A novel erythrocytosis-associated PHD2 mutation suggests the location of a HIF binding groove

Running title: Erythrocytosis and the PHD2 Arg371His mutation

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Abstract
The molecular basis of the erythrocytosis group of red cell disorders is incompletely defined. Some cases are due to dysregulation of Epo synthesis. The Hypoxia Inducible transcription Factor (HIF) tightly regulates Epo synthesis. HIF in turn is regulated through its α-subunit, which under normoxic conditions is hydroxylated on specific prolines and targeted for degradation by the von Hippel Lindau (VHL) protein. Several mutations in VHL have been reported in erythrocytosis, but only one mutation in the HIF prolyl hydroxylase Prolyl Hydroxylase Domain protein 2 (PHD2) has been described. Here we report a novel PHD2 mutation, Arg371His, which causes decreased HIF binding, HIF hydroxylase, and HIF inhibitory activities. In the tertiary structure of PHD2, Arg-371 lies close to the previously described Pro317Arg mutation site. These findings substantiate PHD2 as a critical enzyme controlling HIF and therefore Epo in humans, and furthermore suggest the location of an active site groove in PHD2 that binds HIF.

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**Introduction**

Idiopathic erythrocytosis (IE) is a rare condition characterized by an increase in red cell mass and is assumed to be caused by a heterogeneous group of unidentified genetic mechanisms.\(^1\) Erythrocytosis (also referred to in the literature as polycythemia) has been defined as primary or secondary. In primary erythrocytosis a genetic defect in the erythroid progenitor cells leads to enhanced production of red blood cells, as exemplified by truncation of the erythropoietin receptor (EpoR)\(^2,3\) and JAK2 mutations\(^4-7\). It is associated with sub normal Epo levels. In contrast secondary erythrocytosis is driven by increased production of Epo, resulting in increased or inappropriately normal levels of Epo.

An autosomal recessively inherited form of erythrocytosis, endemic in the Chuvash population in Russia, is caused by a homozygous mutation, Arg200Trp, in VHL. The mutation also exists in families of Asian and Caucasian ancestry\(^8-12\). The VHL protein is part of an E3 ubiquitin ligase complex that targets HIF-\(\alpha\) for proteasomal degradation. This is dependent on hydroxylation of specific proline residues in the oxygen dependent degradation domain (ODD) of HIF-\(\alpha\) mediated by the PHD (also known as HPH and EGLN) family of prolyl hydroxylases.\(^13-15\) Under hypoxic conditions, this modification is inhibited, thereby stabilizing HIF-\(\alpha\). Recently, we identified a heterozygous mutation in PHD2, Pro317Arg, in a family with erythrocytosis\(^16\) thus confirming PHD2 as a major regulator of HIF-\(\alpha\) in oxygen homeostasis. Consequently, PHD2 associated erythrocytosis has been designated as a separate disease entity by Online Mendelian Inheritance in Man (OMIM #609820). Moreover, this raises the possibility that other patients with idiopathic erythrocytosis may harbor mutations in the PHD2 gene, and we report one such individual here. The findings not only lend further support to a critical role for PHD2 in red blood cell control, but also point to a potential substrate binding site in PHD2, which has been previously undefined.

**Patients, Materials and Methods**

**Patients.** 181 patients, who did not fulfil the PV diagnostic criteria proposed by the British Committee for Standards in Haematology\(^17\), and had a raised red cell mass have been investigated. All patients gave informed written consent on entering the study,
which had been approved by the Queen’s University, Belfast Research Ethics Committee, according to the Helsinki protocol.

**Mutation Screening.** Using PCR-direct sequencing PHD1 (exons 1-5), PHD2 (exons 1-4) and PHD3 (exons 1-3) were examined as described previously. A group of 200 normal control samples (Human Random Control DNA panels, ECACC, Salisbury, UK) was screened for the G1112A base change by restriction digest with Tsp45I.

**Plasmids and proteins.** pcDNA3-FlagPHD2 Arg371His and pGEX-HIF-2α (516-549) were constructed by standard recombinant DNA methods. The sources of all other plasmids, as well as the HEK293 cells employed in the reporter gene assays have been described. GST-HIF-2α (516-549) and GST-HIF-1α (531-575) were purified as described previously. 35S-labeled, *in vitro* translated FlagPHD2 or FlagPHD2 Arg371His was prepared using either rabbit reticulocyte lysate (binding assays) or wheat germ (hydroxylase assays) TnT kits (Promega, Madison, Wisconsin, USA). FeCl₂ (50 μM) was included in the reaction mixture. 35S-labeled, *in vitro* translated Flag-VHL was prepared using a rabbit reticulocyte lysate TnT Quick kit.

**Assays.** GST pulldown assays, prolyl hydroxylase, and luciferase assays were performed essentially as previously described.

**Case history.**

A young man at the age of 29 years was diagnosed with a raised hemoglobin (18.8 g/dL) and an elevated hematocrit (0.56). His red cell mass was 139% of the predicted value. White cell (8.2 x10⁹/L) and platelet counts (190 x10⁹/L) were in the normal range. The Epo level was inappropriately normal at 12 mIU/ml, (normal range of the assay was 5-25 mIU/ml). His spleen was normal on ultrasound. Both bone marrow aspirate and trephine had normal appearance. At 35 years of age, he had head CT and MRI studies in which an acute sagittal sinus thrombosis was seen; no other abnormality was noted. He has also had a normal chest X-ray and abdominal ultrasound. There is no history of wound healing problems. He has been managed for the last 9 years by regular venesection and remains well at the age of 38 years.
Results and Discussion
The EpoR, VHL and PHD2 genes, as reflected in the current OMIM classification of erythrocytosis, were screened in all IE cases. Consequently, a novel mutation, G1112A, in PHD2 exon 3 (Figure 1A) was detected in one individual with wild type EpoR or VHL. In addition, screening the other HIF prolyl hydroxylases, PHD1 and 3, did not reveal any further defects. To eliminate the possibility that the base change was a single nucleotide polymorphism, a group of 200 normal control samples was examined by restriction digest, as the presence of an A at base 1112 forms a new restriction site for the enzyme Tsp45I. The G1112A base change was absent in this group of controls (data not shown). Furthermore, screening the mother who had normal Hct (0.436) and Hb (13.5 g/dL) levels did not detect this mutation (Figure 1B). Unfortunately, the father was not available for testing.

The G1112A mutation is predicted to result in loss of arginine at amino acid 371 and replacement with histidine. Residue 371 is three amino acids away from the His-374 iron-chelating residue (Figure 1C). This parallels the previously reported PHD2 mutation, Pro317Arg, which was also located close to an iron chelating residue, His-313. Arg-371 is conserved in all three PHD isoforms, as well as the single HIF prolyl hydroxylases from D. melanogaster and C. elegans (Figure 1C).

To investigate the effect that the Arg371His mutation may exert on PHD2 function, both wild type and mutant protein were prepared by in vitro translation and examined by in vitro binding and enzymatic assays. We found the mutant bound substantially more weakly than wild type to HIF-2α (516-549), which contains the primary hydroxylacceptor prolyl residue, Pro-531 (Figure 2A, lanes 3 and 6). Similar assays showed that, compared to wild type PHD2, the mutant also had a significantly decreased ability to bind HIF-1α (531-575), which contains the primary hydroxylation site, Pro-564 (Figure 2B). We assayed prolyl hydroxylase activity towards HIF-2α (516-549) and observed mutated PHD2 displays significantly less HIF hydroxylase activity than does wild type (Figure 2C, lanes 3 and 4). Furthermore in transfection assays, Arg371His PHD2 was less effective than wild type PHD2 in suppression of HIF-induced activation.
of a hypoxia response element reporter gene (Figure 2D). Taken together, the functional data all point towards a significant loss of PHD2 activity in these assays arising from the mutation.

The present findings provide further support for a central role for PHD2 in the control of red cell mass in humans. The individual in the present study shares certain features with the affected members of the family previously studied. First, the erythrocytosis is modest. Second, the Epo levels were normal, although as before, this should be regarded as inappropriately normal given the elevated hematocrit. Third, the patient is heterozygous for the PHD2 mutation suggesting that partial loss of total cellular PHD2 activity is sufficient to induce this phenotype.

From the recently resolved three-dimensional structure of PHD2, Arg-371 is in the vicinity of Pro-317 (Figure 2E). This therefore suggests that in PHD2, both Arg-371 and Pro-317 may contribute to a HIF-α substrate binding groove. The primary site of hydroxylation in HIF-α is rich in acidic residues both N- and C-terminal to the hydroxylacceptor proline. Therefore, it will also be of interest to determine if Arg-371 contacts such a residue.

References


Figure 1. Identification of the 1112 G > A Mutation in the PHD2 Gene.
(A) Detection of the G1112A mutation by PCR-direct sequencing. PCR was performed on total peripheral blood DNA using a set of primers to specifically amplify exon 3. Sequencing detected a heterozygous G to A change at base 1112 as indicated by an arrow (Upper panel) as compared to wild type sequence (Lower panel). Shown are nucleotides 1093-1131. Bases are as follows: G = black; A = green; T = red; C = blue.
(B) Screening family members for G1112A mutation. The presence of A at base 1112 creates a restriction site for the enzyme Tsp45 I to give 2 products of 246 bp and 273 bp from an exon 3 PCR product from the patient (Lane 4). The mother of the patient (Lane 3) was screened and the absence of the 246 bp and 273 bp bands indicated she did not possess the G1112A mutation. Lane 1 contains a 100 bp DNA size marker, Lane 2 nondigested exon 3 PCR product of 519 bp and Lane 5 Tsp45 I-digested exon 3 PCR product from a control sample negative for the mutation as detected by sequencing. (C) Comparison of amino acid sequence from residues 366-379 (hPHD2 nomenclature) in human HIF prolyl hydroxylases with those from D. melanogaster (DmHPH) and C. elegans (CeEGL9). Sequence shading indicates completely conserved residues, asterisk indicates iron chelating residue His-374, and inverted triangle indicates Arg-371 of human PHD2, which is predicted to be changed to His by the 1112 G > A mutation. Also shown is hPHD2 sequence from residues 307-323, with asterisks indicating iron-chelating residues His-313 and Asp-315, and closed circle indicating Pro-317. The positions of these sequences in full length hPHD2 is shown, with shading indicating prolyl hydroxylase domain. Numbers at bottom indicate residues.

Figure 2. Functional Characterization of the Arg371His PHD2 Mutant.
(A) Association of HIF-2α with Arg371His PHD2. 35S labeled, in vitro translated wild type or Arg371His FlagPHD2 was incubated with 1 µg of either GST or GST-HIF-2α (516-549) immobilized on GSH-agarose. The resins were washed, eluted, and the eluates subjected to SDS-PAGE and autoradiography. Input represents 10% of the total. The relative recovery of wild type PHD2 from three replicates is 100 ± 24 (arbitrary units ± SD) whereas that of Arg371His PHD2 is 2.4 ± 4 (P < 0.005). (B) Association of HIF-1α with Arg371His PHD2. Binding assays with GST-HIF-1α (531-575) were
performed as described in (A). Input represents 5% of total. The relative recovery of wild type PHD2 from three replicates is 100 ± 0.1 (arbitrary units ± SD) whereas that of Arg371His PHD2 is 0.8 ± 0.0001 (P < 0.005). (C) Hydroxylase activity of Arg371His PHD2. Equal quantities (as determined by phosphorimager analysis) of in vitro translated wild type or Arg371His FlagPHD2, or mock in vitro translation reaction, was incubated with 0.75 µg of GST-HIF-2α (516-549) for 1 hr in the presence of 2-oxoglutarate, ascorbic acid and FeCl₂. The GST-HIF-2α (516-549) was isolated using GSH-agarose, washed, and then the degree of HIF hydroxylation assessed by subsequent incubation with 35S labeled, in vitro translated VHL. Input represents 5% of the total. Under the conditions of the assay, the recovery of 35S labeled, in vitro translated VHL using wild type PHD2 from three independent experiments is 100 ± 38 (arbitrary units ± SD) while that using Arg371His PHD2 is 12 ± 7.6 (p < 0.05). (D) HIF-inhibitory activity of Arg371His PHD2. HEK293 cells were cotransfected with 150 ng of (eHRE)₃-Luc, 150 ng of pRL-TK, 300 ng of either pcDNA3 or pSV-Sport-HA-hHIF-2α, and either 0, 0.3, or 0.6 ng of pcDNA3-FlagPHD2 (wild type or P317R). The total DNA dose was normalized with pcDNA3. Forty eight hours after transfection, the cells were harvested and assayed for luciferase activity. Activities were normalized to that of the Renilla luciferase internal transfection control. Shown are results performed in triplicate ± SD. * indicates P < 0.05, ** indicates P < 0.01 when comparing results of wild type and mutant PHD2 at the same dose. In separate experiments, HEK293 cells were transfected with 2 µg of wild type or Arg371His pcDNA3-FlagPHD2, and forty-eight hours later, extracts (15 µg) analyzed by Western blotting with anti-Flag (M2, Sigma) or anti-β tubulin (H-235, Santa Cruz Biotechnology, Santa Cruz, California) antibodies. The position of PHD2, β-tubulin, and a molecular weight marker are as indicated. (E) Three-dimensional structure of PHD2. The structures were generated using Cn3D from PDB coordinates (2G1M) deposited by McDonough et al. Arg-371 and Pro-317 are highlighted in yellow. Compound A (a 2-oxoglutarate competitive inhibitor) is shown in brown.
Figure 1

A

B

C

Fig. 1
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Figure 2

A

B

C

D

E

Arg-371

Pro-317

Fig. 2
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