ERYTHROCYTOSIS-ASSOCIATED HIF-2α MUTATIONS DEMONSTRATE A CRITICAL ROLE FOR RESIDUES C-TERMINAL TO THE HYDROXYLACCEP TOR PROLINE*

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Running Title: Functional Characterization of Erythrocytosis-Associated HIF-2α Mutations

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A classic physiologic response to hypoxia in humans is the upregulation of the ERYTHROPOIETIN (EPO) gene, which is the central regulator of red blood cell mass. The EPO gene, in turn, is activated by Hypoxia Inducible Factor (HIF). HIF is a transcription factor consisting of an α subunit (HIF-α) and a β subunit (HIF-β). Under normoxic conditions, Prolyl Hydroxylase Domain protein (PHD, also known as HIF Prolyl Hydroxylase and Egg Laying Defective Nine protein) site-specifically hydroxylates HIF-α in a conserved LXXLAP motif (where underlining indicates the hydroxylacceptor proline). This provides a recognition motif for the von Hippel Lindau (VHL) protein, a component of an E3 ubiquitin ligase complex that targets hydroxylated HIF-α for degradation. Under hypoxic conditions, this inherently oxygen-dependent modification is arrested, thereby stabilizing HIF-α and allowing it to activate the EPO gene. We previously identified and characterized an erythrocytosis-associated HIF2A mutation, G537W. More recently, we reported two additional erythrocytosis-associated HIF2A mutations, G537R and M535V. Here, we describe the functional characterization of these two mutants as well as a third novel erythrocytosis-associated mutation, P534L. These mutations affect residues C-terminal to the LXXLAP motif. We find that all result in impaired degradation and thus aberrant stabilization of HIF-2α. However, each exhibits a distinct profile with respect to their effects on PHD2 binding and VHL interaction. These findings reinforce the importance of HIF-2α in human EPO regulation, demonstrate heterogeneity of functional defects arising from these mutations, and point to a critical role for residues C-terminal to the LXXLAP motif in HIF-α.

Gene transcriptional responses to hypoxia in mammals are in large part mediated by HIF (1). While the core mechanism—that is, PHD-induced hydroxylation of HIF-α leading to VHL-mediated degradation—is relatively simple, an additional layer of complexity is provided by the multiplicity of isoforms at different levels of this pathway (2,3). Thus, there are three HIF-α isoforms (HIF-1α, HIF-2α, and HIF-3α) and three PHD isoforms (PHD1, PHD2, and PHD3) (1-4). The role of these isoforms in oxygen sensing is an area of active investigation.

HIF binds to the promoters and enhancers of a broad range of genes involved in cellular, local, and systemic responses to hypoxia. They include genes that facilitate glucose uptake, enhance glycolysis, inhibit the Krebs cycle, promote angiogenesis, and augment red blood cell production (4-6). The latter is mediated by EPO, a glycoprotein that is the product of the EPO gene, which is considered a paradigm of oxygen-regulated gene expression because of its high dynamic range of inducibility and the central importance of this gene for oxygen delivery to tissues (7,8).

Aside from addressing the fundamental biologic interest in oxygen-dependent regulation of EPO, this pathway has also provided a framework for understanding certain rare forms of erythrocytosis in which there is abnormally increased red blood cell production due to aberrant upregulation of EPO (9-12). The first gene of the oxygen-sensing pathway in which erythrocytosis-associated mutations were identified was the VHL gene (13), followed by mutations in the PHD2 gene (14). Subsequently, we described a mutation
of the \textit{HIF2A} gene in a family with hereditary erythrocytosis (15). This mutation, G537W, affects a residue in close proximity to the primary prolyl hydroxylation site in HIF-2\(\alpha\), Pro-531. Functional studies indicated that it impairs both the ability of PHD2 to bind to and hydroxylate HIF-2\(\alpha\), as well as the subsequent capacity of VHL to recognize it.

We more recently reported two additional erythrocytosis-associated \textit{HIF2A} mutations, G537R and M535V (16). One of these mutations, G537R, has subsequently been independently identified in other patients with erythrocytosis (17,18). The existence of these new mutations raises questions as to whether they lead to stabilization of HIF-2\(\alpha\), and if so, whether this is due to altered interactions with PHD2 and/or VHL. Such altered interactions might also raise the possibility of dominant negative mechanisms. Our analysis of these mutations as well as a third novel erythrocytosis-associated \textit{HIF2A} mutation, P534L (19), reveals that while all mutations result in the aberrant stabilization of HIF-2\(\alpha\), they do so in ways that are not entirely identical. Moreover, while initial attention on HIF prolyl hydroxylation focused on a highly conserved LXXLAP motif containing residues N-terminal to the hydroxylacceptor proline, these results actually highlight a critical role for residues that are in fact C-terminal to it.

**EXPERIMENTAL PROCEDURES**

**Patients and Mutational Analysis of the \textit{HIF2A} Gene**—Individuals with inappropriately normal or elevated serum Epo levels were selected from a registry of idiopathic erythrocytosis patients for analysis of \textit{HIF2A} (14,20). Previous investigations revealed the absence of defects in the \textit{VHL} and \textit{PHD2} genes. Exon 12 of \textit{HIF2A} was amplified by PCR and sequenced as described previously (15).

**Plasmids**—The pcDNA5/FRT/TO-3xFlag hHIF-2\(\alpha\) wild type and P531A plasmids have been described previously (15). Corresponding constructs for the P534L, M535V, and G537R mutations were prepared as follows. First, a 1.6 kb PCR product was obtained using IMAGE Consortium clone 6305604 as a template and the following primers: 5’ GTA CGG ATC CAG ATG ACA GCT GAC AAG GAG-3’ and 5’-GTA CGC GGC CGC ATG CTC CAG CTA AGC GGC CAG CAG CTC-3’. The PCR product was digested with BamH I and Not I and subcloned into the BamH I/Not I site of pcDNAs5/FRT/TO-3xFlag to yield pcDNA5/FRT/TO-3xFlag-hHIF-2\(\alpha\)-N. Then, 1.0 kb PCR products were obtained using the same IMAGE clone as a template, the primer 5’-GTC AGC GGC CGC TCA GGT GGC CTG AGG AGT AG-3’ and one of the following primers. P534L: 5’- GAG ACG CTA GCA CCC TAT ATC CTC ATG GAC GGG GAA GAC TTC CAG C-3’. M535V: 5’- GAG ACG CTA GCA CCC TAT ATC CCG GTG GAC GGG GAA GAC TTC CAG C-3’. G537R: 5’- GAG ACG CTA GCA CCC TAT ATC CCG ATG GAC AGG GAA GAC TTC CAG CTA AGC-3’. The PCR products were digested with Nhe I/Not I and subcloned into the Nhe I/Not I site of pcDNA3-HA. The 1.0 kb bands from Nhe I/Not I digests of the resulting plasmids were then subcloned into the Nhe I/Not I site of pcDNA5/FRT/TO-3xFlag-hHIF-2\(\alpha\) P534L, M535V, and G537R, respectively.

\textit{pcDNA3-GAL4-HIF-2\(\alpha\)} (516-549) has been described previously (15). \textit{pcDNA3-GAL4-HIF-2\(\alpha\)} constructs with amino acid substitutions were prepared by subcloning into the Eco RI/Xba I site of \textit{pcDNA3-GAL4} oligonucleotide duplexes comprised of the following sequences. P531A: 5’- AAT TCA GTA CCC AGG ACT TCG AGC TGG ACT TGG AGA CAC TGG CAC CCT ATA TCC CCA TGG ACG GGG AAG ACT TCC AAT TGA GCC CCA TCT GCC CGG AGG AGT AG-3’ and 5’- CTG AAT TCC CCT GCC AGG AGT AG-3’. P534L: 5’- AAT TCA GTA CCC AGG ACT TCG AGC TGG ACT TGG AGA CAC TGG CAC CCT ATA TCC CCG TGG ACG GGG AAG ACT TCC AAT TGA GCC CCA TCT GCC CGG AGG AGT AG-3’ and 5’- CTG AAT TCC CCT GCC AGG AGT AG-3’.

\textit{M535V}: 5’- AAT TCA GTA CCC AGG ACT TCG AGC TGG ACT TGG AGA CAC TGG CAC CCT ATA TCC CCA TGG ACG GGG AAG ACT TCC AAT TGA GCC CCA TCT GCC CGG AGG AGT AG-3’ and 5’- CTG AAT TCC CCT GCC AGG AGT AG-3’.
CAT TGA AAT CCG TCT GGG TAC TG-3'.

G537R: 5’-AAT TCA GTA CCC AGA CGG ATT TCA ATG AGC TGG ACT TGG AGA CAC TGG CAC CCT ATA TCC CCA TGG ACA GGG AAG ACT TCC AAT TGA GCC CCA TCT GCC CCG AGG AGT AG-3’ and 5’- CTA GCT ACT CCT CGG GGC AGA TGG GGC TCA ATT GGA ATG CTT CCC TGT CCA TGG GGA TAT AGG GTG CCA GTG TCT CCA AGT CCA GCT CAT TGA AAT CCG TCT GGG TAC TG-3’.

Additional pcDNA-GAL4-HIF constructs were prepared in an analogous manner using duplexes comprised of the following sequences:

HIF-2

Sec (residues 390-423): 5’- AAT TCT TCA CCA AGC TAA AGG AGG AGC CCG AGG AGC TAG CCC AGG TGG CCA CCC CCG GGG ACG CCA TCA TCT CTC TGG ATT TCG GGA ATC AGA ACT TCG AGG AGT AG-3’ and 5’- CTA GCT ACT CCT CGA AGT TCT GAT TCC CGA AAT CCA GAG AGA TGA TGG CCT CCC CCG GGG ACG CCA TCA TCT CTC TGG ATT TCG GGA ATC AGA ACT TCG AGG AGT AG-3’ and 5’- CTA GCT ACT CCT CGA AGT TCT GAT TCC CGA AAT CCA GAG AGA TGA TGG CCT CCC CCG GGG ACG CCA TCA TCT CTC TGG ATT TCG GGA ATC AGA ACT TCG AGG AGT AG-3’ and 5’- CTA GCT ACT CCT CGA AGT TCT GAT TCC CGA AAT CCA GAG AGA TGA TGG CCT CCC CCG GGG ACG CCA TCA TCT CTC TGG ATT TCG GGA ATC AGA ACT TCG AGG AGT AG-3’ and 5’- CTA GCT ACT CCT CGA AGT TCT GAT TCC CGA AAT CCA GAG AGA TGA TGG CCT CCC CCG GGG ACG CCA TCA TCT CTC TGG ATT TCG GGA ATC AGA ACT TCG AGG AGT AG-3’ and 5’- CTA GCT ACT CCT CGA AGT TCT GAT TCC CGA AAT CCA GAG AGA TGA TGG CCT CCC CCG GGG ACG CCA TCA TCT CTC TGG ATT TCG GGA ATC AGA ACT TCG AGG AGT AG-3’.

All constructs were verified by DNA sequencing.

Proteins and Peptides—For binding or hydroxylase assays, (His)_6FlagPHD2 was either purified as previously described (21) and stored at -20 °C, or freshly immunoprecipitated from 10 cm plates of baculovirus-infected Sf9 cells using anti-Flag (M2) agarose and then divided into equal aliquots for the subsequent assays. 35S-labeled, in vitro translated GAL4-HIF fusion proteins were prepared using the appropriate pcDNA3-GAL4 construct and wheat germ extract TnT kits (Promega). 35S-labeled, in vitro translated FlagVHL was prepared using pcDNA3-FlagVHL and rabbit reticulocyte TnT Quik kits (Promega). GST-HIF-1α (531-575) was purified from E. coli as described previously (22). Hydroxyproline-531 (Hyp-531)-substituted or unmodified wild type, P534L, M535V, or G536R HIF-2α (527-542) peptides were obtained from Genscript. The following peptides were also synthesized by Genscript: Hyp-531 HIF-2α Prim (residues 527-542), Hyp-405 HIF-2α Sec (residues 401-416), Hyp HIF-2α Sec/Prim: ETLa{HYP}TPGDAIISLDF, Hyp HIF-2α Prim/Sec: AQLa{HYP}YIPMDGEDFQL, and N-terminal biotinylated Hyp-564 HIF-1α (556-574).

PHD2 Binding Assays—(His)_6FlagPHD2 immobilized on 10 μl of M2-agarose was incubated with 35S-labeled, in vitro translated GAL4-HIF fusion proteins as described (21). The resins were then washed, eluted with 2xSDS loading buffer, and the eluates subjected to SDS-PAGE and phosphorimager analysis on an ABI Storm 860 phosphorimagerr.

2-Oxoglutarate Decarboxylation Assays—(His)_6FlagPHD2 immobilized on 10 μl of M2-agarose was washed and assayed for activity essentially by the method of Kivirikko and Myllyla (23) as modified by Linke et al. (24). Reactions were performed in a total volume of 45 μl of 0.6 mM Hapes, pH 7, 45 mM NaCl, 7.5 mM β-glycophosphate, 0.6 mM sodium pyrophosphate, 3% glycerol, 0.3% Triton X-100, 0.3 mM DTT, with 2.4 mM ascorbate, 120 μM FeCl2, and 0.5 μl of 14C 2-oxoglutarate (50 mCi/mmol; 0.1 mCi/ml) in the absence or presence of 25 μM of wild type or mutant HIF-2α (527-542) peptides. Reactions were conducted for 1 hr at 37 °C in 1.5 ml screw top tubes fitted with filter papers saturated with Ca(OH)2. The 14CO2 liberated and captured on the filter paper was quantitated by scintillation counting. Uncoupled decarboxylation measurements performed in the absence of HIF peptide were subtracted from that in its presence to obtain HIF hydroxylase activity.

Mass Spectrometry Assays—(His)_6FlagPHD2 immobilized on 10 μl of M2-agarose was washed and incubated with 50 mM NH4HCO3 containing 0.5 mM DTT and incubated at 37 °C in a volume of 40 μl of the same buffer supplemented with 1 mM ascorbate and 0.5 mM 2-oxoglutarate in the absence or presence of 10 μM HIF-2α (527-542) peptides. At various
times, aliquots of 5 μl were suspended in 50:50 MeOH:H2O with 0.1% formic acid and subjected to electrospray ionization with an Advion TriVersa NanoMate spray source. All mass spectrometry analysis was performed on a Thermo LTQ Orbitrap hybrid mass spectrometer. Scans accumulated at high resolution (100,000) with 10 microscan accumulation. Typical total accumulations were 15 to 20 sec.

**VHL Binding Assays**—Prolyl hydroxylated GST-HIF-1α (531-575) was prepared by incubating GST-HIF-1α (531-575) immobilized on GSH-agarose with (His)6FlagPHD2 in the presence of ascorbate and 2-oxoglutarate, and then washing the resin. Alternatively, 0.12 μg of biotinylated Hyp-564 HIF-1α (556-574) peptide was incubated with 10 μl of streptavidin-agarose (Sigma). The resin was then incubated with 32P-labeled, in vitro translated FlagVHL as described previously in the absence or presence of Hyp-containing peptide. The resins were washed, eluted with 2xSDS loading buffer, and the eluates subjected to SDS-PAGE and phosphorimager analysis.

**Cell Culture and Generation of Stable Transfectants**—Stably transfected HEK293 Flp-In TRex cell lines (Invitrogen), in which the pcDNA5/FRT/TO construct is integrated into a single defined locus, were generated using Flp recombinase according to the manufacturer’s instructions. Those expression wild type and P531A 3xFlag-HIF-2α have been previously described (15). These cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. HIF-2α expression was induced by treating cells with 0.12 μg/mL doxycycline for 16 hours. For protein stability assays, cycloheximide was employed at a concentration of 20 μg/ml. Hypoxia treatment was performed in an In Vivo 200 Hypoxia Workstation (Ruskinn Technologies).

**Western Blotting**—Cells were lysed in ice cold buffer containing 20 mM Hapes, pH 7, 150 mM NaCl, 25 mM β-glycophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM PMSF and mammalian extract protease inhibitor cocktail (Sigma). Extracts were then centrifuged at 13,000 x g for 15 minutes at 4°C. Protein concentrations were quantified by DC Assay (Bio-Rad) according to the manufacturer’s instructions. Equal amounts of protein were then separated by SDS-PAGE and transferred to Immobilon-P Membranes (Millipore). The membrane was blocked with PBS containing 0.5% Tween and 5% non-fat milk. For 3xFlag-HIF-2α detection, membranes were incubated with alkaline phosphatase-conjugated anti-Flag antibodies (M2, Sigma). For β-tubulin detection, membranes were incubated with rabbit polyclonal anti-β-tubulin antibodies (H-235, Santa Cruz) followed by alkaline phosphatase-conjugated anti-rabbit secondary antibodies. CDP-Star (Roche) was employed as a substrate for detection.

**Real Time PCR**—Total RNA was isolated from stably transfected HEK293 Flp-In T-Rex cell lines induced with doxycycline for 16 hours using TRIzol (Invitrogen) according to the manufacturer’s protocol. cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real Time-PCR analysis was performed using SYBR Green Master mix (ABI) and an Applied Biosystems 7300 Real Time PCR System. Target gene levels were normalized to that of β-actin. SYBR Green primers, designed with the use of Primer Express software, version 3.0 (Applied Biosystems), were as follows. 3xFlag: 5'- TGG TAC CAT GGA CTA CAA AGA-3' and 5'- CGT CAT CCT TGT AAT CAG TGT CA-3'. hVEGF: 5'- AGA CTC CGG CGG AAG CAT-3' and 5'- AAT GGC GAA TCC AAT TCC AA-3'. hNDRG1: 5'- AGT GCG GCT GCC AGG TT-3' and 5'- AAT GGC GAA TCC AAT TCC AA-3'. hADM: 5'- ACC GCC AGA GCA TGA ACA A-3' and 5'- AAG CGG CAG CCA AAG CT-3'. Dissociation curve analysis for each set of probes revealed single peaks. The sequences of the β-actin primers have been previously described (25). Relative quantification was performed using the ΔΔCT method and β-actin as the endogenous control.

**Luciferase Assays**—The HEK293 Flp-In TRex cell lines were cotransfected with (eHRE)3-luciferase (26) and the Renilla-luciferase internal control pRL-TK using FuGENE6 (Roche), and then treated with 0.1 μg/mL doxycycline for 16 hours. In some cases, cells were exposed to hypoxia (0.2% O2). Cells were harvested with Passive Lysis Buffer (Promega) and then clarified by centrifugation at 13,000 x g for 15 minutes at 4°C. Luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) and a Berthold Lumat LB9507 luminometer.

**Statistical Analysis**—Student’s t-test and analysis of variance were used for statistical
RESULTS

Following our identification and functional characterization of the initial erythrocytosis-associated G537W mutation in the HIF2A gene (15), we subsequently reported on two additional HIF2A mutations, M535V and G537R (16). In this report, we describe the functional characterization of these latter two HIF-α mutants as well as a third novel heterozygous erythrocytosis-associated mutant, P534L (19). We initially performed binding assays in which we incubated recombinant PHD2 with 35S-labeled, in vitro translated, wild type or mutant GAL4-HIF-2α (516-549), and then we immunoprecipitated the PHD2 and assessed for 35S-GAL4-HIF-2α (516-549) binding (Figure 1A). Compared to wild type HIF-2α (516-549), the mutants all bound more weakly (compare lanes 3, 6, 9 and 12). The mean (±SD) relative recoveries of P534L, M535V, and G537R HIF-2α (516-549) were 34 ± 6%, 14 ± 4%, and 9 ± 2%, respectively, of that of wild type (P < 0.001 in all cases).

Impaired binding to PHD2 might be reflected in decreased PHD2-catalyzed hydroxylation. To assess this, we incubated recombinant PHD2 with wild type or mutant HIF-2α (527-542) peptides in the presence of 14C labeled 2-oxoglutarate, and then measured the liberated 14C labeled CO2 as a measure of HIF hydroxylation. To assess this, we incubated recombinant PHD2 with 35S-labeled, in vitro translated, wild type or mutant GAL4-HIF-2α (527-542) peptides in the presence of 14C labeled 2-oxoglutarate, and then measured the liberated 14C labeled CO2 as a measure of HIF hydroxylation. Relative to PHD2 towards P534L, M535V, and G537R HIF-2α (516-549) were 34 ± 6%, 14 ± 4%, and 9 ± 2%, respectively, of that of wild type (P < 0.05 in all cases).

The 14C 2-oxoglutarate decarboxylation assay indirectly measures hydroxylation. As an independent means of assessing whether the mutations impair PHD2-induced hydroxylation, we incubated recombinant PHD2 with wild type, P534L, M535V, or G537R HIF-2α (527-542) peptide, and then assessed the extent of hydroxylation by electrospray ionization mass spectrometry (Figure 2). Under conditions in which the wild type peptide was very efficiently hydroxylated (Figure 2A), all three mutant peptides were hydroxylated substantially less so (Figure 2B-D), evident at both the 30 min and 60 min time points.

In addition to effects on PHD2 binding and hydroxylation, these mutations may also affect subsequent recognition by VHL. Towards this end, we evaluated the capacity of wild type and mutant Hyp-HIF-2α (527-542) peptides to compete with hydroxylated GST-HIF-1α (531-575) for binding to 35S-labeled, in vitro translated VHL (15). In control experiments, hydroxylated GST-HIF-1α (531-575) efficiently captures VHL, while the non-hydroxylated counterpart does not (Figure 3A, lanes 1 and 2). In addition, wild type Hyp-HIF-2α (527-542) very efficiently competes for VHL binding, as reflected by diminished binding to GST-HIF-1α (531-575) (lanes 4-6). We find that the P534L and the G537R Hyp-HIF-2α (527-542) peptides are substantially less potent in competing for VHL binding (compare lanes 7 and 13 with lane 4). The M535V peptide, in contrast, is equally effective as the wild type peptide. This is reflected by comparable titration results to wild type peptide at both high peptide concentrations (Figure 3A, compare lanes 4-6 with lanes 10-12) and low peptide concentrations (Figure 3B, compare lanes 4-6 with lanes 7-9).

We next generated stably transfected, Flp-In TRex HEK293 cells that express doxycycline-inducible Flag-tagged P534L, M535V, and G537 HIF-2α (Figure 4A). The constructs encoding for the HIF-2α mutants integrate into a single FRT site in this cell line so as to generate isogenic cell lines. As a reflection of this, two independent clones for each of these three mutants revealed comparable levels of steady state, doxycycline-induced protein levels, as assessed by Western blotting with anti-Flag antibodies (Figure 4A). When compared to wild type Flag-tagged HIF-2α expressed from the same system, we find that the protein levels for each mutant is higher. Another cell line expressing a hydroxylation-defective P531A HIF-2α also displays higher steady state levels of protein than wild type, as reported previously (15). For subsequent experiments, we selected a single clone from each pair of the P534L, M535V, and G537R mutants. Real Time PCR employing primers specific for the nucleotide sequence encoding the Flag tag show that the wild type, P534L, M535V, and G537R mRNA levels are all comparable (Figure 4B).

To assess the stability of these proteins in more detail, we arrested protein synthesis by treatment of cells with cycloheximide, and then
monitored protein level by Western blotting using anti-Flag antibodies (Figure 5). As a control, wild type HIF-2α is rapidly degraded (left hand side of each panel). We find that the P534L, M535V and G537R mutants of HIF-2α mutants are each degraded more slowly than wild type HIF-2α (top three sets of panels), though perhaps not as slowly as the P531A hydroxylation defective control (bottom set of panels).

We next examined, by Real Time PCR, the expression of the HIF target genes adrenomedullin (ADM), N-myc downstream regulated gene 1 (NDRG1) and vascular endothelial growth factor (VEGF) (27) in the wild type and mutant HIF-2α expressing HEK293 cells (Figure 6). The expression of M535V and G537R HIF-2α, as well as P531A HIF-2α, induced significantly increased mRNA transcript levels from the ADM gene (Figure 6A) as compared to wild type HIF-2α. The expression of P534L and G537R HIF-2α, as well as P531A HIF-2α, induced significantly increased mRNA transcript levels from the NDRG1 and VEGF genes as compared to wild type (Figure 6B,C). The baseline levels of a given target gene showed slight variation among the different cell lines. We therefore also calculated the induction ratios of each HIF target gene (Figure 6D-F). When analyzed in this manner, the induction ratios of all three genes are higher with all of the HIF-2α mutants than that obtained with wild type HIF-2α.

The higher levels of HIF target genes induced by the HIF-2α mutants is consistent with increased transcriptional activity. To examine this independently, we transfected the HIF-2α-expressing HEK293 cell lines with a Hypoxia Response Element reporter gene (Figure 7). Under normoxic conditions, and consistent with the previous data (Figure 6), we find that reporter gene activity is increased with all three mutants as compared to wild type. In contrast, hypoxic conditions, which would be expected to stabilize wild type HIF-2α, leads to levels of reporter gene activity that are similar to that of wild type.

All three of the mutations examined here affect residues C-terminal to primary site of hydroxylation in HIF-2α, Pro-531, suggesting a critically important role for these residues. The secondary site of hydroxylation in HIF-2α, Pro-405 is dissimilar in this region (Figure 8A, top, compare HIF-2α Prim and HIF-2α Sec). Indeed, it differs at all three of the residues that are mutated in HIF2A-associated erythrocytosis patients (indicated by asterisks). To explore this further, we prepared [35S labeled, in vitro translated, HIF-2α Sec (residues 390-423), corresponding to the secondary site of hydroxylation. We find that it binds more weakly to PHD2 than the corresponding sequence (residues 516-549) from the primary site of hydroxylation (Figure 8A, bottom, compare lanes 3 and 12). We next prepared a chimera containing the N-terminal residues from the primary site of hydroxylation and C-terminal residues from the secondary site of hydroxylation (Prim/Sec) and find that it too also binds more weakly (lane 6). Conversely, and in contrast, a peptide with the N-terminal residues from the secondary site of hydroxylation and C-terminal residues from the primary site of hydroxylation (Sec/Prim) binds with substantial affinity to PHD2 (lane 9).

In other experiments, we also compared the ability of Hyp-containing peptides corresponding to the primary and secondary sites of hydroxylation to bind to VHL (Figure 8B, top). In this case, we used the previously described competition assay. As shown in Figure 8B (bottom), a hydroxylated peptide containing the primary site of HIF-2α hydroxylation binds with high affinity to VHL, as reflected by its capacity to inhibit VHL binding to hydroxylated HIF-1α (556-574) (compare lane 4 with lane 3). The secondary site of hydroxylation binds with substantially lower affinity to VHL (lane 7). Notably, a chimera containing the C-terminal residues from the primary site of hydroxylation and N-terminal residues from the secondary site binds with substantial affinity to VHL (lane 6), and more tightly than that of the chimera with the converse switch (lane 5). These observations therefore reinforce the importance of residues C-terminal to the hydroxyacceptor proline in HIF-2α, both in the interaction with PHD2 and in the interaction with VHL.

DISCUSSION

After the initial identification and characterization of an erythrocytosis-associated HIF2A mutation (15), we have subsequently identified three additional distinct HIF2A mutations (16,19). The present characterization of these mutations provides new observations and insights into this disease.

First, all three mutations lead to decreased degradation and hence, stabilization of HIF-2α. Consequently, all display a gain of function...
phenotype. Dominant negative mechanisms—such as, for example, increased mutant binding to PHD2 leading to impaired hydroxylation of the wild type HIF-2α, or alternatively increased mutant binding to VHL leading to impaired degradation of wild type HIF-2α—are not supported by the present data. None of the mutants was found to have increased affinity for either PHD2 or VHL, and indeed, in most cases there were significant decreases in affinity.

Second, while all mutations act to stabilize HIF-2α, they do so in ways that are not identical. Thus, while the P534L and G537R mutations impair both binding to PHD2 and to VHL, we find that the M535V mutation impairs only binding to PHD2. In the three-dimensional structure of a Hyp HIF-1α peptide bound to VHL, HIF-1α Met-568 (which corresponds to HIF-2α Met-535) is not a contact residue with VHL, and its side chain points away from VHL (28,29). In contrast, Pro-567 (which corresponds to HIF-2α Pro-534) forms a van der Waals contact with VHL. Furthermore, Gly-537, which is not conserved in HIF-1α, is just C-terminal to a residue that corresponds to HIF-1α Asp-569, which also makes a van der Waals contact with VHL (28,29).

This therefore suggests that the M535V mutation is pathogenic mainly through effects on PHD2 binding and PHD2-catalyzed hydroxylation. This in turn suggests that impairment of the PHD2:HIF-2α interaction alone is sufficient to induce erythrocytosis, and is consistent with the fact that mutations in PHD2 alone comprise a distinct cause of erythrocytosis (10,12,14,30,31). That impairment of the HIF-α:VHL interaction alone is sufficient to induce erythrocytosis is independently supported by the existence of erythrocytosis-associated VHL mutations (10,13,20,32-34). This, in turn, raises the possibility that there might exist yet to be identified HIF2A mutations that selectively impair the interaction of HIF-2α with VHL, but not with PHD2.

A third notable result of these studies is that all erythrocytosis-associated HIF2A mutations identified to date affect residues C-terminal to the hydroxylacceptor proline. This is somewhat of an unexpected finding, since much of the attention on sequence determinants in HIF-α initially focused on residues in a highly conserved LXXLAP motif (where underlining indicates the hydroxylacceptor proline) that lie N-terminal to the hydroxylacceptor proline (35-37). Residues C-terminal to the hydroxylacceptor proline are substantially less well conserved, but these naturally occurring missense mutations now highlight the fact that they are indeed functionally critical. This is reinforced by the observations that chimeras between the primary and secondary sites of hydroxylation of HIF-2α bind with high affinity to PHD2 and to VHL in a manner that primarily tracks with the C-terminal, as opposed to N-terminal, residues (Figure 8). It will in this regard be of interest to examine the three-dimensional structure of HIF-2α bound to PHD2.

Collectively, these studies reinforce the importance of prolyl hydroxylation as the key posttranslational modification regulating the HIF pathway in response to changes in oxygen tension, reaffirm the role of the primary site of hydroxylation in HIF-2α in regulating its protein stability, and support the importance of HIF-2α in the control of EPO and hence erythropoiesis in humans.
REFERENCES


**FOOTNOTES**

*This work was supported by NIH R01-CA090261 (to FSL) and a University of Pennsylvania Research Foundation grant (to FSL).

2The abbreviations used are: HIF, Hypoxia Inducible Factor; Hyp, hydroxylproline; PCR, polymerase chain reaction; PHD, Prolyl Hydroxylase Domain protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VHL, von Hippel Lindau protein.

**ACKNOWLEDGMENTS**

We thank the clinicians who have referred patients with erythrocytosis from the United Kingdom and Ireland to our registry and provided samples.
FIGURE LEGENDS

FIGURE 1. Effects of HIF-2α mutations on PHD2 binding and PHD2-catalyzed hydroxylation. (A) PHD2 binding assays. Anti-Flag agarose with or without immobilized recombinant (His)_6FlagPHD2 was incubated with [35S] labeled, in vitro translated GAL4-HIF-2α (516-549), washed, eluted, and the eluates subjected to SDS-PAGE. Input represents 2% of the total amount of wild-type or mutant GAL4-HIF-2α (516-549). Relative recovery is as indicated. (B) HIF prolyl hydroxylase assays. (His)_6FlagPHD2 was incubated with the indicated HIF-2α (527-542) peptides. Activity was determined by the amount of [14C] labeled CO₂ liberated from [14C] 2-oxoglutarate.

FIGURE 2. Effects of HIF-2α mutations on PHD2-catalyzed hydroxylation. Recombinant (His)_6FlagPHD2 immobilized on anti-Flag agarose beads was incubated with the indicated HIF-2α (527-542) peptides. At various time points, reaction products were analyzed by electrospray ionization mass spectrometry. Monoisotopic m/z values for the unmodified HIF-2α (527-542) peptides are indicated by the open triangles and are 938.9 for wild type, 946.9 for P534L, 922.9 for M535V, and 988.4 for G537R. The predicted values are 938.9, 946.9, 922.9, and 988.4, respectively. Monoisotopic m/z values for the hydroxylated peptides are indicated by the filled arrows and are 946.9 for wild type, 954.9 for P534L, 930.9 for M535V, and 996.4 for G537R. [M + H + K]²⁺ adducts are shown; thus, hydroxylation is reflected by an m/z change of 8.

FIGURE 3. Effects of HIF-2α mutations on VHL binding. (A) Unmodified or PHD2-hydroxylated GST-HIF-1α (531-575) immobilized on GSH-agarose was incubated with [35S] labeled, in vitro translated VHL in absence or presence of 1, 5, or 25 nM hydroxylated wild type or mutant HIF-2α (527-542) peptide. Resins were washed, eluted, and the eluates subjected to SDS-PAGE. (B) The same assay was employed to examine wild type and M535V HIF-2α (527-542) at lower peptide concentrations of 0.04, 0.2, and 1 nM. In (A) and (B), Input represents 25% of the total amount of VHL employed in each reaction.

FIGURE 4. Generation of Flp-In TRex HEK293 cells stably transfected with expression constructs for wild type or mutant HIF-2α. (A) Flp-In TRex 293 cells were induced with doxycycline (Dox) as shown, and then extracts prepared and subjected to Western blotting using anti-Flag antibodies. Two independent clones for each of the P534L, M535V, and G537R mutants were examined and are shown. HIF2-α P534L, M535V, and G537R clones depicted in lanes 4, 5, and 7, respectively were chosen for further analysis. Western blot for β-tubulin is also shown. (B) Real-time PCR of messenger RNA transcripts from the 3xFlagHIF-2α gene in wild type or mutant 3xFlagHIF-2α cell lines. P values for each mutant (as compared to wild type) were > 0.05.

FIGURE 5. Effects of HIF-2α mutations on protein stability. Flp-In TRex 293 cell lines were induced to express wild type or mutant 3xFlagHIF-2α, treated with 20 μg/ml cycloheximide (CHX), and then harvested at the indicated time points. The first lane in each blot shows results for the parental (untransfected) cell line.

FIGURE 6. HIF target gene analysis for HIF-2α mutants. Flp-In TRex 293 cells were induced to express 3xFlagHIF-2α with doxycycline (Dox) as indicated. RNA was harvested, reverse transcribed, and then Real Time PCR performed to measure the levels of transcripts from the (A) adrenomedullin (ADM), (B) N-myc downstream regulated gene 1 (NDRG1), and (C) vascular endothelial growth factor gene (VEGF) genes. Means of relative quantification (RQ, normalized to β-actin) from three separate experiments are shown. T bars indicate standard deviations. Asterisks indicate P < 0.05 in comparison to levels achieved by doxycycline-induced wild type HIF-2α. (D-F) The doxycycline-inducibility of messenger RNA transcripts from HIF-2α target genes when compared to non-induced levels in each cell line is shown. Asterisks indicate P < 0.05 in comparison to the induction ratio for wild type HIF-2α.

FIGURE 7. HRE reporter gene assays for HIF-2α mutants. Flp-In TRex 293 cells were transfected with an HRE-Luciferase reporter construct. Some cells were induced with doxycycline (Dox), and some
were subjected to hypoxia (0.2% O\textsubscript{2}) as shown. Luciferase activities were measured and normalized to that of Renilla luciferase expressed from a cotransfected pRL-TK internal control. Means from three separate experiments are shown. T bars indicate standard deviations. Asterisks indicate $P < 0.05$ for comparisons of activities from induced mutant 3xFlagHIF-2α with induced wild type 3xFlagHIF-2α under normoxia.

FIGURE 8. Analysis of HIF-2α chimeras. (A) PHD2 binding. Top, comparison of HIF-2α sequences at the primary (amino acids 516-549; Prim) and secondary (amino acids 390-423; Sec) sites of prolyl hydroxylation. Shading denotes sequences at primary site of hydroxylation. Hydroxylation site is indicated by triangle, and Pro-534, Met-535, and Gly-537 are indicated by asterisks. Sequences of chimeras (Prim/Sec and Sec/Prim) are also shown. Bottom, anti-Flag agarose with or without immobilized recombinant (His)$_\text{6}$FlagPHD2 was incubated with $^{35}$S labeled, in vitro translated GAL4-HIF-2α polypeptides, washed, eluted, and the eluates subjected to SDS-PAGE. Input represents 10% of the total amount of GAL4-HIF-2α polypeptide. (B) VHL binding. Top, comparison of HIF-2α peptide sequences at the primary (amino acids 527-542; Prim) and secondary (amino acids 401-416; Sec) sites of prolyl hydroxylation. Shading denotes sequences at primary site of hydroxylation. Pro-534, Met-535, and Gly-537 are indicated by asterisks. Sequences of chimeras (Prim/Sec and Sec/Prim) are also shown. Bottom, streptavidin-agarose with or without biotinylated Hyp-564 HIF-1α (556-574) was incubated with $^{35}$S labeled, in vitro translated VHL in absence or presence of 50 nM of the indicated hydroxylated HIF-2α peptides. Resins were washed, eluted, and the eluates subjected to SDS-PAGE. Input represent 10% of the total amount of VHL employed in each reaction.
Figure 1

A

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GAL4-HIF-2α (516-549) Relative Recovery

B

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<td>G537R</td>
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Figure 1
Figure 3

A

GST-HIF-1α (531-575) +
GST-Hyp-HIF-1α (531-575)
Hyp-HIF-2α (527-542)

Input

Wild Type
P534L
M535V
G537R

Relative Recovery

100 20 14 6 88 23 7 29 10 11 12 13 14 15

B

GST-HIF-1α (531-575) +
GST-Hyp-HIF-1α (531-575)
Hyp-HIF-2α (527-542)

Input

Wild Type
M535V

Relative Recovery

100 77 56 31 69 62 36

Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

Relative Luciferase Activity

Dox

- WT

+ WT

+ P534L

+ M568V

+ G53TR

+ P531A

- WT

+ WT

+ P534L

+ M568V

+ G53TR

+ P531A

21% O₂

0.2% O₂

*
Figure 8