An Endoplasmic Reticulum Transmembrane Prolyl 4-Hydroxylase Is Induced by Hypoxia and Acts on Hypoxia-inducible Factor α

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The C-P4Hs act on Xaa-Pro-Gly sequences in collagens and more than 20 collagen-like proteins, the 4-hydroxyproline residues forming being essential for the assembly of triple-helical molecules (1–3). All vertebrate C-P4Hs are αβ2 tetramers in which the enzyme and chaperone protein disulfide-isomerase (PDI) acts as the β subunit (1–3). Three isoforms of the catalytic α subunit have been cloned and characterized and hydroxylated by HIF-1α, HIF-2α, and [α(III)]2β2 tetramers with PDI, known as C-P4Hs I, II, and III, respectively (3, 8, 9).

The HIF-P4Hs regulate the hypoxia-inducible factors (HIFs) by hydroxylating proline residues in Leu-Xaa-Xaa-Leu-Ala-Pro sequences at two separate sites in their α subunits (10, 11). The human HIF-P4Hs have three isoenzymes, HIF-P4Hs 1-3 (also known as PHDs 1-3, HPHs 3-1, and EGLNs 2, 1, and 3, respectively), which show a 42–59% sequence identity to each other but essentially no sequence similarity to the C-P4Hs except for the catalytically critical residues (12–14). HIFs are αβ heterodimers that act as master regulators of the transcription of more than 100 hypoxia-regulated genes (5–7). The human HIF-α subunit has three isoforms, HIF-1α–HIF-3α. HIF-1α and HIF-2α are synthesized constitutively, and hydroxylation of at least one of two critical proline residues in their oxygen-dependent degradation domain (ODDD), Pro402 and Pro564 in HIF-1α, generates a binding site for the von Hippel-Lindau E3 ubiquitin ligase complex that targets HIF-α for rapid proteasomal degradation under normoxic conditions. This hydroxylation is inhibited in hypoxia, so that the HIF-α escape degradation, translocate to the nucleus, and dimerize with HIF-β. The dimer then recognizes the HIF-responsive elements in various hypoxia-regulated genes (5–7).

All P4Hs are 2-oxoglutarate dioxygenases that require Fe2+, 2-oxoglutarate, O2, and ascorbate. The C-terminal region of the C-P4H α subunits contains four conserved residues, two histidines, and one aspartate that bind the Fe2+ atom and a lysine that binds the 2-oxoglutarate (15). The HIF-P4Hs also contain the three Fe3+-binding residues, whereas the lysine that binds the 2-oxoglutarate is replaced by an arginine (12–14).

We report here on the characterization of a novel human P4H (P4H-TM), which, unlike any other P4H characterized so far, possesses a transmembrane domain. Oehme et al. (16) have studied this same protein, noted its sequence similarity to the C-P4Hs but not the HIF-P4Hs, shown it to be associated with...
the ER, suggested an orientation in which the catalytic site may be on the cytosolic surface, and shown that its overexpression suppressed cellular HIF-1α and HIF-2α reporter transactivation activity and reduced the HIF-2α protein level. P4H-TM was inactive in vitro, however, suggesting that it may need a modification to become active or may modify a member of the HIF degradation pathway without directly acting on HIF-α (16). Correspondingly it was not possible to demonstrate directly whether P4H-TM is indeed a P4H (16). Our surveys of gene bank sequences indicated that the gene encoding P4H-TM is found only in vertebrates, including zebrafish, but not in flies and nematodes. Its sequence resembles more closely those of the C-P4Hs than the HIF-P4Hs, but P4H-TM lacks sequences corresponding to those of the peptide substrate-binding domain of the C-P4Hs. P4H-TM is a hypoxia-inducible homodimer, it is N-glycosylated and is located in the ER membrane in an orientation in which the catalytic site is inside the lumen. Despite this location, its overexpression in cultured human neuroblastoma Kelly cells reduced HIF-α-ODDD reporter construct levels, and its siRNA increased the HIF-1α protein level. Furthermore, recombinant human P4H-TM produced in insect cells hydroxylated the two critical prolines in HIF-1α and its ODDD in vitro, with a preference for the C-terminal hydroxylation site, whereas it did not hydroxylate any prolines in recombinant type I procollagen chains.

**EXPERIMENTAL PROCEDURES**

Cloning, Expression, and Analysis of Recombinant Human P4H-TM—Full-length human P4H-TM cDNA was cloned into the baculovirus expression vector pVL1392 (for details see supplemental material). In addition, baculovirus vectors encoding the signal sequence of PDI or GP67, a His6 tag, and P4H-TM (for details see supplemental material). In addition, baculovirus vectors encoding the signal sequence of PDI or GP67, a His6 tag, and P4H-TM (for details see supplemental material). In addition, baculovirus vectors encoding the signal sequence of PDI or GP67, a His6 tag, and P4H-TM were cotransfected into Sf9 cells with BaculoGold DNA (PharMingen), and the resultant viruses were amplified in Dulbecco’s (Biochrom) or Eagle’s (Sigma) minimal essential medium or in RPMI 1640 (Sigma) medium with 10% fetal bovine serum (BioClear) and 50 μg/ml ascorbate. The cells were exposed to hypoxia in 1% O2 balanced with 5% CO2 and 95% N2 for 20 h in an InVivo2 400 hypoxia work station (Ruskinn Technologies). The culture medium was removed, and cell extracts were obtained by lysing the cells in 150 mM NaCl, 0.1% SDS, 20 mM Tris-HCl, pH 6.8, followed by analysis on SDS-PAGE and Western blotting with P4H-TM and β-actin antibodies (Sigma). P4H Activity Assays—P4H activity was assayed by the hydroxylation-coupled decarboxylation of 2-oxo[1-14C]glutarate (17) or determination of the amount of radioactive 4-hydroxyproline formed when wild-type, Pro^{302} → Ala, Pro^{566} → Ala, or double mutant HIF-1α ODDD, full-length HIF-1α, or proa1 chains of type I procollagen translated in the presence of [2,3,4,5-3H]proline in a rabbit reticuloocyte lysate were used as a substrate (18, 19) (for details see supplemental material).

**Analysis of the Expression of Endogenous P4H-TM in Various Human Cell Lines**—Human adult fibroblasts (CRL-2068) and embryonic kidney (HEK293), hepatocellular carcinoma (Hep3B), fibrosarcoma (HT1080), and neuroblastoma (Kelly) cells were obtained from the American Type Culture Collection or the German Resource Centre for Biological Material and cultured in Dulbecco’s (Biochrom) or Eagle’s (Sigma) minimal essential medium or in RPMI 1640 (Sigma) medium with 10% fetal bovine serum (BioClear) and 50 μg/ml ascorbate. The cells were exposed to hypoxia in 1% O2 balanced with 5% CO2 and 95% N2 for 20 h in an InVivo2 400 hypoxia work station (Ruskinn Technologies). The culture medium was removed, and cell extracts were obtained by lysing the cells in 150 mM NaCl, 0.1% SDS, 20 mM Tris-HCl, pH 6.8, followed by analysis on SDS-PAGE and Western blotting with P4H-TM and α-tubulin (Sigma) antibodies.

**Immunofluorescence and Immuno-EM**—Cellular location of endogenous P4H-TM in normoxic and hypoxic HEK293 cells was studied by immunofluorescence staining and the ultrastuctural location of endogenous and recombinant P4H-TM in HEK293 and S9 cells by immuno-EM staining with the P4H-TM antibody (for details see supplemental material).

**Transient Overexpressions and ODDD Reporter Gene Assays**—Triplicate wells of 4 × 10^4 Kelly cells seeded in 96-well plates were cotransfected with 37.5 ng of HIF-ODDD reporter plasmids in which the N-terminal or C-terminal region of the
HIF-1α or HIF-2α ODDDS (20) is cloned between an NF-κB activation domain and a Gal4 DNA-binding domain (Stratagene) followed by a Gal4-dependent luciferase reporter, 12.5 ng of pSV-βGal (Promega), and 0–100 ng of pCDNA-HIF-P4H-2 or pCDNA-P4H-TM with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. The empty vector pCDNA3.1 (Invitrogen) was used to obtain equal amounts of DNA in each cotransfection. The cells were cultured in the presence of the transfection mixture in RPMI and 10% fetal bovine serum for 24 h, after which cell extracts were prepared and analyzed for β-galactosidase and luciferase activities (Tropix). Luciferase levels were normalized to β-galactosidase activity to assess the transfection efficiency.

siRNA Transfections and HIF-1α Protein Determination—Kelly cells (1.6 × 10⁶) were transfected with 120 pmol of siRNA for HIF-P4H-1, 2, or 3 (Ambion) or P4H-TM (Dharmacon) or a nontargeting control siRNA (siCNTL, Dharmacon) with an Amaxa nucleoplatinator according to the manufacturer’s instructions (Solution V, Program D-23) and seeded to 50% confluency. The cells were cultured in RPMI and 10% fetal bovine serum for 24 h, after which cell extracts were prepared and analyzed for HIF-1α protein levels using a HIF-1α whole cell lysate kit (MesoScale Discovery). The amount of HIF-1α was normalized to mg of total protein as determined by a BCA assay (Pierce). The efficiency of silencing by siRNA was monitored by 18 S normalized quantitative PCR on Cells-to-Signal (Ambion) lysates with an ABI7000 machine, and the values were found to be 78, 82, 51, and 93% for HIF-P4H-1, HIF-P4H-2, HIF-P4H-3, and P4H-TM mRNAs, respectively.

Intron-spanning probes for HIF-P4H-1 (Hs00363196_m1), HIF-P4H-2 (Hs00254392_m1), HIF-P4H-3 (Hs00222966_m1), and P4H-TM (Hs00214665_m1) from Applied Biosystems were used with QuantiTect Multiplex reverse transcription-PCR reagent (Qiagen) according to the manufacturer’s recommendations.

RESULTS

The P4H-TM Sequence Is More Closely Related to the C-P4H than the HIF-P4H Sequences but Lacks Sequences Corresponding to the Peptide Substrate-binding Domain of the C-P4Hs—The human P4H-TM cDNA encodes a 502-residue polypeptide with no signal sequence but a putative transmembrane domain (Fig. 1, residues 59–82). Our data base searches indicated that P4H-TM is also found in other verte-
brates and that a polypeptide with 51% identity to the human sequence is present in the zebrafish (accession number ENSDART0000012024.3) but not in Caenorhabditis elegans or Drosophila melanogaster. The human P4H-TM sequence is 14–15% identical to those of the human C-P4H α(I)–α(III) subunits and 10–13% identical to those of the human HIF-P4Hs 1–3 (Fig. 1). The identity is highest within the catalytically important C-terminal region, P4H-TM residues 314–502 being 26–28% and 13–15% identical to the corresponding residues in the C-P4H α subunits and the HIF-P4Hs, respectively (Fig. 1). The two histidines and one aspartate that bind the C-5 carboxyl group of 2-oxoglutarate are conserved (stars in Fig. 1), the basic residue being a lysine, as in the C-P4Hs, whereas it is an arginine in the HIF-P4Hs (12–15, 21, 22). The catalytically critical C-terminal region of P4H-TM is thus more closely related to those of the C-P4Hs than the HIF-P4Hs, both in terms of overall identity and on account of the 2-oxoglutarate-binding residue. The C terminus of the polypeptide has a sequence Arg-Val-Glu-Leu that is a functional variant of the Lys-Asp-Glu-Leu ER retention signal.³ The human polypeptide has two potential N-glycosylation sites, Asn-Val-Thr (residues 368–370) and Asn-Arg-Thr (residues 382–384).

³ Lloyd Ruddock, personal communication.

The P4H-TM sequence shows no similarity to those of the C-P4H α subunits, and none of the seven tyrosines is conserved in the human P4H-TM sequence (Fig. 1). As pointed out (16), a calcium-binding EF hand motif is found between P4H-TM residues 192 and 249.

P4H-TM mRNA Is Expressed in Many Human Tissues, with the Highest Levels in the Pancreas, Heart, Skeletal Muscle, Brain, Placenta, and Kidney—Northern analysis indicated that P4H-TM mRNA is expressed in many human tissues, with adult tissues showing higher levels than fetal tissues (Fig. 2, A–C). Three mRNA species of 2.7, 2.3, and 1.8 kb were seen in several tissues, the 1.8-kb species being the main form in all except the brain. Additional experiments using PCR indicated that the mRNA is also expressed at low levels in epiphyseal cartilage, where C-P4H II is the main C-P4H form (2), and in fibroblasts (Fig. 2D). The expression pattern differs markedly from those of the C-P4H mRNAs. The C-P4H α(I)-(III) mRNAs, for example, show very little expression in the adult brain where P4H-TM mRNA was expressed at high levels. PCR analysis showed that P4H-TM mRNA is also expressed in all of the tumor samples studied (Fig. 2E).

Recombinant Human P4H-TM Is a Homodimer and Is in Part Cleaved after the Transmembrane Domain—A recombinant baculovirus encoding human P4H-TM was generated and used to infect Sf9 insect cells. The cells were homogenized in a buffer containing Triton X-100 and centrifuged, and the pellets were solubilized in 1% SDS. The samples were analyzed by SDS-PAGE under reducing conditions followed by Coomassie Blue staining or Western blotting (Fig. 3A). The recombinant polypeptide was mainly found in the SDS extract, but significant amounts were also seen in the Triton X-100 fraction (Fig. 3A, lanes 1, 2, 7, and 8). The polypeptide was found in two forms (Fig. 3A, lanes 7 and 8). N-terminal sequencing indicated that these began with Met and Asp (not shown), indicating that some of the full-length polypeptides become cleaved after the transmembrane domain. Western blot analysis of the soluble fraction of human embryonic kidney HEK293 cells transfected with a plasmid encoding full-length P4H-TM likewise showed the presence of two immunoreactive polypeptides (Fig. 3A, lane 11) with mobilities identical to those in the insect cells, indicating that the cleavage also occurs in mammalian cells. The molecular composition of recombinant P4H-TM was studied by gel filtration of the soluble fraction from insect cells expressing the full-length polypeptide. P4H-TM was found in fractions corresponding to molecular masses of ~105–120 kDa and 85–90 kDa, suggesting that both the full-length P4H-TM and its cleaved form exist as homodimers (details not shown).
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To overcome difficulties that are common in the purification of membrane proteins, two additional viruses were generated to produce truncated P4H-TM polypeptides starting from Asp^{88}, thus lacking the transmembrane domain. One of them contained a baculovirus GP67 signal sequence followed by a His tag in its N terminus. The majority of this recombinant polypeptide was secreted into the culture medium by the H5 insect cells but not by the Sf9 cells. The other virus encoded a polypeptide beginning at Asp^{88} and contained a PDI signal sequence and a His tag, the majority of this polypeptide remaining inside both cell types (Fig. 3A, lanes 3, 4, 9, and 10). Both polypeptides could be purified to homogeneity from the culture medium or the soluble fraction of the cell lysate, depending on the signal sequence, using a metal chelate resin and imidazole elution (Fig. 3A, lanes 5 and 6). N-terminal sequencing indicated that the signal peptide had been cleaved from both polypeptides (not shown).

To study whether recombinant P4H-TM is glycosylated, purified P4H-TM starting from Asp^{88} was treated with N-glycosidase F and analyzed by SDS-PAGE followed by Coomassie Blue staining. A distinct increase was found in the mobility of the polypeptide after the treatment, indicating that it had been synthesized in the insect cells in an N-glycosylated form (Fig. 3B, lanes 1 and 2). Recombinant full-length P4H-TM and its cleaved form were also found to be glycosylated in HEK293 cells, because an increase in their mobility was seen after N-glycosidase F treatment (Fig. 3B, lanes 3–6). The CD spectrum of the polypeptide beginning at Asp^{88} was typical of a folded protein (details not shown).

P4H-TM Is Expressed in Many Human Cell Lines and Is Induced by Hypoxia.—We studied expression of the P4H-TM polypeptide in cultured human adult fibroblasts and embryonic kidney (HEK293), hepatoma (Hep3B), fibrosarcoma (HT1080), and neuroblastoma (Kelly) cells by Western blotting. Two immunoreactive polypeptides with molecular masses corresponding to those of glycosylated and nonglycosylated recombinant full-length P4H-TM (see above) were seen in all of the cell lines studied (Fig. 4). Interestingly, the amount of P4H-TM was greatly increased by hypoxia in all the cell lines except the HT1080 cells, where the increase was only moderate, and the Kelly cells, where no increase was observed (Fig. 4). The normoxic expression level of P4H-TM was highest in the HT1080 cells, whereas P4H-TM was not detected in normoxic HEK293 and Hep3B cells at all (Fig. 4). In agreement with the induction by hypoxia, the 5' untranslated sequence of the gene encoding human P4H-TM was found to contain five hypoxia-responsive elements 5'-RCGTG-3', at positions −1961, −1952, −874, −757, and −197 with respect to the translation start site.

P4H-TM Is an ER Transmembrane Protein with its Catalytic Site Inside the Lumen.—To study the cellular location of endogenous P4H-TM, normoxic and hypoxic HEK293 cells were analyzed by immunofluorescence staining with the P4H-TM antibody. P4H-TM was located in the cytoplasm and showed a reticular patterning resembling the arrangement of the ER (details not shown). The staining was markedly increased in the hypoxic cells, whereas hypoxia did not affect the location (details not shown).

To study the ultrastructural localization of P4H-TM, Sf9 insect cells were infected with a baculovirus encoding the full-length enzyme and analyzed by immuno-EM. The antibody used had been generated against a polypeptide beginning after the transmembrane domain at Asp^{88} and thus reveals the location of the catalytic C-terminal region. Gold particles were seen almost exclusively in close contact with ER membranes, either inside the lumen or on the membranes (Fig. 5A). Counting of more than 1000 particles in 15 randomly chosen 0.18-μm^{2} squares indicated that 69% of them were inside the lumen, 29% on the membranes, and only 2% on the cytoplasmic side. Because the diameter of the gold particles is 10 nm, and the spacer arm of the secondary antibody is ~5 nm, the grains appearing on the membranes may also represent protein that is
located inside the ER. An identical location was found in HEK293 cells that were stably transfected to express full-length P4H-TM (Fig. 5B). Counting of these grains revealed that 76% of them were inside the ER, 22% on its membranes, and 1% in the cytoplasm. To verify that this result was not an artifact caused by the high expression levels of the recombinant protein, immuno-EM was repeated with nontransfected HEK293 cells (Fig. 5C). The gold particle density was markedly lower in these cells than in the Sf9 and HEK293 cells expressing the recombinant P4H-TM, but the distribution of the endogenous P4H-TM was identical. Counting of 120 gold particles indicated that 67% of the grains were inside the ER, 33% on its membranes, and 0% in the cytoplasm. Our data thus indicate that P4H-TM is an ER membrane protein located in an orientation in which its catalytic site is inside the lumen. This location is in agreement with the presence of the Arg-Val-Glu-Leu variant of the ER retention signal in the C terminus of the polypeptide and with our data (above), indicating that P4H-TM is N-glycosylated.

P4H-TM Overexpression Reduces Cellular Recombinant HIF-α ODDD Levels, but Silencing of P4H-TM Increases HIF-1α Protein Level—Cultured Kelly cells were transiently cotransfected with (a) a constant amount of a reporter plasmid for the N-terminal or C-terminal region of the HIF-1α or HIF-2α ODDD and (b) increasing amounts of a plasmid encoding full-length P4H-TM or HIF-P4H-2. Overexpression of either enzyme reduced the levels of all four ODDD polypeptides in a similar dose-dependent manner and with approximately equal efficiency, although there was some variation in detail between the individual experiments (a typical experiment is shown in Fig. 6A). In additional experiments, Kelly cells were transfected with siRNA oligonucleotides for P4H-TM or any of the three HIF-P4Hs. The cells were cultured for 48 h, the amount of HIF-1α was quantitated in cell extracts, and the values were normalized per mg total amount of soluble cell protein. Silencing of HIF-P4H-2 increased the HIF-1α level to ~2.1-fold, whereas silencing of HIF-P4H-1 or HIF-P4H-3 increased the HIF-1α level to ~1.5–1.8-fold (Fig. 6B). Silencing of P4H-TM likewise increased the HIF-1α level to ~1.8-fold (Fig. 6B). Our data thus indicate that overexpression and silencing of P4H-TM influence the stability of HIF-1α in a similar manner to those of the HIF-P4Hs.

Purified Recombinant Human P4H-TM Hydroxylates the Two Critical Prolines in HIF-1α, with a Strong Preference for the C-terminal Proline—The C-P4Hs and HIF-P4Hs, in the absence of any peptide substrate, catalyze an uncoupled decarboxylation of 2-oxoglutarate at a rate of ~3 mol/mol enzyme/min in the case of C-P4H I (15) and 1.3–1.6 mol/mol/min in the cases of the three HIF-P4Hs (26). P4H-TM was also found to catalyze an uncoupled 2-oxoglutarate decarboxylation, but the rate varied markedly between enzyme preparations, presumably because of rapid inactivation of the enzyme during purification. Inactivation is typical also for many other hydroxylases that belong to the 2-oxoglutarate dioxygenase family, e.g. the
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C-P4Hs (17), lysyl hydroxylases (27), and HIF-P4Hs (26), but P4H-TM seems to become inactivated especially easily. The highest uncoupled decarboxylation rate observed was 0.2 mol/mol/min, being thus markedly lower than with the other P4Hs.

Hydroxylation of synthetic peptides was studied by using the nonpurified full-length P4H-TM and the purified recombinant P4H-TM beginning at Asp⁸⁸. The enzyme reaction was carried out as reported either for the C-P4Hs or the HIF-P4Hs, and the assay used was based on measurement of the hydroxylation-coupled stoichiometric release of ¹⁴CO₂ from 2-oxo[¹-¹⁴C]glutarate (17, 28). The substrates were produced in the presence of L-2,3,4,5-³H proline-labeled HIF-1α ODDDs by P4H-TM, HIF-P4H-2 and -3, and C-P4H-I. The amount of 4-hydroxy[³H]proline formed was analyzed by a specific radiochemical assay for radioactive 4-hydroxyproline.

Because it was recently found that HIF-P4Hs have much lower Kᵥ values for recombinant HIF-1α and HIF-2α ODDDs of 248 and 215 residues, respectively, than for synthetic 19–20- and 35-residue peptides (18), we decided to study the hydroxylation of the above HIF-1α ODDDs, its mutants, and the full-length HIF-1α polypeptide produced in rabbit reticulocyte lysates as recombinant [L-2,3,4,5-³H]proline-labeled polypeptides. Aliquots of the translation products were used as substrates for the purified recombinant P4H-TM beginning at Asp⁸⁸ or purified recombinant HIF-P4H-1, 2, or 3 produced in insect cells (18, 26). The reaction was carried out as reported for the HIF-P4Hs (18), and the 4-hydroxy[³H]proline formed was assayed in hydrolyzed samples (19).

The full-length HIF-1α polypeptide and its ODDD were found to serve as P4H-TM substrates in five independent experiments with separate enzyme preparations, but the amount of 4-hydroxy[³H]proline formed varied considerably between experiments, and many other experiments gave essentially no hydroxylation, presumably due to inactivation of the enzyme. The amount of 4-hydroxy[³H]proline formed by P4H-TM in the best experiment was ~8000 dpm/2 × 10⁶ dpm incorporated [³H]proline in the full-length HIF-1α and 17200 dpm/2 × 10⁶ dpm in its ODDD, both values being ~20% of those obtained with an equal amount of HIF-P4H-2 and 50% of those obtained with an equal amount of HIF-P4H-3 (Table 1). The amount of 4-hydroxy[³H]proline formed by P4H-TM in the HIF-1α ODDD in another experiment was 6200 dpm/2 × 10⁶ dpm, being 13% of that obtained with an equal amount of HIF-P4H-1 in the same experiment (details not shown). A P402A mutant HIF-1α ODDD in which the Pro⁵⁶⁴ hydroxylation site remained intact was almost as good a P4H-TM substrate as the wild-type ODDD, whereas a P564A mutant ODDD, possessing only the Pro⁴⁰₂ hydroxylation site, gave a 4-hydroxy[³H]proline value that was only ~10% of that obtained with the wild type (Table 1). This hydroxylation pattern is very similar to that seen with HIF-P4H-3 (Table 1). We did not see a corresponding difference in the hydroxylation-mediated degradation of the N-terminal and C-terminal regions of the HIF-1α ODDD by P4H-TM in Kelly cells, however (Fig. 6A), probably because the expression level of the N-terminal region of the HIF-1α ODDD was only ~10–13% of that of the C-terminal region. The amount of overexpressed P4H-TM may therefore have been sufficient for the hydroxylation of the N-terminal region to the same relative extent as the C-terminal region.

A low level of 4-hydroxy[³H]proline formation was obtained with P4H-TM even with a P402A, P564A double mutant ODDD (Table 1). Our data thus indicate that P4H-TM also hydroxylated at a low rate some additional proline(s) that are not recognized by the three HIF-P4Hs. No 4-hydroxy[³H]proline formation was detected when the recombinant HIF-1α ODDD was tested as a substrate for C-P4H-I (Table 1) or when [³H]proline-labeled recombinant type I procollagen chains were used as substrates for P4H-TM (details not shown).

**DISCUSSION**

Our data indicate that the actions of P4H-TM resemble closely those of the three well characterized HIF-P4Hs. Purified recombinant P4H-TM hydroxylated the two critical prolines in [³H]proline-labeled HIF-1α and its ODDD in vitro, but P4H-TM diverged from the three HIF-P4Hs in that it also acted at a low rate on some of the other proline residue(s) in these polypeptides. Furthermore, overexpression of P4H-TM reduced the cellular HIF-α ODDD polypeptide levels in a manner very similar to that produced by overexpression of HIF-P4H-2, and silencing of endogenous P4H-TM by siRNA increased the cellular HIF-1α level in a manner very similar to that achieved by silencing of HIF-P4Hs.

Although the C-terminal catalytic region of P4H-TM is more closely related to those of the C-P4Hs than the HIF-P4Hs, P4H-TM is not a C-P4H, because P4H-TM failed to hydroxylate procollagen polypeptides in vitro. Furthermore, P4H-TM lacks any sequences corresponding to the peptide substrate-binding domain (23) of the C-P4Hs. The substrate requirements of the two known P4H families are distinctly different, because the C-P4Hs act almost exclusively on proline residues.

| TABLE 1  | 4-Hydroxy[³H]proline formed in full-length HIF-1α and wild-type and mutant HIF-1α ODDDs by P4H-TM, HIF-P4H-2 and -3, and C-P4H-I |
| --- | --- |
| l-[2,3,4,5-³H]Proline-labeled substrate | Enzyme | 4-Hydroxy[³H]proline formed per 2 × 10⁶ dpm of incorporated proline |
| HIF-1α | P4H-TM | 8,000 |
|  | HIF-P4H-2 | 42,100 |
|  | HIF-P4H-3 | 15,900 |
| HIF-1α ODDD | C-P4H-I | 0 |
|  | P4H-TM | 17,200 |
|  | HIF-P4H-2 | 76,400 |
|  | HIF-P4H-3 | 36,100 |
| HIF-1α ODDD P402A | C-P4H-I | 0 |
|  | P4H-TM | 15,400 |
|  | HIF-P4H-2 | 44,800 |
|  | HIF-P4H-3 | 38,100 |
| HIF-1α ODDD P564A | C-P4H-I | 0 |
|  | P4H-TM | 2,100 |
|  | HIF-P4H-2 | 38,000 |
|  | HIF-P4H-3 | 3,100 |
| HIF-1α ODDD P402A,P564A | C-P4H-I | 0 |
|  | P4H-TM | 1,200 |
|  | HIF-P4H-2 | 0 |
|  | HIF-P4H-3 | 200 |
in Xaa-Pro-Gly sequences, whereas the two prolines to be hydroxylated in various HIF-αs are present in Leu-Xaa-Xaa-Leu-Ala-Pro-Xaa sequences (2, 5, 7). The substrate requirements of the HIF-P4Hs are nevertheless highly redundant in that alanine is the only relatively but not absolutely strict requirement in addition to the proline itself, the substrate specificity being probably achieved by multiple interactions, none of which is absolutely critical (30, 31).

Although P4H-TM acted on HIF-1α and its ODDD in vitro and influenced the HIF-α ODDD reporter construct and HIF-1α levels in the same way as HIF-P4Hs in intact cells, its cellular location was distinctly different from those of the HIF-P4Hs. HIF-P4H-1 is exclusively found in the nucleus, the majority of HIF-P4H-2 is found in the cytoplasm, and HIF-P4H-3 is evenly distributed in the cytoplasm and nucleus, suggesting that HIF-α is hydroxylated in the cytoplasm and nucleus (32, 33). Our data demonstrate that P4H-TM is located in ER membranes in an orientation in which its catalytic sites are inside the lumen. The levels of HIF-αs in normoxic cells are so low that they are rarely detected in normoxia, whereas numerous studies have demonstrated that HIF-α is located in the nucleus in hypoxia. It is therefore not clear how our transfected P4H-TM, which had a location identical to that of the endogenous enzyme, was able to act on HIF-α ODDDs in the same way as transfected HIF-P4H-2, and how the silencing of endogenous P4H-TM by siRNA influenced the HIF-1α protein level in a similar manner to silencing of HIF-P4Hs. Some studies have reported localization of substantial amounts of HIF-α in the ER in normoxia, possibly because of interaction with pVHL on the cytosolic surface of ER membranes (34). Interestingly, HIF-1α and HIF hydroxylases have been shown to be targeted to peroxisomes in hypoxic primary hepatocytes, indicating that these polypeptides can also be found in membrane-ous cytoplasmic organelles (35).

Hypoxia increased the P4H-TM level in several cultured human cell lines, whereas the P4H-TM location was not affected, as shown by immunofluorescence staining in hypoxic HEK293 cells. Nevertheless, no significant hypoxic induction was seen in Kelly cells, nor has any been previously found in A549 human lung carcinoma cells (16). HIF-P4H-2 and 3 mRNAs are likewise induced by hypoxia, whereas expression of the HIF-P4H-1 mRNA is oxygen-independent (36, 37).

In addition to the major P4H-TM form located in the ER membranes, cells were also found to contain a N-terminally truncated polypeptide. It is currently unknown whether a fraction of the shed P4H-TM form is somehow transported to the cytoplasm or nucleus, but our immunofluorescence and immuno-EM data gave no evidence for at least any major presence of P4H-TM in either of these locations. Furthermore, because the polypeptide has an ER retention signal, the shed form is likely to be retained within the ER. It is therefore unknown whether the shed form has any specific function that is different from those of the full-length enzyme.

All three established HIF-P4Hs contribute to the regulation of the HIF-α isoforms, the contributions of the individual enzymes being strongly dependent on their cellular abundance, but the enzymes also appear to have specificity in their functions, such as HIF-P4H-3 possibly being more active on HIF-2α than HIF-1α (20). P4H-TM was also found to act on HIF-αs, but its different cellular location suggests that it may also have specific functions, and the two enzyme families may work in a complimentary fashion. Furthermore, it has often been speculated that the HIF-P4Hs may have additional physiological substrates besides the HIF-αs (6, 32, 37), this suggestion being supported by their high degree of substrate sequence redundancy (30, 31). Nevertheless, no other proteins have so far been reported to be hydroxylated by them, although indirect evidence has been reported for the hydroxylation of the large subunit of RNA polymerase II and IκB kinase-β by HIF-P4Hs and the activating transcription factor ATF-4 by HIF-P4H-3 (38–40). The HIF asparaginyl hydroxylase factor inhibiting HIF was recently shown to hydroxylate ankyrin repeats in IκB proteins in addition to the C-terminal transactivation domain of HIF-1α and HIF-2α (41). Our data do not exclude the possibility that P4H-TM may have additional physiological substrates and that this ER transmembrane enzyme may play an even more important role in the hydroxylation of those substrates than in the regulation of HIF-αs. HIF-P4Hs are found in nematodes and flies as well as in vertebrates (3, 5), whereas we did not find P4H-TM sequences in nematodes or flies, although a polypeptide with 51% sequence identity was present in zebrafish. If P4H-TM has substrates other than HIF-αs, they are thus likely to be found only in vertebrates.

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REFERENCES

1. Kivirikko, K. I., and Pihlajaniemi, T. (1998) Adv. Enzymol. Rel. Areas Mol. Biol. 72, 325–398
2. Myllyharju, J. (2003) Matrix Biol. 22, 15–24
3. Myllyharju, J., and Kivirikko, K. I. (2004) Trends Genet. 20, 33–43
4. Semenza, G. L. (2003) Nat. Rev. Cancer 3, 721–732
5. Schofield, C. J., and Ratcliffe, P. J. (2004) Nat. Rev. Mol. Cell. Biol. 5, 343–354
6. Dann, C. E., III, and Bruck, R. K. (2005) Biochem. Biophys. Res. Commun. 338, 639–647
7. Kaelin, W. G., Jr. (2005) Annu. Rev. Biochem. 74, 115–128
8. Kukkola, L., Hieta, R., Kivirikko, K. I., and Myllyharju, J. (2003) J. Biol. Chem. 278, 47685–47693
9. Van Den Diepstraten, C., Papay, K., Bolender, Z., Brown, A., and Pickering, J. G. (2003) Circulation 108, 508–511
10. Ivan, M., Kondo, K., Yang, H., Kim, W., Vialiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G., Jr. (2001) Science 292, 461–468
11. Jaakkola, P., Mole, D. R., Tian, Y.-M., Wilson, M. I., Gielbert, J., Gaskell, S. J., von Kriegsheim, A., Abbestret, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) Science 292, 468–472
12. Bruck, R. K., and McKnight, S. L. (2001) Science 294, 1337–1340
13. Epstein, A. C. R., Gleadle, J. M., McNell, L. A., Hewitson, K. S., O’Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhandha, A., Tian, Y.-M., Masson, N., Hamilton, D. L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P. H., Pugh, C. W., Schofield, C. J., and Ratcliffe, P. J. (2001) Cell 107, 43–54
14. Ivan, M., Haberberger, T., Gervasi, D. C., Michelson, K. S., Günzler, V., Kondo, K., Yang, H., Sorokina, I., Conaway, R. C., Conaway, J. C., and Kaelin, W. G., Jr. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 13459–13464
15. Myllyharju, J., and Kivirikko, K. I. (1997) EMBO J. 16, 1173–1180
16. Oehme, F., Ellinghaus, P., Kolhkop, P., Smith, T. J., Ramakrishnan, S., Hütter, J., Schramm, M., and Flamme, I. (2002) Biochem. Biophys. Res. Commun. 296, 343–349
17. Kivirikko, K. I., and Myllylä, R. (1982) Methods Enzymol. 82, 245–304
18. Koivunen, P., Hirslilä, M., Kivirikko, K. I., and Myllyharju, J. (2006) J. Biol. Chem. 281, 28712–28720
19. Juva, K., and Prockop, D. J. (1966) Anal. Biochem. 15, 77–83
20. Appelhoff, R. J., Tian, Y.-M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Gleadle, J. M. (2004) J. Biol. Chem. 279, 38458–38465
21. Clifton, I. J., McDonough, M. A., Ehrismann, D., Kershaw, N. J., Granatino, N., and Schofield, C. J. (2006) J. Inorg. Biochem. 100, 644–669
22. McDonough, M. A., Li, V., Flashman, E., Chowdhury, R., Mohn, C., Liénard, B. M. R., Zondlo, J., Oldham, N. J., Clifton, I. J., Lewis, J., McNeill, L. A., Kurzeja, R. J. M., Hewitson, K. S., Yang, E., Jordan, S., Syed, R. S., and Schofield, C. J. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 9814–9819
23. Myllyharju, J., and Kivirikko, K. I. (1999) EMBO J. 18, 306–312
24. Hieta, R., Kukkola, L., Permi, P., Pirilä, P., Kivirikko, K. I., Kilpeläinen, L., and Myllyharju, J. (2003) J. Biol. Chem. 278, 34966–34974
25. Pekkala, M., Hieta, R., Bergmann, U., Kivirikko, K. I., Wierenga, R. K., and Myllyharju, J. (2004) J. Biol. Chem. 279, 52255–52261
26. Hirslilä, M., Koivunen, P., Xu, L., Seeley, T., Kivirikko, K. I., and Myllyharju, J. (2005) FASEB J. 19, 1308–1310
27. Rautavuoma, K., Takaluoma, K., Passoja, K., Pirskanen, A., Kvist, A.-P., Kivirikko, K. I., and Myllyharju, J. (2002) J. Biol. Chem. 277, 23084–23091
28. Hirslilä, M., Koivunen, P., Günzler, V., Kivirikko, K. I., and Myllyharju, J. (2003) J. Biol. Chem. 278, 30772–30780
29. Hieta, R., and Myllyharju, J. (2002) J. Biol. Chem. 277, 23965–23971
30. Huang, L. E., Pete, E. A., Schau, M., Milligan, J., and Gu, J. (2002) J. Biol. Chem. 277, 41750–41755
31. Li, D., Hirslilä, M., Koivunen, P., Brenner, M. C., Xu, L., Yang, C., Kivirikko, K. I., and Myllyharju, J. (2004) J. Biol. Chem. 279, 55051–55059
32. Huang, J., Zhao, Q., Mooney, S. M., and Lee, F. S. (2002) J. Biol. Chem. 277, 39792–39800
33. Metzen, E., Berchner-Pfannschmidt, U., Stengel, P., Marxsen, J. H., Stolze, I., Klinger, M., Huang, W. Q., Wotzlaf, C., Hellwig-Bürgel, T., Jelkmann, W., Acker, H., and Fandrey, J. (2003) J. Cell Sci. 116, 1319–1326
34. Liu, Q., Berchner-Pfannschmidt, U., Möller, U., Brecht, M., Wotzlaf, C., Acker, H., Jungermann, K., and Kietzmann, T. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 4302–4307
35. Khan, Z., Michalopoulos, G. K., and Stolz, D. B. (2006) Am. J. Pathol. 169, 1251–1269
36. Berra, E., Benizri, E., Ginouvès, A., Volmat, V., Roux, D., and Pouységur, J. (2003) EMBO J. 22, 4082–4090
37. Metzen, E., and Ratcliffe, P. J. (2004) Biol. Chem. 385, 223–230
38. Kuznetsova, A. V., Müller, J., Schnell, P. O., Nash, J. A., Ignacak, M. L., Sanchez, Y., Conaway, R. C., and Czyzyk-Krzeska, M. F. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2706–2711
39. Cummins, E. P., Berra, E., Comerford, K. M., Ginouvès, A., Fitzgerlad, K. T., Seeballuck, F., Godson, C., Nielsen, J. E., Moynagh, P., Pouységur, J., and Taylor, C. T. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 18154–18159
40. Köditz, J., Nesper, J., Wottawa, M., Stiehl, D. P., Camenisch, G., Franke, C., Myllyharju, J., Wenger, R. H., and Katschinski, D. (2007) Blood, in press
41. Cockman, M. E., Lancaster, D. E., Stolze, I. P., Hewitson, K. S., McDonough, M. A., Coleman, M. L., Coles, C. H., Yu, X., Hay, R. T., Ley, S. C., Pugh, C. W., Oldham, N. J., Masson, N., Schofield, C. J., and Ratcliffe, P. J. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 14767–14772