrade N-TAD at normoxia. The major effect of the M561A/L562A mutation was observed in gene reporter assays where a drastic reduction in transactivation activity was measured, indicating a critical role of these residues for transcriptional activation.

In addition to the P563A mutation, two other point mutants of the PYI motif, Y564G and I565G, also proved to be critical for pVHL binding and pVHL-mediated degradation. In transactivation assays these two mutants generate a weak and constitutively active transactivation response. Interestingly, the introduction of the Y564G mutation into the full-length mHIF-1α resulted in a drastic reduction in transactivation potential, suggesting an important role of this residue in the N-TAD-mediated transactivation function. Previous studies using in vitro assays of the effect of the mutation Y565A in hHIF-1α (corresponding to Tyr565 in mHIF-1α) have yielded contradictory results with regard to the importance of this residue for binding of pVHL (21, 22). On the other hand, structural studies (34, 35) have shown that residue Tyr565 interacts with His110 of pVHL, and the integrity of this tyrosine has been suggested to be important for the hydroxylation of Pro564 by two of the prolyl 4-hydroxylases (26). The present results obtained with Y564G mutation demonstrated that the tyrosine residue is critical both for degradation function mediated by pVHL at normoxia and the transactivation function of the N-TAD. The residue Ile695 in hHIF-1α (corresponding to Ile660 in mHIF-1α) has been mutated previously to alanine and shown not to affect the interaction with pVHL (22, 23). However, following mutation of Ile660 to glycine, both in vitro and in vivo assays indicated that this residue is also critical for binding of pVHL. In excellent agreement with this data, one of the studies of the co-crystal of an N-TAD peptide and the pVHL-BC complex showed Ile565 to interact through hydrogen bonds with two residues, Pro661 and Ile660, of pVHL (34). Thus, Ile660 constitutes the residue in the vicinity of Pro564 that makes most contacts with pVHL.

We also mutated several residues located in the C terminus of the PYI motif, and we observed that mutants D568A/D569A/D570A, F571A, and L573A were critical for the in vitro interaction with the pVHL. The D568A/D569A/D570A triple mutant was resistant to pVHL-mediated degradation. However, in the subsequent analysis of single amino acid point mutants only the mutation D570A resulted in disruption of the binding in vitro, whereas it was still degraded in vivo at normoxia. This residue has been shown in the structural analysis to interact with the pVHL by van der Waals contacts (34). Phe672 in hHIF-1α (corresponding to Phe571 in mHIF-1α) contacts with Ile660 and Gly670 of pVHL, whereas Leu574 in hHIF-1α (Leu573 in mHIF-1α) contacts with Cys575 and Arg579 (34). Two of these residues Gly670 and Arg579 are frequent tumor-derived pVHL missense mutations (32). Thus, our data suggest that the integrity of both Phe571 and Leu573 is critical for interaction between the N-TAD and pVHL. In conclusion, we have identified several residues in the N-TAD that are important for degradation mediated by pVHL and the transactivation function of HIF-1α.

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