The Novel WD-repeat Protein Morg1 Acts as a Molecular Scaffold for Hypoxia-inducible Factor Prolyl Hydroxylase 3 (PHD3) *

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Abstract

Hypoxia-inducible factor-1 (HIF-1), a transcriptional complex composed of an oxygen-sensitive α- and a β-subunit, plays a pivotal role in cellular adaptation to low oxygen availability. Under normoxia, the α-subunit of HIF-1 is hydroxylated by a family of prolyl hydroxylases (PHDs) and consequently targeted for proteasomal degradation. Three different PHDs have been identified, but the difference among their in vivo roles remain unclear. PHD3 is strikingly expressed by hypoxia, displays high substrate specificity, and has been identified in other signaling pathways. PHD3 may therefore hydroxylate divergent substrates and/or connect divergent cellular responses with HIF. We identified a novel WD-repeat protein, recently designated Morg1 (MAPK organizer 1), by screening a cDNA library with yeast two-hybrid assays. The interaction between PHD3 and Morg1 was confirmed in vitro and in vivo. We found seven WD-repeat domains by cloning the full-length cDNA of Morg1. By confocal microscopy both proteins co-localize within the cytoplasm and the nucleus and display a similar tissue expression pattern in Northern blots. Binding occurs at a conserved region predicted to the top surface of one propeller blade. Finally, HIF-mediated reporter gene activity is decreased by Morg1 and reduced to basal levels when Morg1 is co-expressed with PHD3. Suppression of Morg1 or PHD3 by stealth RNA leads to a marked increase of HIF-1 activity. These results indicate that Morg1 specifically interacts with PHD3 most likely by acting as a molecular scaffold. This interaction may provide a molecular framework between HIF regulation and other signaling pathways.

Mammalian cells respond to reduced oxygen tensions by the expression of several dozens of genes, most of them are directly or indirectly induced by the transcriptional complex hypoxia-inducible factor (HIF)2 (1, 2). HIFs are heterodimeric basic helix-loop-helix proteins belonging to the family of PAS (PER-ARNT-SIM) domain transcription factors (3, 4). They consist of an α subunit of which three isoforms have been identified in human (HIF-1α, -2α, and -3α) and a β subunit (HIF-1β/ARNT) (5–7). Under normoxia, HIF-1α is continuously expressed in the cell but immediately degraded via the proteasomal pathway after ubiquitination (8). The von Hippel-Lindau protein acts as a particle recognition protein of the responsible E3 ubiquitin-ligase complex if two distinct prolyl residues within a region, referred to as the oxygen-dependent degradation domain of HIF-1α, Pro302 and/or Pro564, are hydroxylated (9–13). The site-specific hydroxylation of HIF prolyl residues is catalyzed by a conserved class of 2-oxoglutarate- and Fe(II)-dependent dioxygenases, designated prolyl hydroxylase domain-containing enzymes PHD1 (also named HPH3, EGLN2, and Falkor), PHD2 (HPH2 and EGLN1), and PHD3 (HPH1, EGLN3, and SM-20) (13–16). The Km value of the three PHDs for O2 is slightly above its atmospheric concentration, indicating that they are effective oxygen sensors and therefore represent a link between oxygen tension and HIF stability (17). PHD1, PHD2, and PHD3 share highly conserved COOH-terminal regions responsible for hydroxylase activity but differ greatly at the N terminus (16, 18, 19). Each isoform displays its own tissue and cell line-specific expression pattern as well as its particular subcellular distribution (15, 17, 20–25).

PHD3 is distinct in many ways because it can mediate diverse cellular outcomes depending on the cell type and the extracellular cues. In the hypoxic pathway it displays high substrate specificity because it exclusively hydroxylates Pro564, contributes to the regulation of HIF-2α and HIF-1α, and is strikingly induced by hypoxia so that it may serve as a feedback loop by limiting physiological activation of HIF in hypoxia (17, 20, 21, 26–29). Alternative splicing in various adult and fetal human tissues suggests a complex regulation, in particular because major transcripts encode catalytically inactive polypeptides on HIF-1α substrates. Interestingly, PHD3 is significantly shorter than either PHD1 or PHD2, and a possible additional binding partner required for full activity was discussed (30). Moreover, no rigid consensus sequence for hydroxylation of HIF-1α was found and a long peptide binding site with multiple interactions is likely (8, 17, 27). Finally, PHD3 was found as a growth factor responsive gene involved in growth regulation and/or apoptosis in smooth muscle, neuronal, and PC12 cells (23, 31–37).

These results suggest that additional cofactors, other prolyl hydroxylase substrates, and/or molecular scaffolds are required to allow multiple interactions. In the present work we identified Morg1, a WD-repeat protein, as a molecular scaffold that directly binds PHD3 in vitro and in vivo and may link PHD3 to different pathways. PHD3 and Morg1 perfectly co-localize in the cytoplasm of mammalian cells and display a similar tissue expression pattern in different tissues. Binding of Morg1 occurs within the β-sheet of strand b, involving residues predicted to the top of one WD-propeller blade. Finally, we show that Morg1 decreases HIF-mediated reporter gene activity and that this effect is additive by co-expression of PHD3. Vice versa, suppression of Morg1 or...
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PHD3 by stealth RNA results in a marked increase of HIF-1 activity. Morg1 may therefore serve as a scaffold for protein interactions and represent a link between PHD3 and diverse cellular outcomes.

EXPERIMENTAL PROCEDURES

All chemicals other than indicated were purchased from Sigma, restriction and modifying enzymes from New England Biolabs, the yeast two-hybrid system vectors, primers, yeast strain, yeast plasmid purification products, cDNA library, and co-immunoprecipitation products were from Clontech. Cell culture reagents were purchased from Invitrogen.

Yeast Two-hybrid Screening

pGBK7 was expressed as a fusion protein to a GAL4 DNA binding domain and a c-Myc epitope tag using tryptophan as selection marker. A rat brain cDNA library cloned into pACT2 was expressed as a fusion protein to the GAL4 activation domain and a HA epitope tag using leucine as selection marker. All yeast transformations were performed according to the high transformation efficiency protocol of yeast transformation system 2 (Clontech). Expression of PHD3 in yeast was confirmed by immunoblotting protein extracts using monoclonal antibodies against the c-Myc epitope. Protein extracts were obtained by the urea/SDS methods according to the yeast protocol handbook (Clontech). To check for autonomous activation, PHD3-transformed yeasts were plated onto SD-agar plates lacking tryptophan and containing X-α-galactosidase. To increase transformation efficiency both plasmids were sequentially transformed into yeast strain AH109. AH109 transformants were selected using three different reporters: adenine (ADE2), histidine (HIS3), and α-galactosidase (MEL1), under the control of distinct GAL4 upstream activating sequences and TATA boxes. Transformants were screened on SD-agar plates lacking adenine, histidine, leucine, tryptophan, and containing X-α-galactosidase (high stringency selection). SD-agar plates lacking tryptophan and leucine were used to calculate the cotransformation efficiency and the number of clones screened. To eliminate false positive clones yeast plasmids were isolated using a yeast plasmid isolation KIT (Clontech), transformed into Escherichia coli, purified, retransformed into the yeast strain expressing PHD3, and streaked out twice. Positive clones from this screening were subjected to direct sequencing. Deletion constructs were screened accordingly.

Plasmid Construction

Generation of full-length rat PHD3, Morg1, deletion constructs, and the 6X HIF responsive element (HRE) is described as supplemental data.

Northern Blot, RNA Extraction, 5’ RACE, and 3’ RACE

Northern Blots—RNA extraction and Northern blotting were performed as described (38). Morg1 and PHD3 cDNAs were labeled with [32P]dATP (3000 Ci/mmol; Amersham Biosciences) using random hexamer primers. Probes were hybridized overnight at 42 °C in rapid-hybridization buffer (Amersham Biosciences), washed several times in 2x SSC, 0.1% SDS and 0.1x SSC, 0.1 SDS and exposed to a Hyperfilm ECL (Amersham Biosciences). Morg1 cDNA was used as a probe to label a rat multiple tissue Northern blot (Clontech).

5’ RACE—Total rat brain and testis RNA were extracted using TRI Reagent and DNase I treated (DNA-free; Ambion, Austin, TX). First strand cDNA was synthesized using primer CCCTGTGTCTT-TATCTAGAA and Superscript II reverse transcriptase (Invitrogen). The original mRNA template was removed by treatment with RNase H/T1 (Invitrogen), and unincorporated dNTPs, primer, and proteins were separated from the cDNA by Qiaquick® PCR purification (Qiagen). A polymeric tail was then added to the 3’ end of the cDNA using terminal deoxynucleotidyl transferase (Invitrogen) and dCTP. One nested round of PCR was sufficient to reveal the full 5’ end (5’-GGCC-ACGGTCGTCACTAGTACGGGIIIGGGGIIG-3’ (Invitrogen) and 5’-GGTGATGCGCGTCCCGCAGC-3’). PCR products were cloned into pGEM T-easy and subjected to direct sequencing.

3’ RACE—First strand cDNA synthesis was catalyzed by SuperScript II reverse transcriptase using DNaI I-treated rat brain and testis RNA and adapter primer (5’-GGCCACCCGTTCGACTAGTACGTATTTTTTTTTTTTTTTTT-3’ (Invitrogen)). Residual mRNA was degraded by RNase H/T1 and target cDNA was amplified using primer AUAP (5’-GGCCACCCGTTCGACTAGTAC-3’ (Invitrogen) and 5’-GGTTCTA-TATGTTGTCAC-3’). PCR products were ligated into pGEM T-easy and subjected to direct sequencing.

Cell Culture, Transfection, and Immunoblotting

PC12 cells (European Collection of Cell Cultures) were maintained in RPMI1640 containing 10% fetal bovine serum, 1 mM minimal essential medium sodium pyruvate, 15 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin. HEK293 cells (Invitrogen) were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 units/ml penicillin, and 100 g/ml streptomycin. All transfections were performed with Superfect® (Qiagen) if not otherwise stated. To determine the ERK phosphorylation status, cells were transfected with either Morg1 and pCEP4, PHD3 and pCEP4 (to achieve equimolar concentrations), or PHD3 and Morg1, serum-starved overnight and stimulated with 10% serum for 10 min. Cells were lysed in 150 mM Tris-HCl, pH 6.8, 6.6% SDS, and denatured in loading buffer (5% glycerol, 0.03% bromphenol blue, 10 mM/liter dithiothreitol) at 95 °C for 5 min.

Yeast extracts were prepared according to the urea/SDS method following the yeast protocol handbook (Clontech). Immunoprecipitation products, yeast, or cell lysates were separated by 12% SDS-PAGE (NuPAGE® precast gel, Invitrogen), electrophoresed onto polyvinylidene difluoride filters (Amersham Biosciences) with an Invitrogen semi-dry blotting apparatus. Filters were blocked with 7.5% (w/v) dried milk in PBS, 0.1% Tween 20 (PBS-T), then incubated in rabbit polyclonal anti-α-tubulin, nonimmune rabbit serum, or monoclonal antibodies for 1 h at room temperature (dilutions: anti-Morg1 and anti-PHD3, 1:500 (36); anti-T7 (Novagen), 1:10000; anti-c-Myc (Clontech), 2 μg/ml; anti-HA (Roche), 1 μg/ml; anti-p44/42 MAP kinase and anti-phospho-p44/42 MAPK (Cell Signaling), 1:1000). After three washes in PBS-T, filters were incubated with a horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (BD Bioscience; 1:2000 in PBS-T, 1 h at room temperature) and washed three times in PBS-T. Signal detection was performed by chemiluminescence (Amersham Biosciences). Anti-phospho-p44/42 MAP kinase was blocked and incubated in Tris-buffered saline, 0.1% Tween with 5% bovine serum albumin, washed, and incubated with an AP conjugated anti-mouse antibody (Southern Biotech; 1:2500). Detection was performed by CDP-Star (Tropix).

In Vitro Transcription/Translation

pGADT7/-Morg1 (HA-epitope tagged) and pGBK7/PHD3 (c-Myc epitope-tagged), both containing T7 polymerase promoter sequences, were linearized and purified using Qiaquick PCR purification columns (Qiagen). pGBK7-53 (expressing c-Myc epitope-tagged p53) and pGADT7-T (expressing HA epitope-tagged large T-antigen) were used as controls. Each plasmid was mixed with rabbit reticulocyte lysate, reaction buffer containing 1 mM of all amino acids, except methionine,
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For co-localization experiments, PC12 and HEK293 cells were cultured in Lab-TekII® chamber slides (Nalge Nunc Co.) and transiently transfected with the chimeric pEGFP-PHD3 (gift of Robert S. Freeman) and Morg1-HA pCEP4. After 48 h PC12 cells were attached to Cell-Tak® (BD Biosciences)-treated chamber slides for 1 h. Fixation and immunostaining were performed as described (39). In brief, cells were washed with PBS and fixed with methanol for 10 min on ice. Immunostaining was performed at room temperature using the mouse monoclonal anti-HA antibody 12CA5 (Roche) and rabbit anti-EGFP antibodies (Molecular Probes) with nonimmune rabbit IgG as a control. Secondary antibodies were Texas Red anti-mouse IgG and fluorescein anti-rabbit IgG (DAKO). The subcellular distribution of fluorescent activity was examined by conventional fluorescence (Axioscope, Zeiss) and confocal laser scanning microscopy (LSM 510 META, Zeiss). For tryptic cleavage, HEK293 cells were transfected with Morg1-pCEP4-4 using Superfect® reagent. Cells were lysed in trypsin reaction buffer (50 mM Tris-HCl, 20 mM CaCl₂, pH 8.0) and incubated with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (New England Biolabs) for 10 min at 30 °C as described (40). The reaction was stopped by adding Laemmli sample buffer, immediately heated at 95 °C for 5 min, and analyzed by 12% SDS-PAGE.

Luciferase Assays

PHD3, Morg1, 6 × HRE, and a normalizing pCMV-β-galactosidase plasmid were transfected into HEK293 cells using Superfect® transfection reagent (Qiagen) according to the manufacturer’s protocol. An empty pCEP4 vector was transfected along with all transfections to achieve equimolar amounts of plasmids. The PGL3-promotor plasmid (Promega) was used as a control. After 8 h, 800 μl l-mimosine was added as indicated. 24 h post-transfection cells were washed with PBS, lysed (25 mM glycylglycin, pH 8, 15 mM MgSO₄, 4 mM EGTA, 1% Triton X-100, 1 mM dithiothreitol), and luciferase assays were performed as described (41, 42). All transfections were performed in triplicate for each construct, and all transfection sets were repeated at least three times. Transfection results were averaged, normalized with the β-galactosidase results, and expressed as the means.

Production of Anti-Morg1 Antisera and Enzyme-linked Immunosorbent Assay

Two polyclonal antisera to the synthetic peptide, CRSRKPEP-VQTLDEA of Morg1 (comprising amino acids 138–152), coupled to hemocyanin of limulus polyphemus were raised in New Zealand White rabbits. Specific IgGs were purified by affinity chromatography (Biotrend). Antibodies were characterized by immunoblotting and indirect enzyme-linked immunosorbent assay as described (43). In brief,
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FIGURE 2. Tissue expression and cloning of full-length Morg1. A, a rat multiple tissue Northern blot was hybridized with the Morg1 cDNA as a probe. Rat Morg1 cDNA comprises ~1200 bp. Expression is strong in testis and brain, moderate in heart, liver, and kidney, weak in spleen and lung, and absent in muscle. Rat brain and testis RNA was probed for the 5’ and 3’ sequence of Morg1. B, 5’ RACE yielded a 612-bp product that was highly homologous to the 5’ sequence of murine Morg1. C, 3’ RACE revealed a 274-bp product.

96-well plates were coated with 100 ng/well peptide in 0.1 M NaHCO₃, pH 8.2, overnight, washed with PBS-T, blocked with 5% goat serum in PBS-T, 2 h at room temperature, washed with PBS-T, and incubated with the appropriate concentration of sera (diluted in 5% goat serum) for 2 h at 37°C. After four additional wash steps plates were incubated with an alkaline phosphatase-labeled antibody (Southern Biotechnologies, dilution 1:2500, 1 h at room temperature). Detection was performed with p-nitrophenyl phosphate for 30 min at 37°C.

Stealth RNA Gene-silencing Assay and Real-time PCR Analyses

A stealth RNA targeting the 25-nucleotide sequence, ACUCUACUCUAGGCUCUAGAUAA of Morg1, the Morg1 control stealth RNA, ACUCUCAGAAGUUCGGAUCUCAA, a stealth RNA targeting the PHD3 gene, TGGAGCAACTGACGCTTGACDTGATTAT, and the corresponding PHD3 control RNA, TGGAGCAACTGACGCTTGACDTGATTAT, were obtained from Invitrogen. 100 nM stealth RNA was synthesized using 200 ng of RNA, random hexamers, and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Real-time PCRs were performed as described (44) using primers Morg1-F, GCCGATACAACGCTTGTGTGACG; Morg1-R, CCCAGAAGAACACTTTGCCG; PHD3-F, TGGGAGCAACTGACGCTTGACDTGATTAT; PHD3-R, CGATGTACCGTACAGGTCCGCATTC. Transfection efficiencies were optimized by fluorescence-activated cell sorter analysis using BLOCK-it Fluorescent Oligo (Invitrogen). For luciferase assays the indicated plasmids were transfected with Lipofectamine. A PHD3 vector revealed no intrinsic DNA binding or transcriptional activation properties, indicated by a lack of growth on high stringency agar (Fig. 1, G and H). Co-transfection efficiencies were tested by growth of AH109 yeast on agar lacking leucine (selection marker of the cDNA library) and tryptophan (selection marker of PHD3) (Fig. 1, B, D, F, and H). PHD3 was properly expressed by yeast (Fig. 1I) and showed no autonomous activation as indicated by white colonies on galactosidase agar lacking tryptophan.

Sequence Analysis, Identification of the 3’ and 5’ Ends of Morg1, and Tissue Expression—Sequence alignments revealed that the identified clone of 715 bp comprised four WD-repeat motives of Morg1 in contrast to seven repeats in mouse and human homologues. A rat multiple tissue Northern blot was next hybridized with the cDNA as a probe revealing that rat Morg1 consists of an ~1200 bp cDNA. Expression was strong in testis and brain, moderate in heart, liver, and kidney, weak in spleen and lung, and absent in muscle (Fig. 2A). Rat brain and testis RNA was probed for the 5’ sequence of rat Morg1. 5’ RACE yielded a 612-bp product that was highly homologous to the 5’ sequence of murine Morg1 (Fig. 2B). 3’ RACE using brain and testis RNA as templates revealed a 274-bp product (Fig. 2C). Taken together, rat Morg1 comprises a cDNA of 1137 bp (Fig. 3). The full-length rat clone was constructed by ligating a 5’ PCR product to the 3’ end of rat Morg1. Full-length rat Morg1 is 92/87% identical at the nucleotide level and 97/93% identical at the amino acid level to murine Morg1 and human MORG1, respectively. The ATG (nucleotides 37–39) is likely to be the start site for translation for several reasons: (a) the presence of three TAA upstream terminator codons, (b) the first ATG codon lies in a strong Kozak context (A⁻³ and G⁻¹¹), and (c) translation usually initiates uniquely at the first ATG codon in an adequate context. The second ATG (nucleotides 580–583) (with an equally good context) unlikely serves as the initiation site simply because it is internal and thus inaccessible to the scanning 40 S ribosomal subunit, which advances from the 5’ end (46). Therefore, the rat Morg1 cDNA predicts a protein of 316 amino acids and a molecular mass of 34,380 daltons (GenBank accession number AY940050). Seven WD-repeat domains span the entire length of the sequence with neither a putative signal peptide nor a predicted mitochondrial targeting signal (Fig. 3).

PHD3 and Morg1 Co-localize in the Cytoplasm—cDNAs of PHD3 and Morg1 were next utilized to probe RNAs of different cell lines to identify cells expressing both proteins. Northern blots revealed a strong expression of PHD3 and Morg1 in PC12 cells and a weak expression of

3 U. Hopfer and G. Wolf, unpublished observation.
FIGURE 3. Rat Morg1 comprises a cDNA of 1137 bp. The ATG (nucleotides 37–39) is likely to be the start site for translation. Double underlined, strong Kozak context (A^−1 and G^+3). Rat Morg1 cDNA predicts a protein of 316 amino acids and a molecular mass of 34,380 daltons (GenBank accession number AY940050). Seven WD-repeat domains (underlined) span the entire length of the sequence. Bold, peptide used for generation of anti-sera.
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Both proteins in HEK293 cells (Fig. 4A). Therefore, PC12 and HEK293 cells were used for in vivo interaction assays. Additionally, HEK293 cells were chosen because preparations of PHD3 in HEK293 cells were proven to be catalytically active (47).

To examine the subcellular distribution of PHD3 and Morg1, HEK293 and PC12 cells were transiently transfected with HA-tagged Morg1 and a PHD3-GFP fusion protein, stained with anti-HA/anti-mouse Texas Red and anti-GFP/anti-rabbit fluorescein antibodies, and examined by confocal microscopy. PHD3 occurred in a punctuate pattern throughout the cytoplasm and within the nucleus of PC12 cells. Immunostaining of Morg1 showed a punctuate staining pattern predominantly within the cytoplasm and to a lesser extend within the nucleus of PC12 cells. In double staining experiments Morg1 co-localized with PHD3. In HEK293 cells both PHD3 and Morg1 localized primarily in the cytoplasm and less in the nucleus. Morg1 immunofluorescence co-localized perfectly with PHD3. C, Morg1 expressed in HEK293 cells is resistant to trypsin cleavage. Despite 32 potential trypsin cleavage sites Morg1 showed resistance to proteolysis.

To explore whether the interaction between PHD3 and Morg1 detected in yeast and in vitro translated products occurs in intact mammalian cells, immunoprecipitation experiments were performed in HEK293 cell lysates co-transfected with PHD3 and Morg1. Immunoprecipitation of either anti-Morg1 (Fig. 6, A and B), anti-PHD3 (Fig. 6C), or anti-T7 (protein tag of PHD3, Fig. 6D) led to recovery of both proteins in HEK293 cells, supporting an in vivo interaction between PHD3 and Morg1. Immunoprecipitations of co-transfected cell lysates with either preimmune rabbit serum or Protein G plus-agarose did not lead to recovery of either protein. Specific Morg1 antiserum used in immunoprecipitation experiments were tested by immunoblotting lysates from HEK293 cells transfected with full-length Morg1 cDNA. Both antiseras recognized Morg1 at a mobility consistent with the predicted molecular mass of 34 kDa (Fig. 6, E and F). Immunoblotting of lysates using an anti-HA antibody served as control. An indirect enzyme-linked immunosorbent assay against the immunogen was used to estimate the sensitivity of both antibodies and revealed specific binding of the crude sera and purified IgGs above the 128-fold dilution and no binding of pre-immune sera (Fig. 6G).
FIGURE 6. In vivo interaction of Morg1 and PHD3. Immunoprecipitation (IP) experiments were performed in HEK293 cell lysates co-transfected with PHD3 and Morg1. Immunoprecipitation of anti-Morg1 (A and B), anti-PHD3 (C), or anti-T7 (protein-tag of PHD3) (D) led to recovery of both proteins in HEK293 cells. Immunoprecipitations of co-transfected cell lysates with either preimmune rabbit serum or Protein G plus-agarose did not lead to recovery of either protein. P, Protein G plus-agarose; M, anti-Morg1; S, anti-PHD3; T7, anti-T7.

Morg1 antisera were tested by immunoblotting lysates from HEK293 cells transfected with full-length Morg1 cDNA. Both antisera (E1 and F1) recognized Morg1 at a mobility consistent with the predicted molecular mass of 34 kDa. Immunoblotting of crude lysates served as control (F2 and G2). G, enzyme-linked immunosorbent assay against the immunogen was used to estimate the sensitivity of both antibodies and revealed specific binding of crude sera and purified IgGs above dilutions of 1:128.
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**Characterization of the Binding Region of Morg1**—The Morg1/PHD3 interaction was explored further using truncated Morg1 and full-length PHD3 constructs in yeast two-hybrid assays. PHD3 and all truncated constructs were sufficiently transfected in yeast as represented by growth on agar plates lacking leucine and tryptophan (AA–LL). Strong interaction was accomplished by expression of all seven WD-repeats (Fig. 7A). Truncation of WD5 to WD7 did not change the interaction (Fig. 7B–E). However, yeast failed to grow by truncation of Δaa 167–318 (Fig. 7F), indicating that this variant was not able to continue to interact efficiently with PHD3. Truncations of Δaa 172–318 (G), Δaa 177–318 (H), Δaa 181–318 (I), or Δaa 1–128 (J) led to growth of yeast, suggesting that the interaction at least requires aa 166 and 167. Partial (K) or complete deletion (L) of the conserved jelly roll core of PHD3 did not hamper the interaction, suggesting binding within the unique NH2-terminal tail.

Morg1 Decreases HIF-mediated Reporter Gene Activity—PHD3 was reported to suppress HRE-mediated reporter gene activity when over-expressed in cells (8, 28). To test whether Morg1 influences HIF-mediated reporter gene expression we fused six copies of the HRE of the EPO gene to a SV40 promotor and a luciferase gene. Transfection of this construct into PC12 (Fig. 8A) or HEK293 cells (Fig. 8B) significantly induced luciferase activity compared with the empty SV40 promotor/luciferase gene (n = 6, p < 0.05) indicating sufficient HIF activity in these cells. Inhibition of PHDs by L-mimosine as described (49) super-induced the reporter gene activity in both cell lines (p < 0.01). In conformity to a high PHD3 expression (Fig. 4) this further implicates PHD3 activity in these cells. As reported previously, overexpression of PHD3 decreased HRE-mediated luciferase activity. Luciferase activity was also decreased when Morg1 was co-expressed with the reporter gene, suggesting an interaction of internal PHD3 and Morg1. Co-expression of PHD3 and Morg1 reduced reporter gene response to almost basal levels (p < 0.05). These results suggest that Morg1 promotes PHD3 action on HIF-mediated reporter gene activity.

To assess whether Morg1 indeed affects HIF-mediated reporter gene activity and to show that PHD3 is truly active under this condition we knocked down both mRNAs with stealth RNA oligonucleotides. We either targeted mock plasmid-transfected PC12 cells (for endogenous protein) or Morg1- or PHD3-transfected PC12 cells (for exogenous protein) with stealth RNA oligonucleotides and determined the amount of Morg1 and PHD3 mRNAs by quantitative real-time PCR. Morg1 was silenced by
Regulated protein degradation is a key event in many cellular processes. Ubiquitin-dependent degradation uses covalent attachment of a polyubiquitin chain on lysine residues of the substrate, which mediates its recognition and subsequent degradation by the 26 S proteasome.

The activity of the transcriptional complex of HIF-1α is regulated by oxygen-dependent post-translational modifications that are mediated by specific 2-oxoglutarate and Fe(II)-dependent dioxygenases (PHDs). PHDs hydroxylate two conserved prolines of HIF-1α within its oxygen-dependent degradation, which leads to capture by its corresponding E3 ubiquitin-ligase complex (9–13, 50–55). Interestingly, PHD3 also affects growth control and growth factor-dependent cell survival, but little functional data are available (31–34, 36).

How does PHD3 act in these divergent pathways? Substrates other than HIF-1α for hydroxylation in various pathways may be involved, however, a WD-repeat protein as a molecular scaffold for different interaction partners or other substrates provide a good explanation. We identified Morg1 as an interaction partner of PHD3 by yeast two-hybrid assays and described seven WD-repeat domains by cloning the full-length cDNA. Morg1 displays in part a similar tissue expression pattern to PHD3 that is expressed in brain, heart, skeletal muscle, kidney, liver, spleen, and lung (56).

The defining feature of proteins in the WD-repeat family is the presence of 4–8 repeating units containing a conserved core of 27–45 amino acids that are bracketed by two characteristic dipeptide sequences, GH and WD, but neither the GH nor the WD dipeptide is absolutely conserved (57–59). The crystal structure of one WD-repeat protein, the signal transducing G protein β subunit of heterotrimeric G proteins, shows that the repeating units form a circular, propeller-like structure with each blade made up of four β strands (60, 61). If there is a common functional theme, it appears to be that the WD-repeat propeller structures create a stable platform that can reversibly form complexes with several proteins, thus coordinating sequential and/or simultaneous interactions involving several sets of proteins. Gβ, for example, interacts tightly with Gγ and, simultaneously, interacts with one of >15 different proteins (59, 62). Given that Morg1 possesses the three-dimensional structure seen with other members of the WD40 protein family, it is likely that PHD3 interacts with multiple partners. It was suggested that, as with non-WD-repeat propellers, the top surface of WD-repeat proteins, including the central tunnel opening, coordinates interaction with other proteins and/or small ligands (60, 61, 63, 64). We were able to define short sequences within Morg1, which were sufficient for recognition by PHD3. Interestingly, these sequences correspond to a region of striking conservation and localize to the top surface of the protein near the center of the top tunnel opening. WD-repeat β-propeller domains may be fused to domains that are predicted to have enzymatic activity, e.g. kinase activity (65, 66), but no catalytic activity has been attributed to the propeller portion of a WD-repeat protein (58). As in Gβ the repeat of Morg1 spans the entire length of the sequence, to this end catalytic activities seem unlikely.

PHD1, -2, and -3 share a conserved COOH-terminal region responsible for hydroxylase activity but differ greatly at the NH2 terminus (18). Interestingly, deletion of the conserved COOH terminus of PHD3 did not affect its ability to hydroxylate HIF-1α but instead enhanced HIF-1α stability (19). Morg1 Interacts with PHD3

FIGURE 8. HIF-mediated reporter gene activity is decreased by Morg1. Transfection of a 6 × HRE (HIF-responsive element) reporter construct into PC12 (A) or HEK293 cells (B) significantly induced luciferase activity (n = 6, p < 0.05) compared with the empty SV40 promoter/luciferase gene (designated 100%), indicating sufficient HIF activity in these cells. Inhibition of PHDs by l-mimosine (L-Mim) superinduced reporter gene activity in both cell lines (p < 0.01), which implicates PHD activity in these cells. Expression of PHD3 decreased HRE-mediated luciferase activity. Luciferase activity was also decreased when Morg1 was co-expressed with the reporter gene, suggesting an interaction of internal PHD3 and Morg1. Co-expression of PHD3 and Morg1 reduced reporter gene response to almost basal levels (p < 0.05). C, knock-down of endogenous Morg1 noticeably induced reporter gene activity in PC12 cells. Luciferase activity was also superinduced by silencing exogenous Morg1 alone or co-expressed with PHD3. D, by silencing either endogenous PHD3, exogenous PHD3 or PHD3 cotransfected with Morg1, luciferase activity increased markedly in PC12 cells. E, to determine the impact of PHD3 upon ERK phosphorylation, cells were transfected with Morg1 (lanes 1 and 2), PHD3 (lanes 3 and 4), PHD3 and Morg1 (lanes 5 and 6), mock plasmid (lanes 7 and 8), or left untreated (lanes 9 and 10). Cells were stimulated with 10% serum (lanes 1, 3, 5, 7, and 9). Western blots probed with anti-ppERK1/2 showed that overexpression of neither PHD3 nor PHD3 and Morg1 notably changed the status of pERK1/2 phosphorylation when compared with mock transfected cells. Anti-pERK1/2 was used as loading control. To control for transfection efficiency a second blot was probed against anti-T7-PHD3, anti-HA-Morg1, and β-actin.

~85% in mock-transfected cells and ~90% in cells overexpressing Morg1. A control stealth RNA did not change Morg1 gene expression (expression ±10%). PHD3 was silenced by ~50% in mock and ~60% in PHD3-transfected cells. No significant difference was detected by transfection of the control stealth RNA (expression ±10%).

Knock-down of endogenous Morg1 noticeably induced reporter gene activity in PC12 cells (Fig. 8C). Luciferase activity was also superinduced by silencing exogenous Morg1 alone or co-expressed with PHD3. By silencing endogenous PHD3, exogenous PHD3, or PHD3 cotransfected with Morg1, luciferase activity increased markedly (Fig. 8D).

Overexpression of PHD3 Has No Impact on the Status of pERK1 or pERK2 in HEK293 Cells—Morg1 facilitates ERK activation after stimulation with fetal bovine serum (45). To determine the impact of PHD3 upon ERK phosphorylation, 293 cells were transfected with Morg1, PHD3, or both and stimulated with 10% fetal bovine serum. Western
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