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Expression, Purification and Characterization of Human PHD1 in *Escherichia coli*

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The hypoxia-inducible factors (HIFs) play a central role in oxygen homeostasis. HIF prolyl hydroxylases (PHDs) modify HIFα subunits and thereby target them for proteasomal degradation. Mammalian PHDs comprise three isozymes, PHD1, PHD2 and PHD3, and belong to the iron(II)-2-oxoglutarate-dependent dioxygenase family. We have expressed full-length human PHD1 in *Escherichia coli*, and purified it to apparent homogeneity by immobilized Ni-affinity chromatography, cation-exchange HPLC followed by gel filtration. Fe^{2+} was found to have EC_{50} value of 0.64 μM and the purified enzyme showed maximal activity at 10 μM Fe^{2+}. The IC_{50} values for transition metal ions, Co^{2+}, Ni^{2+} and Cu^{2+}, were 58, 35 and 220 μM, respectively, in the presence of 100 μM Fe^{2+}. Mn^{2+} did not affect the activity <1 mM. Many transcription-related proteins are regulated by phosphorylation. Thus, recombinant PHD1 was examined for *in vitro* phosphorylation using protein kinase A, protein kinase Cα, casein kinase I and II and Erk2. The protein was most strongly phosphorylated by protein kinase Cα, and the phosphorylation sites were found to be Ser-132, Ser-226 and Ser-234. Mutation of Ser-132 or Ser-234 to Asp or Glu diminished the enzymatic activity to 25–60%, while mutation of Ser-226 scarcely influenced the activity.

Key words: HIF-1α stabilization, HIF prolyl hydroxylase, protein kinase Cα, recombinant PHD1, transition metal ions.

Abbreviations: DTT, dithiothreitol; HIF, hypoxia-inducible factor; IPTG, isopropyl-β-d(-)-thiogalactopyranoside; MALDI-TOF/TOFMS, matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry; OD_{600}, optical density at 600 nm; PHD, HIF prolyl hydroxylase; PKCα, protein kinase Cα; PMSF, phenylmethanesulphonyl fluoride.

The hypoxia-inducible transcription factors (HIFs) play a central role in the regulation of oxygen homeostasis. HIFs are heterodimers of two subunits; an oxygen-regulated α subunit (HIFα) and a common subunit HIF-1β (also known as Arnt) (*1–3*). The HIF-1α and HLF (EPAS1 or HIF-2α) subunits show similar DNA-binding specificity and dimerization property. In the presence of oxygen, HIFα proteins are hydroxylated at specific proline residues by HIF prolyl hydroxylases (PHDs). The resulting hydroxyproline residues are recognized by the VHL tumour suppressor protein in the E3 ubiquitin ligase complex. HIFα proteins are subsequently ubiquitylated and degraded by the proteasome (*2–6*). This hydroxylation, which requires oxygen, ceases under hypoxic conditions so that HIFα escapes degradation and forms a dimer with Arnt, and the dimer then becomes to bind to the hypoxia-response elements (HRE) in a number of hypoxia-inducible genes. Thus, PHDs have been characterized as cellular oxygen sensors (*7*, *8*). In mammalian cells, three PHD isozymes, PHD1, PHD2 and PHD3, have been identified (*6*). Although PHD1 mRNA level was highest in the testis, whereas PHD2 mRNA and PHD3 mRNA levels were highest in the heart (*9*), the mRNAs are shown to be ubiquitously expressed in all tissues examined (*10*, *11*) suggesting that they must have distinctive roles. Indeed, PHD2 was demonstrated recently to be the main oxygen sensor in mammalian cells by using siRNA-mediated gene silencing (*12*). Subcellular localization of the three enzymes is different from each other; PHD1 is predominantly localized to the nucleus, whereas PHD2 is present in the cytoplasm (*10*). PHD3 distributes evenly in both nucleus and cytoplasm (*10*). PHD2 and PHD3, but not PHD1, have been reported to be hypoxically induced in a HIF-1-dependent manner (*6*). While *Phd1*$^{-/-}$ and *Phd3*$^{-/-}$ mice survive normally, *Phd2*$^{-/-}$ embryos die at mid-gestation stages (*13*). Aragones *et al*. (*14*) reported that PHD1-deficient myofibers showed hypoxia tolerance and preserved mitochondrial respiration, while hypoxia tolerance was not observed in myofibres of heterozygous PHD2-deficient or homozygous PHD3-deficient mice. Although PHDs share considerable sequence similarity in the C-terminal catalytic domain, there are substantial differences in their N-terminal sequences (*6*). The function of these differing sequences is obscure. In order to characterize the enzymes in detail, large quantities of highly purified enzyme are required.

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Purification of PHDs with high purity from *Escherichia coli* is troublesome because of their unstability and/or insolubility. Detailed purification procedures for recombinant PHDs expressed in *E. coli* as a hexahistidine-tagged catalytic domain of PHD2 (*15*) and the hexahistidine-tagged PHD3 (*16*) were reported, but their enzymatic properties were not elucidated in detail. Here, we report a procedure for expression and purification of PHD1. We also report the characterization of the purified enzyme.

MATERIALS AND METHODS

Plasmids, Enzymes and Other Materials—pRSETA vector (Invitrogen, San Diego, CA, USA), pET-28a(+) vector (Novagen, Darmstadt, Germany), pCold I vector (Takara, Otu, Japan), Ni-NTA agarose (Qiagen, Valencia, CA, USA), protein kinase A (bovine, Sigma, St Louis, MO, USA), protein kinase Cα (recombinant, Wako, Osaka, Japan), casein kinase I (CKI) (recombinant, BioLabs, Ipswich, MA, USA), casein kinase II (CKII) (recombinant, BioLabs), p42 MAP kinase (Erk2) (recombinant, BioLabs), restriction enzymes (Takara), IPTG (Wako), ampicillin (Wako), kanamycin (Sigma), benzamidine (Sigma), PMSF (Sigma), thrombin (Itoham, Moriya, Japan), oligonucleotides (Nihon Gene Inc., Sendai, Japan) and a substrate peptide DLDLEALAPYIPADDDFQL (NAD-19, Genenet Co., Fukuoka, Japan) were purchased. pCP 2 vector was described previously (*17*).

Construction of Expression Plasmids—Plasmid pBos-PHD1-FLAG containing a full-length PHD1 cDNA was obtained by PCR with a Hep3B cDNA library, and details will be described elsewhere (K. Sogawa and J. Sugiyama, submitted for publication). The *Bam*HI/*Hin*dIII fragment of pBos-PHD1-FLAG was ligated to the *Hin*dIII/*Bam*HI fragments of pRSETA or pET-28a(+), resulting pRSETA-PHD1-FLAG or pET-28a(+)-PHD1-FLAG, respectively. pCP2-His6-PHD1-FLAG was generated by the ligation between the *Hin*dIII/*Nde*I fragment of pCP2 and the *Nde*I/*Hin*dIII fragment of pRSETA-PHD1-FLAG.

Preparation of Plasmids for Deletion Mutants—Preparation of expression plasmids for deletion mutants was carried out by PCR using Pfu Turbo (Stratagene, West Cedar Creek, TX, USA) DNA polymerase with the plasmid template, pRSETA-PHD1-FLAG. Used primers were as follows; for PHD1Δ1-161, GGTGAATTCAGTGCTGGGCTGATGG (forward) and ACCGAATTCACCATGATGATGATGA (reverse); for PHD1Δ123-161, GGTGAATTCAGTGCTGGGCTGATGG (forward) and ACCGAATTCGGCCCATTTCCGTTTG (reverse). The PCR products were digested with *Eco*RI and self-ligated, and *Nde*I/*Hin*dIII fragments were inserted between the *Hin*dIII and *Nde*I sites of the pCP2 vector. Authenticity of the produced DNA constructs was confirmed by DNA sequencing.

Introduction of Point Mutation—Introduction of point mutation was carried out by PCR using Pfu Turbo with the template, pRSETA-PHD1-FLAG. Used primers were as follows; for PHD1S132D, ATGCCCCTTCACCCGATA AACGGCCCTGGGCCA (forward) and TGGCCCAGGGC CGTTTATCGGGTGAAGGGGCAT (reverse); for PHD1S132E, ATGCCCCTTCACCCGAAAAACGGCCCTGGGCCA (forward) and TGGCCCAGGGCCGTTTTTCGGGTGAAG GGGCAT (reverse); for PHD1S226D, GAGACGGGCAGC TAGTGGATCAGAGGGCGATCCC (forward) and GGGA TCGCCCTCTGATCCACTAGCTGCCCGTCTC (reverse); for PHD1S226E, GAGACGGGCAGCTAGTGGAACAGAG GGCGATCCC (forward) and GGGATCGCCCTCTGTTCC ACTAGCTGCCCGTCTC (reverse); for PHD1S234D, GCG ATCCCGCCGCGCGATATCCGTGGGGACCAG (forward) and CTGGTCCCCACGGATATCGCGCGGCGGGATCGC (reverse); and for PHD1S234E, GCGATCCCGCCGCG CGAAATCCGTGGGGACCAG (forward) and CTGGTCC CCACGGATTTCGCGCGGCGGGATCGC (reverse). The PCR products were digested with *Dpn*I to remove the template DNA and then transformed to *E. coli* XL1. Authenticity of the obtained DNA constructs was confirmed by restriction analysis and DNA sequencing. Mutated pRSETA-PHD1 plasmids were digested with *Nde*I and *Hin*dIII, and the *Nde*I/*Hin*dIII fragments containing mutated sites were inserted between the *Hin*dIII and *Nde*I sites of pCP2.

Expression and Purification of Recombinant PHD1—A starter culture of 100 ml LB medium supplemented with 50 mg/l ampicillin was inoculated with a single colony of *E. coli* BL21 (DE3) with pCP2-His6-PHD1-FLAG in a shaker (100 r.p.m.) at 37°C overnight. A main culture of 1 l LB medium supplemented with 50 mg/l ampicillin was inoculated with 20 ml of the starter culture. In the case of pET-28a(+)-PHD1-FLAG, 30 mg/ml kanamycin was used as antibiotic instead of ampicillin. Inoculated culture was incubated about 2 h at 37°C in a shaker to $OD_{600} = 1.0$, then induced with isopropyl-β-D(-)-thiogalactopyranoside (IPTG) (0.2 mM) and grown for additional 4 h at 37°C in a shaker.

Cell pellets from 10 l culture were resuspended in 400 ml lysis buffer [50 mM Tris–HCl buffer, pH 7.8, containing 0.5 M NaCl, 5 mM dithiothreitol (DTT), 1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM benzamidine, 50 μg/ml aprotinin, 0.1% Triton X-100 and 10% glycerol] and sonicated on ice (1 min × 10 at output 5, Sonifier 250, Branson). After centrifugation at 23,000*g* for 30 min, the supernatant was added with ammonium sulphate to 20% saturation, left for 1 h and centrifuged at 23,000*g* for 30 min. The supernatant was added with ammonium sulphate to 50% saturation, left for 1 h and centrifuged at 23,000*g* for 30 min. The sediment was resuspended in 200 ml loading buffer (50 mM phosphate buffer, pH 7.2, containing 0.5 M NaCl, 40 mM imidazole and 5 mM 2-mercaptoethanol) and centrifuged at 23,000*g* for 30 min. The supernatant was applied to the Ni-NTA agarose column (1 ml) at 4°C at the flow rate of 2 ml/min, and the column was washed with loading buffer (20 ml) at 1 ml/min, then the bound proteins were eluted with 4 ml of 200 mM imidazole in 20 mM phosphate, pH 6.8, containing 0.5 M NaCl and 5 mM 2-mercaptoethanol, at 0.3 ml/min. The eluate was filtered through 0.45 μm hydrophilic polypropylene membrane, concentrated to 0.3 ml with Amicon Ultra-30k (Millipore, Billerica, MA, USA) and added with 1.2 ml of 20 mM phosphate buffer, pH 6.8, containing 1 mM DTT. The recombinant protein was further purified by cation exchange HPLC on a *Biofine* IEC-SP column (7.5 × 75 mm, JASCO, Hachioji, Japan) with a linear concentration gradient elution of

NaCl from 0.1 to 0.5 M in 20 mM phosphate buffer, pH 6.8, containing 1 mM DTT over 20 ml at a flow rate of 1 ml/min. Fractions containing the recombinant protein were pooled and concentrated to 0.1 ml with Amicon Ultra-30k. Gel filtration was performed on Superdex^{TM} 75 (10 × 300 mm, GE Healthcare, Buckinghamshire, UK) equilibrated with 20 mM Tris–HCl, pH 7.8, containing 0.5 M NaCl and 1 mM DTT at a flow rate of 0.5 ml/min. The component eluted at 20–22 min was pooled, concentrated and rechromatographed on the same column.

Phosphorylation of PHD1 and Specification of Phosphorylated Residues—Phosphorylation of recombinant PHD1 by PKA, PKCα, CKI, CKII or Erk2 was carried out according to the manufacturer's protocols. The specification of the phosphorylated residues with PKCα was carried out at Hitachi High-Technologies Co. (Hitachinaka City, Japan). Briefly, phosphorylated and untreated proteins were digested with trypsin and resultant peptides were analysed with MALDI-Qq-TOF MS/MS QSTAR Pulsar I (Applied Biosystems, Foster, CA,USA). The +80 *m/z* peaks observed only in PKCα treated tryptic peptides were further analysed by the same apparatus at MS/MS mode.

Activity Assay—The enzymatic activity was assayed by a modified method based on the hydroxylation-coupled decarboxylation of 2-oxo[1-^{14}C]glutarate (*18*). The reaction was performed in a final volume of 50 μl of 100 mM ammonium acetate buffer, pH 8.0, containing 12 μg of recombinant enzyme, 20 nmol of the substrate peptide NAD-19, 20 nmol of 2-oxo[1-^{14}C]glutarate (40,000 d.p.m.), 50 nmol of DTT, 100 nmol of ascorbate, 5 nmol of $Fe(NH_3)_2(SO_4)_2$, 5 μg of catalase and 25 μg of bovine serum albumin. The enzymatic reaction was carried out at 37°C for 1 h.

Other Methods—Protein concentration was estimated spectrophotometrically from absorbance at 280 and 310 nm according to the following equation calculated from the amino acid composition and molecular weight: protein concentration (mg/ml) = $0.96 \times (A_{280} - A_{310})$. Protein purity was assessed by SDS–PAGE (10% acrylamide gel), which was performed according to Laemmli (*19*). The secondary structure prediction was calculated with PAPIA New Joint Method System (Computational Biology Research Center, AIST).

RESULTS AND DISCUSSION

Expression of PHD1 in E. Coli—Human PHD1 with hexahistidine-tag at the N-terminus and FLAG-tag at the C-terminus was expressed in *E. coli* BL21(DE3) using the pCP2 vector. The recombinant protein was successfully expressed as soluble protein. The amount of PHD1 was monitored with temperature dependence and time-course. The expression plasmid pCP2-His6-PHD1-FLAG was transformed to *E. coli* BL21(DE3) and expressed at 37°C for 3, 4, 6, 8 and 16 h (Fig. 1A). The amount of PHD1 was maximal at 4–6 h, which was 10 times higher than that at 16 h. The protein seemed rather unstable in *E. coli* cells and many degraded components were observed when cultured overnight at 37°C. The expression was also carried out at 30°C for 6, 8 and 10 h, and at 25°C for 6, 8, 10 and 16 h but the amounts of PHD1 were less than that at 37°C for 4 h (data not shown). Induction with 0.2 mM IPTG was tested at $OD_{600} = 0.3$, 0.6 and 1.0. Highest expression was obtained at $OD_{600} = 1.0$; the expression was about two times higher than that at $OD_{600} = 0.3$ (data not shown). No significant change in the amount of protein was observed in the range from 0.2 to 1.0 mM IPTG but it was slightly and obviously declined at 0.1 and 0.05 mM, respectively. The amount of the expressed protein was

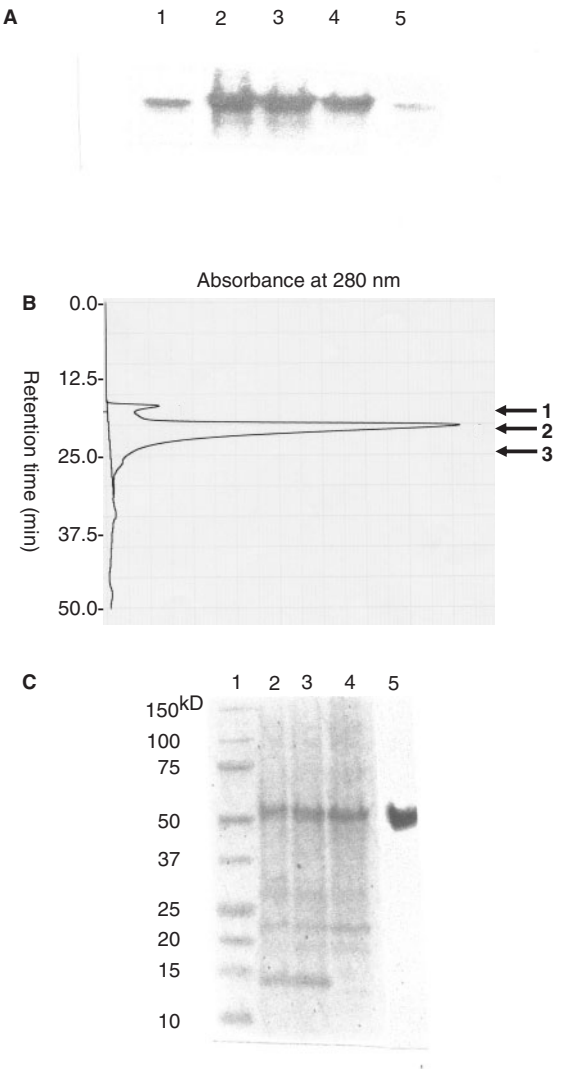

Fig. 1. **Expression and purification of recombinant PHD1.** (A) Time course of recombinant PHD1 expression at 37°C. Western blot against hexahistidine-tag of soluble fractions from equal amount of cells cultured for 3 h (lane 1), 4 h (lane 2), 6 h (lane 3), 8 h (lane 4) and 16 h (lane 5) was shown. (B) HPLC elution profile of recombinant PHD1 from Superdex 75 column. Elution was carried out at a flow rate of 0.5 ml/min; absorbance at 280 nm was monitored and 0.5 ml fractions were collected. Arrows indicate the elution time of standard globular proteins (1; bovine serum albumin, 67 kD: 2; ovalbumin, 45 kD: 3; α-chymotrypsinogen A, 27 kD). (C) Purity of recombinant PHD1 was analysed by SDS–PAGE and Coomassie staining. Molecular weight marker (lane 1), 0.3 μg protein after Ni-affinity chromatography (lane 2), 0.3 μg protein after cation-exchange HPLC (lane 3), 0.3 μg protein after gel filtration (lane 4) and 2 μg protein after rechromatography on Superdex 75 (lane 5).

slightly less when *E. coli* Rosetta(DE3) or *E. coli* BL21(DE3, codon plus) was used instead of *E. coli* BL21(DE3) (data not shown), suggesting that the expression level of PHD1 was not affected by codon usage. PHD1 was also expressed by using other vectors. However, its expression level was approximately five times lower when pET-28a(+) vector was used and 20 times lower when pRSETA and pCold I vector were used (data not shown). Therefore, we adopted the following conditions; pCP2-His6-PHD1-FLAG as the expression plasmid, *E. coli* BL21(DE3) as host strain, induction at $OD_{600} = 1.0$ with 0.2 mM IPTG and incubation for 4 h at 37°C.

Purification of Recombinant PHD1—Recombinant His6-PHD1-FLAG in crude extracts was purified with ammonium sulphate fractionation (precipitated at the range of 20–50% saturated ammonium sulphate) followed by immobilized Ni-affinity chromatography. The partially purified PHD1 was applied to a cation-exchange column, and the protein was eluted from the column at 0.3 M NaCl. The retention time from the gel filtration column indicates that the apparent molecular size of the protein is 52 kD (Fig. 1B), while the calculated molecular weight is 48 kD. This result suggests that the protein is present as a monomer in distinction from that the catalytic domain of PHD was present as a trimer in the crystal (*15*) and PHD3 tends to form aggregates (*16*). After the second gel filtration, ~125 μg purified protein was obtained with 97% purity from 10 l bacterial culture (Fig. 1C and Table 1). At the crude state (before cation-exchange HPLC), the protein tended to form aggregates and precipitate at low NaCl concentrations, but purified protein was stable in 0.15 M NaCl–1 mM DTT over pH 6.5–8.5. The attempt to remove the hexahistidine-epitope with thrombin was unsuccessful because PHD1 was over-sensitive to the protease, resulting in a very low yield.

Inhibitory Effect of Transition Metal Ions—The requirement of Fe^{2+} for PHDs was demonstrated in the observations that iron chelators are able to stabilize HIF-1α (*4*, *5*). The recombinant PHD1 was also inhibited perfectly by desferrioxamine (1 mM) or ethylenediaminetetraacetic acid (1 mM) (data not shown). The enzymatic activity was increased as the Fe^{2+} ion concentration was increased and levelled off at 10 μM Fe^{2+} (Fig. 2A). The EC_{50} value was 0.64 μM. Although the value for other PHDs was not reported, it was one-sixth of that for human collagen prolyl 4-hydroxylase (4 μM) (*20*). Transition metal ions (such as Co^{2+}, Ni^{2+} and Mn^{2+}) are known as hypoxic mimetic agents (*2*, *21*, *22*). We examined the inhibitory effect of transition metal ions on the activity in the presence of 100 μM Fe^{2+} (Fig. 2B). The IC_{50} values for Co^{2+}, Ni^{2+} and Cu^{2+} were 58, 35 and 220 μM, respectively. This result suggests that the affinity of Co^{2+} and Ni^{2+} for PHD1 was slightly higher than that of Fe^{2+}. Mn^{2+} did not affect the activity <1 mM. It is interesting to note that Mn^{2+} showed no inhibition effect on the activity even at 1 mM. This result suggests that PHD1 could be active in Mn^{2+}-treated cells in which hypoxia response was caused by the stabilization of HIF-1α (*22*). This estimation that PHD1 is unable to hydroxylate HIF-1α in the cells is consistent with previous findings that PHD2 but not PHD1 and PHD3 is the main cellular oxygen sensor (*9*).

Table 1. **Typical purification of recombinant PHD1 from 10 l of bacterial culture.**

Step	Total protein (μg)	Purity (%)[a]	Total activity[b]	Recovery (%)
Ni-NTA eluate	420	70	---[c]	---
Biofine-SP eluate	280	85	860	100
Superdex 75 eluate (1st)	200	92	730	85
Superdex 75 eluate (2nd)	125	97	420	49

[a]Purity was estimated from the data of protein density of SDS–PAGE. The proportion of recombinant protein was too small to estimate at the ammonium sulphate precipitation step. [b]Arbitrary units. [c]Not determined.

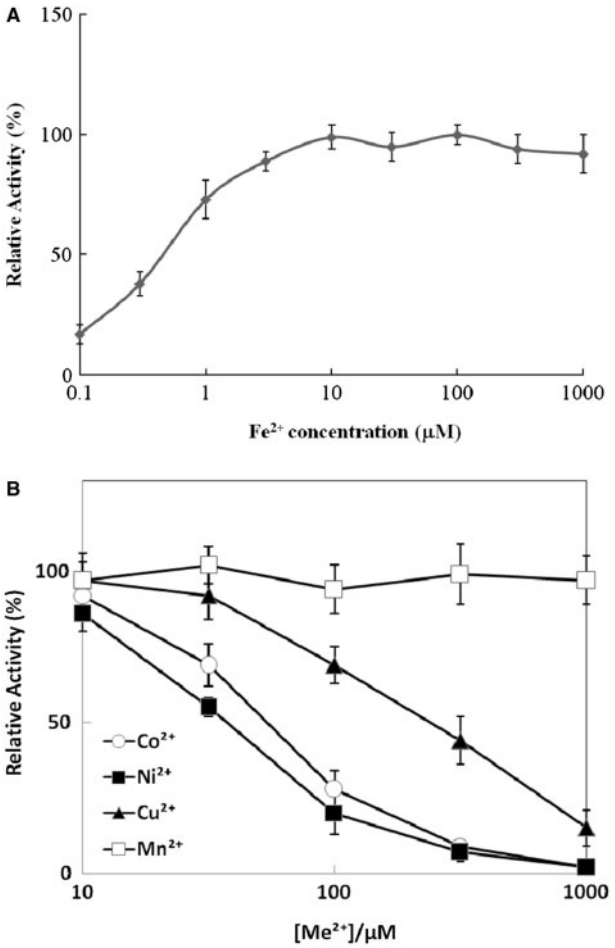

Fig. 2. **Effects of transition metal ions.** (A) Effect of Fe^{2+} concentration on the enzymatic activity of PHD1. Values represent mean ± SD for three measurements. (B) Inhibition of the enzymatic activity by transition metal ions at the presence of 100 μM Fe^{2+}. Values represent mean ± SD for three measurements.

Activity of Deletion Mutants—The amino acid sequence of the C-terminal aa 168–388 of PHD1 shows high homology to those of PHD2 (70.4% homology) and PHD3 (62.4% homology). This region is thought to be the catalytic region. On the other hand, the N-terminal region aa 1–167 of PHD1 does not show any homology to the N-terminal region aa 1–183 of PHD2 at all,

and PHD3 lacks this region. This region is considered to be the regulatory region and in fact, it prevented from the degradation of the protein itself by Siah2 (*23*). Almost no secondary structure was predicted to PHD1 N-terminal region (aa 1–161) except two short helices at the region of aa 36–40 and aa 107–112 (data not shown). Two deletion mutants (PHD1Δ1-161 and PHD1Δ123-161) were expressed in *E. coli* and purified. The yields and purity of the two mutants were similar to those of wild-type. As shown in Fig. 3B, the activity of PHD1Δ1-161, which deleted the whole N-terminal domain, was intensified to 140% of the full-length PHD1, suggesting that the removed N-terminal sequence has an inhibitory function. PHD1Δ123-161, which lacked a part of the domain, was almost inactive for unknown reason. The resultant N-terminal part may block access of peptide substrates to the PHD1 active-site.

Phosphorylation and Specification of Phosphorylated Residues—It is well known that many transcription-related proteins are regulated by phosphorylation. For example, HIF-1α is phosphorylated in the hypoxic cell (*24–26*) and some Ser/Thr kinases regulate HIF-1α stability (*24*) or activate its transcriptional activity (*27*). Therefore, we investigated the possibility that PHD1 activity is also regulated by phosphorylation. The recombinant protein was treated *in vitro* with PKA, PKCα, CKI, CKII or Erk2, using [γ-^{32}P]ATP (Fig. 4A). Phosphorylation by PKA and CKII was not detected. Maximal phosphate incorporation in PHD1 was achieved by PKCα (0.9 mol phosphate/mole protein) and the phosphorylated enzyme diminished its activity to 70% (Fig. 4B). Phosphorylation of the enzyme with CKI (0.7 mol/mol) or Erk2 (0.6 mol/mol) did not change the enzymatic activity as shown in Fig. 4B. In order to identify phosphorylated residues, the phosphorylated PHD1 by PKCα was digested with trypsin, analysed by MALDI-TOF MS and the profile was compared with that of the unphosphorylated protein (data not shown). Four +80 *m/z* peaks were found in the range of 800–3000 *m/z*, and further analysed at MS/MS mode (Table 2). Since phosphoserine residues were detected as dehydroalanine residues at MS/MS mode, they were distinguished from intact serine residues. Peptides I, II and III were phosphorylated at Ser-226, Ser-132 and Ser-234, respectively. Peptide IV was generated from the incomplete tryptic digestion at Arg-233 because it is followed by an acidic residue, phosphoserine-234. In order to confirm the phosphorylation of the serine residues, the phosphorylated protein was also digested with V8 protease or a mixture of trypsin and V8 protease, and resultant peptides were analysed in the same way. Phosphorylation of the three serine residues was confirmed, and phosphorylation of other amino acid residues was not found (data not shown). Peptide sequences around these phosphorylated serine residues satisfied consensus sequences of phosphorylation by PKC, S*/T*-X_{2-0}-R/K (*28*).

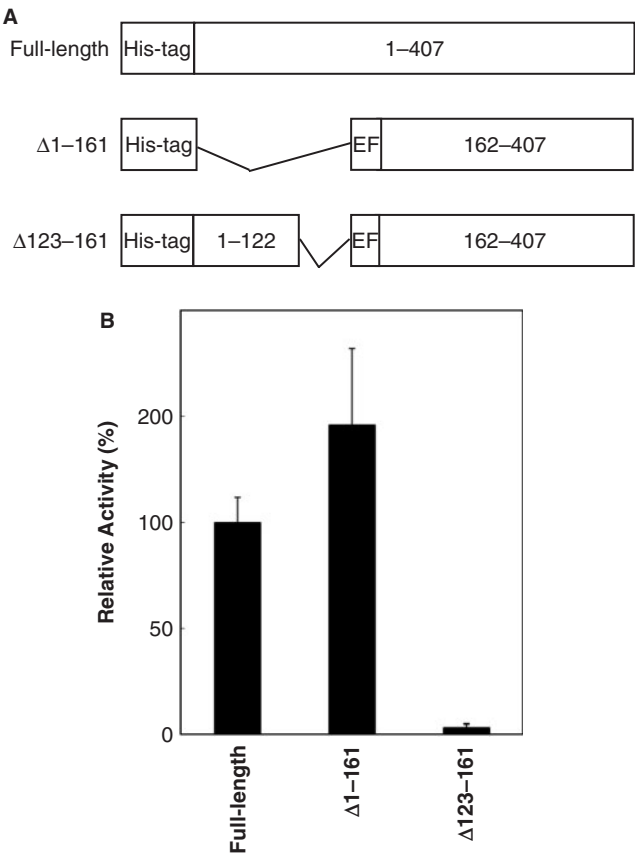

Fig. 3. **Activity of deletion mutants of PHD1.** (A) Schematic diagrams of deletion mutants. Two amino acid residues, EF, were introduced by the *Eco*RI linker site. (B) Comparison of the enzymatic activity of deletion mutants to full-length PHD1. Full-length and deleted PHD1 were assayed by the method of the hydroxylation-coupled decarboxylation of 2-oxo[1-^{14}C]glutarate. Values represent mean ± SD for three measurements.

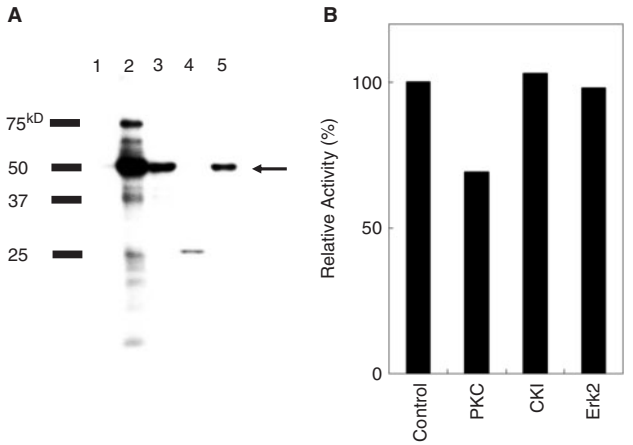

Fig. 4. **Phosphorylation of PHD1.** (A) Autoradiography of phosphorylated recombinant PHD1. Aliquots of the recombinant PHD1 (20 μg in 50 μl reaction mixture) were treated with protein kinases according to the manufacturer's protocols, using [γ-^{32}P]ATP, and 5 μl of the mixture was analysed by SDS–PAGE. Phosphorylated recombinant PHD1 with PKA (lane 1), PKCα (lane 2), CKI (lane 3), CKII (lane 4) or Erk2 (lane 5). The arrow indicates the position of unphosphorylated recombinant PHD1. (B) Comparison of the enzymatic activity of phosphorylated PHD1 to untreated PHD1. Phosphorylated recombinant PHD1 with PKCα, CKI or Erk2 was desalted by gel-filtration on a Sephadex -50 short column, concentrated with Amicon Ultrafree-MC(30k) and assayed at standard conditions ($n = 2$).

Table 2. **Phosphorylated peptides and their sites.**

Peptide	*m/z*	Position	Sequence	Phosphorylated residue
I	1,251	219–228	LRDGQLV**S**QR	Ser-226
II	2,062	121–128	WAEDGGDAPSP **S**KRPWAR	Ser-132
III	2,147	234–251	**S**IRGDQIAWVE GHEPGCR	Ser-234
IV	2,681	229–251	AIPPR **S**IRGDQIAW VEGHEPGCR	Ser-234

Boldfaces indicate the phosphorylated residues which were detected as dehydroalanine residues at MS/MS mode.

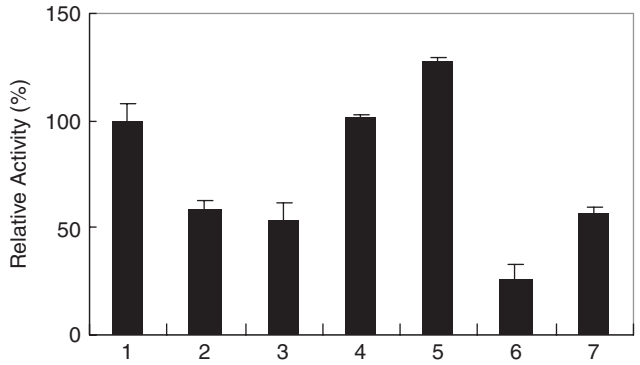

Fig. 5. **Comparison of the enzymatic activities of wild-type and mutated PHD1s.** The enzymatic activity of purified wild-type PHD1 (lane1); and mutated PHD1S132D (lane 2); PHD1S132E (lane 3); PHD1S226D (lane 4); PHD1S226E (lane 5); PHD1S234D (lane 6) and PHD1S234E (lane 7) were assayed at standard conditions. Values represent mean ± SD for three measurements.

Activity of the Mutants—In order to evaluate the effect of phosphorylation of the three serine residues identified as above on the activity, we mutated the serines to Asp or Glu. The mutants were expressed in *E. coli* and purified to assay their enzymatic activity. Purity and yields of the wild-type and mutants were similar to each other. The mutation of Ser-132 to Asp or Glu diminished the enzymatic activity to 50–60% (Fig. 5). Mutation of Ser-226 did not influence the activity. Substitution of aspartic acid for Ser-234 largely diminished the activity to 25% and mutation to Glu also reduced the activity to 50% as shown in Fig. 5. These results suggest that phosphorylation of Ser-234 and to a lesser extent Ser-132 by PKCα plays a role in the down-regulation of PHD1. Ser-234 is suggested to be sited in the β2–β3 loop, which was assigned to the substrate-determining region of the PHD family (*29*). The introduction of negative charge to the site may diminish the affinity to the substrate. It is important to elucidate whether PHD1 is regulated by phosphorylation of Ser-234 and Ser-132 with PKCα in mammalian cells. Experiments along this line are now in progress.

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CONFLICT OF INTEREST

None declared.

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