ANTIOXIDANTS & REDOX SIGNALING
Volume 9, Number 9, 2007

DOI: 10.1089/ars.2007.1683

Forum Original Research Communication

Regulated Function of the Prolyl-4-Hydroxylase Domain (PHD) Oxygen Sensor Proteins

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ABSTRACT

Cellular oxygen is sensed by prolyl-4-hydroxylase domain (PHD) proteins that hydroxylate hypoxia-inducible factor (HIF) α subunits. Under normoxic conditions, hydroxylated HIFα is bound by the von Hippel–Lindau (pVHL) tumor suppressor, leading to ubiquitinylation and proteasomal degradation. Under hypoxic conditions, hydroxylation becomes reduced, leading to HIFα stabilization. The authors recently showed that changes in PHD abundance and activity can regulate HIFα stability under normoxic as well as under hypoxic conditions. Thus, the PHD oxygen sensors themselves represent effectors of cellular signalling pathways as well as potential drug targets. Here, a cell-free *in vitro* microtiter plate-based peptide hydroxylation assay was used to investigate the influence of ferrous iron, Krebs cycle intermediates, transition metals, and vitamin C and other antioxidants on the activity of purified PHD1 to 3. PHD activity depends not only on oxygen availability but is also regulated by iron, vitamin C, and Krebs cycle intermediates, suggesting a physiological relevance of their cellular concentrations. Copper but not iron, cobalt, or nickel salts catalyzed vitamin C oxidation. While vitamin C is essential for PHD activity *in vitro*, *N*-acetyl-L-cysteine had no effect, and gallic acid or *n*-propyl gallate efficiently inhibited the activity of all three PHDs, demonstrating different functions of these antioxidants. *Antioxid. Redox Signal.* 9, 1329–1338.

INTRODUCTION

Oxygen availability affects many physiological and pathophysiological processes, including embryonic development, adaptation to high altitudes, wound healing, inflammation, cancer, and ischemic diseases such as infarction and stroke. Central to the understanding of these processes is the elucidation of the molecular mechanisms by which cells react and adapt to insufficient oxygen supply (hypoxia). Oxygen availability is measured by a family of oxygen-dependent protein hydroxylases that regulate the abundance and activity of hypoxia-inducible transcription factor (HIF) α subunits (47). HIFs in turn control the expression levels of effector genes involved in either anticipatory metabolic changes, adaptive survival, or programmed death of the affected tissue (58). HIFs are heterodimeric transcription factors consisting of one out of three different oxygen-sensitive HIFα subunits (HIF-1α, HIF-2α, or HIF-3α) and a common constitutive HIFβ subunit. While HIF-1 and HIF-2 $\alpha\beta$ heterodimers function as transcriptional activators of oxygen-regulated target genes, the role of HIF-3α is less clear and a short splice variant of HIF-3α, termed inhibitory PAS protein (IPAS), functions as a transcriptional repressor (57).

Dependent upon the cellular oxygen partial pressure (pO_2), a family of prolyl-4-hydroxylase domain (PHD) enzymes covalently modify two proline residues within the oxygen-dependent degradation (ODD) domain of HIFα subunits. The PHD family is comprised of three members called PHD1,

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PHD2, PHD3, or HIF prolyl hydroxylase (HPH) HPH3, HPH2, HPH1, respectively (7, 14). A fourth member, called PH4, regulates HIFα under overexpression conditions only (40). Upon hydroxylation under normoxic conditions, HIFα is bound by the von Hippel–Lindau (VHL) tumor suppressor protein and targeted for proteasomal destruction (35). Thus, the high turnover rate of HIFα subunits allows for an instantaneous stabilization under hypoxic conditions (21). According to the current model, also the asparagine hydroxylase function of the factor inhibiting HIF (FIH) becomes impaired when oxygen availability is further decreased, resulting in a decrease in C-terminal HIFα asparagine hydroxylation (44). This allows for the progressively increased recruitment of p300/CPB transcriptional co-activators, leading to a successively higher transcriptional function of HIF (28, 31).

While all three PHDs can hydroxylate HIFα with similar efficiency, PHD2 has been suggested to play the main role for normoxic HIFα turnover (6). Consistent with these *in vitro* findings, PHD2 but not PHD1 or PHD3 knock-out mice die during embryonic development (51). The three PHDs are expressed in most organs, but there are strikingly high levels of PHD3 mRNA in the heart and of PHD1 mRNA in the testis (50). In addition to HIFα, there is some evidence that the iron regulatory protein IRP2, the RNApol II large subunit Rpb1, the heme synthesis enzyme ALAS2, and IκB kinase-β are regulated by PHDs (1, 10, 27, 54, 55). Of note, these experiments were mainly based on pharmacological inhibition of PHDs and no direct evidence for protein hydroxylation has been provided yet. Interestingly, ankyrin repeats within the NF-κB family (p105) and IκBα were shown to be efficiently hydroxylated by FIH (9). The function of this FIH-dependent hydroxylation, however, is unclear up to date.

The regulation of PHD expression and activity has become of considerable interest in the recent past. Endogenous tricarboxylic acid cycle intermediates and reactive oxygen species (ROS) have been reported to inhibit PHD function and hence link mitochondrial function with PHD-dependent oxygen sensing (12, 15, 43, 48). Small molecule inhibitors that can be added exogenously are currently being developed for clinical tissue protection in diseases associated with oxygen deprivation. However, little is known on the differential regulation of the three PHD family members. We therefore set out to investigate the control of each PHD family member individually by small molecules as well as by newly identified protein interactors and by HIF-dependent feedback regulation.

MATERIALS AND METHODS

Expression and purification of PHDs

GST-PHD1, GST-PHD2, and GST-PHD3 were expressed in baculovirus-infected Sf9 insect cells and purified as described before (50). Briefly, after 80–110 h of infection, Sf9 cells were lysed in ice-cold 0.1% NP-40, 10 m*M* Tris-HCl pH 7.5, 100 m*M* NaCl, 100 m*M* glycine, and 10 μM DTT. Cleared lysates were incubated with equilibrated glutathione-sepharose beads (Amersham, Dübendorf, Switzerland) for 2 h at 4°C with gentle agitation. After washing of the beads three times with PBS, bound protein was eluted with 15 m*M* reduced glutathione, 50 m*M* Tris-HCl pH 8.0, and 2 μM $FeSO_4$. Purity of recombinant fusion-proteins was routinely estimated by SDS-PAGE and coomassie blue staining.

In vitro *prolyl-4-hydroxylation assays*

Enzymatic activity of recombinant PHDs was determined as described before (32, 41). Biotinylated peptides (100 ng/well) derived from human HIF-1α aa 556 to 574 (either wild-type or P564A mutant) were bound to NeutrAvidin-coated 96-well plates (Pierce, Perbio, Lausanne, Switzerland). Purified recombinant PHD enzymes were used to hydroxylate the peptides in the presence of 0.5 m*M* 2-oxoglutarate, 2 m*M* ascorbate, 10 μM $FeSO_4$ in 20 m*M* Tris-HCl pH 7.5, 5 m*M* KCl, 1.5 m*M* $MgCl_2$ for 1 h at room temperature. A polycistronic expression vector for His_6- and thioredoxin-tagged pVHL/elongin B/elongin C (VBC) complex was kindly provided by S. Tan (Pennsylvania, PA). VBC was expressed in bacteria, purified by nickel affinity chromatography, followed by ion exchange chromatography and buffer exchange gelfiltration. VBC complex was allowed to bind to the hydroxylated peptides and bound VBC complex was detected by rabbit anti-thioredoxin antibodies, followed by secondary horseradish peroxidase-coupled anti-rabbit antibodies (Sigma, Buchs, Switzerland), using the TMB (3,3′,5,5′-tetramethylbenzidine) substrate kit (Pierce). The peroxidase reaction was stopped by adding H_2SO_4 to 1 *M* and absorbance was determined at 450 nm in a microplate reader.

Ascorbate oxidation assays

Ascorbate oxidation to dehydroascorbate was measured spectrophotometrically as described (49). Therefore, the decrease in absorbance at 265 nm was measured in a 100 μM ascorbate solution in 20 m*M* Tris-HCl pH 7.5, 5 m*M* KCl, 1.5 m*M* $MgCl_2$ using open cuvettes with free access to air.

Hypoxia reporter cells

The Chinese hamster ovary (CHO) cell line stably transfected with a hypoxia-responsive firefly luciferase reporter gene (termed HRCHO5) has been described before (56). Cell lysis and determination of luciferase activity was performed according to the manufacturer's instructions (Promega, Wallisellen, Switzerland). Relative light units were measured in a 96-well luminometer (Berthold, Regensdorf, Switzerland) and normalized to the protein concentration determined by the Bradford assay (Biorad, Reinach, Switzerland).

RESULTS

Ferrous iron availability is essential for the function of all three PHDs

GST-tagged PHD1, PHD2, and PHD3 were purified from Sf9 insect cells to 80–90% purity and their activities were analyzed by a VBC binding assay. All three PHDs induced VBC binding to the wild-type P564 but not to the mutant P564A-containing peptide derived from the HIF-1α ODD (Fig. 1A). The PHD preparations were calibrated with hydroxyproline

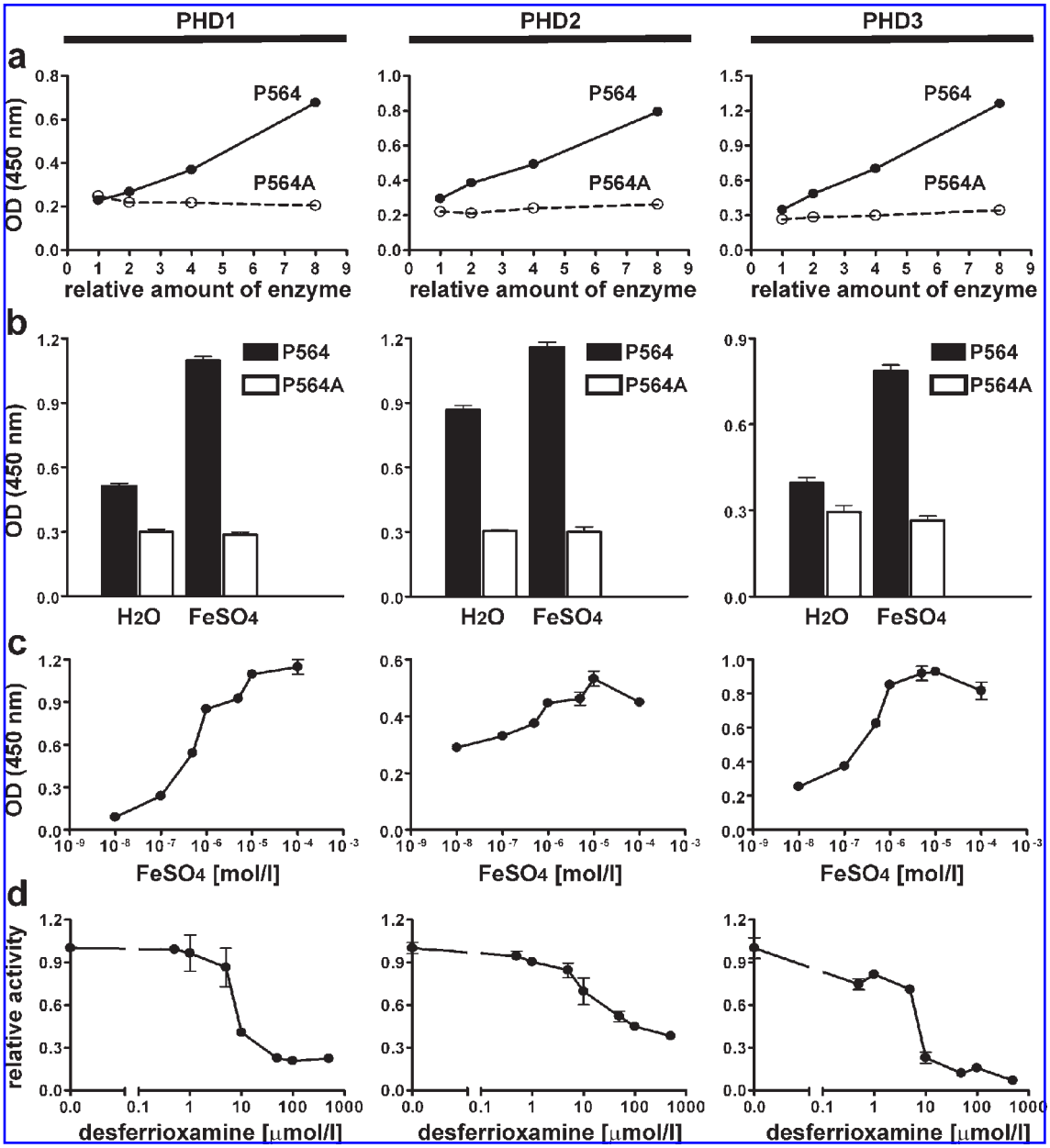

FIG. 1. Regulation of PHD function by iron. PHD1, PHD2, and PHD3 (*from left to right*) were purified as GST-tagged fusion-proteins from Sf9 cells and their hydroxylation activity was estimated in microtiter plate-based VBC binding assays. (**A**) VBC binding is dependent on the presence of both functional PHD enzyme and Pro564. Mutant P564A peptides cannot be hydroxylated. (**B**) Ferrous iron supplementation is essential for full PHD function. (**C**) Titration of ferrous iron reveals that ~10 μM $FeSO_4$ is required for full PHD function. (**D**) Inhibition of PHD function by iron chelation. Mean values ± SEM of representative experiments performed in triplicate are shown.

(Hyp)564 peptide-containing hydroxylation assays and subsequently diluted to obtain solutions with similar specific activities which were then used for all following experiments.

Although 2 μM $FeSO_4$ was initially present in the elution buffer, the PHDs did not display full activity without additional ferrous iron in the reaction buffer (Fig. 1B), suggesting that both active center and added iron was at least partially oxidized. An iron titration experiment revealed that the addition of ~10 μM $FeSO_4$ was required for full induction of the hydroxylation activity of all three PHDs (Fig. 1C). This concentration was hence kept in the following experiments. The addition of ferric iron did not stimulate the activity of the PHDs (data not shown).

The sensitivity of the PHDs to the availability of "free" iron explains the long-known feature of iron chelators to induce the HIFα subunits. Indeed, the hexadentate hydroxamic acid iron chelator desferrioxamine inhibited the *in vitro* hydroxylation activity of all three PHDs (Fig. 1D). Of note, PHD2 was some-

what less sensitive to desferrioxamine than PHD1 or PHD3, which were efficiently inhibited by 10 μM desferrioxamine, corresponding to the concentration of iron in the reaction solution.

Requirement for 2-oxoglutarate and inhibition by succinate

2-Oxoglutarate is used as a co-substrate of all three PHDs which is oxidatively decarboxylated during target protein hydroxylation. Titration experiments with 2-oxoglutarate revealed that all three PHDs required a similar 2-oxoglutarate concentration of ~10–100 μM for full activity (Fig. 2A). These data confirm previous estimates of Km values of 55–60 μM for all three PHDs (17), and a PHD2 binding constant for 2-oxoglutarate of <2 μM (36). Therefore, a concentration of 500 μM 2-oxoglutarate was kept in all further experiments. Higher concentrations of 2-oxoglutarate inhibited the *in vitro* PHD activity in some experiments. Whether this effect is of physiological relevance is currently unknown.

It has been reported previously that succinate can inhibit PHD activity (12, 30, 48). We hence tested the effects of increasing succinate concentration on each PHD enzyme. In the presence of 500 μM 2-oxoglutarate, addition of succinate only moderately inhibited the hydroxylation activity of purified PHDs and this effect was even reversed at higher concentrations (Fig. 2B). However, in the presence of 50 μM 2-oxoglutarate, succinate efficiently inhibited PHD activity with IC_{50} values of ~600 μM (Fig. 2C).

Vitamin C oxidation impairs PHD function

Vitamin C is essential for the function of collagen hydroxylases as well as for HIFα hydroxylases because it protects the enzyme's amino acid residues and/or active center iron from oxidation in reactions uncoupled from target hydroxylation (38). Purified PHDs do not show any activity in the absence of ascorbate and the addition of at least 0.1 mM ascorbate was required to fully induce hydroxylation activity of all three PHDs (Fig. 3A). The addition of oxidized ascorbate (dehydroascorbate) did not induce PHD activity (data not shown). These data confirm previously reported similar ascorbate Km values (140–180 μM) for all three PHDs (17). Therefore, an excess of freshly prepared ascorbate (2 mM) was added to all subsequent

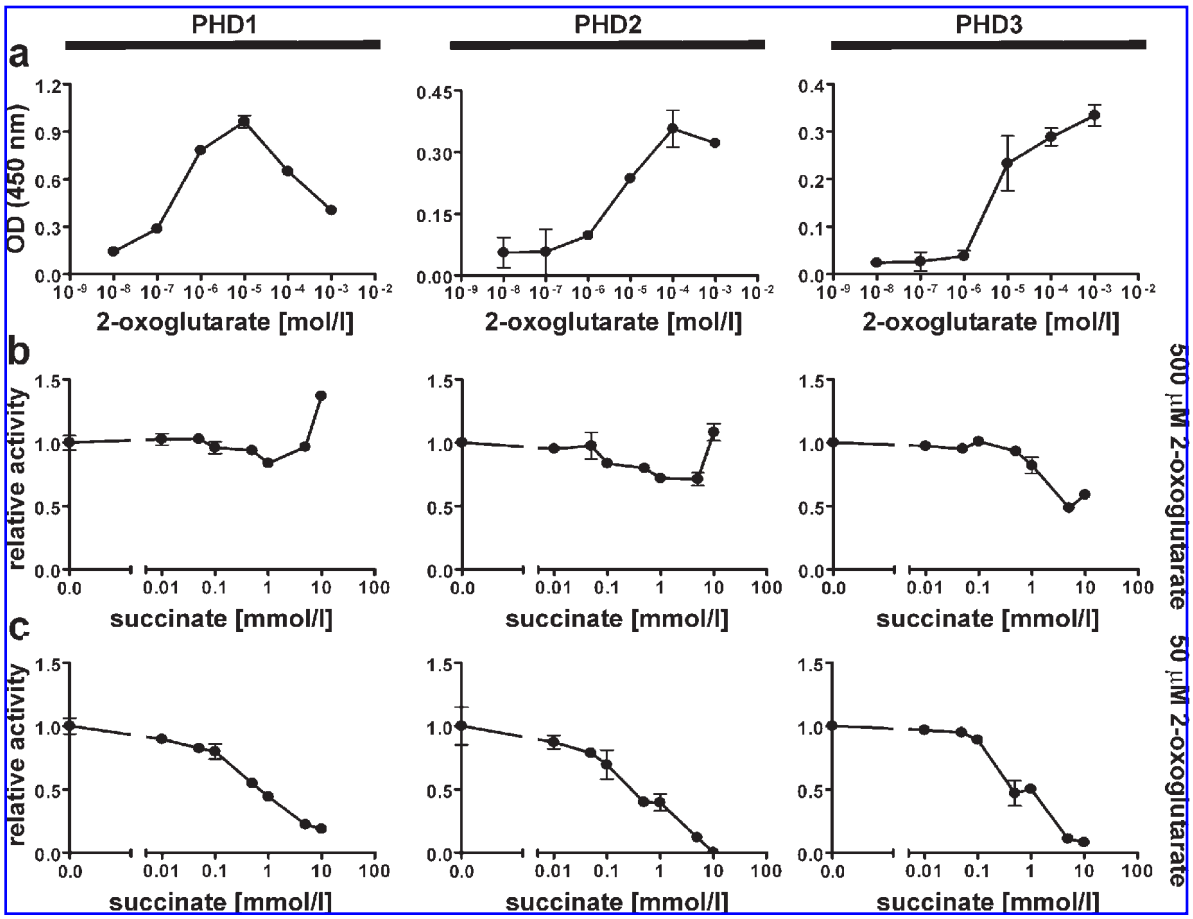

FIG. 2. Regulation of PHD function by Krebs cycle intermediates. PHD1, PHD2, and PHD3 (*from left to right*) hydroxylation activity was estimated in microtiter plate-based VBC binding assays. Titration curves of the cosubstrate 2-oxoglutarate (**A**) or the co-product succinate in the presence of 500 μM (**B**), or 50 μM (**C**) 2-oxoglutarate are shown as mean values ± SEM of representative experiments performed in triplicate.

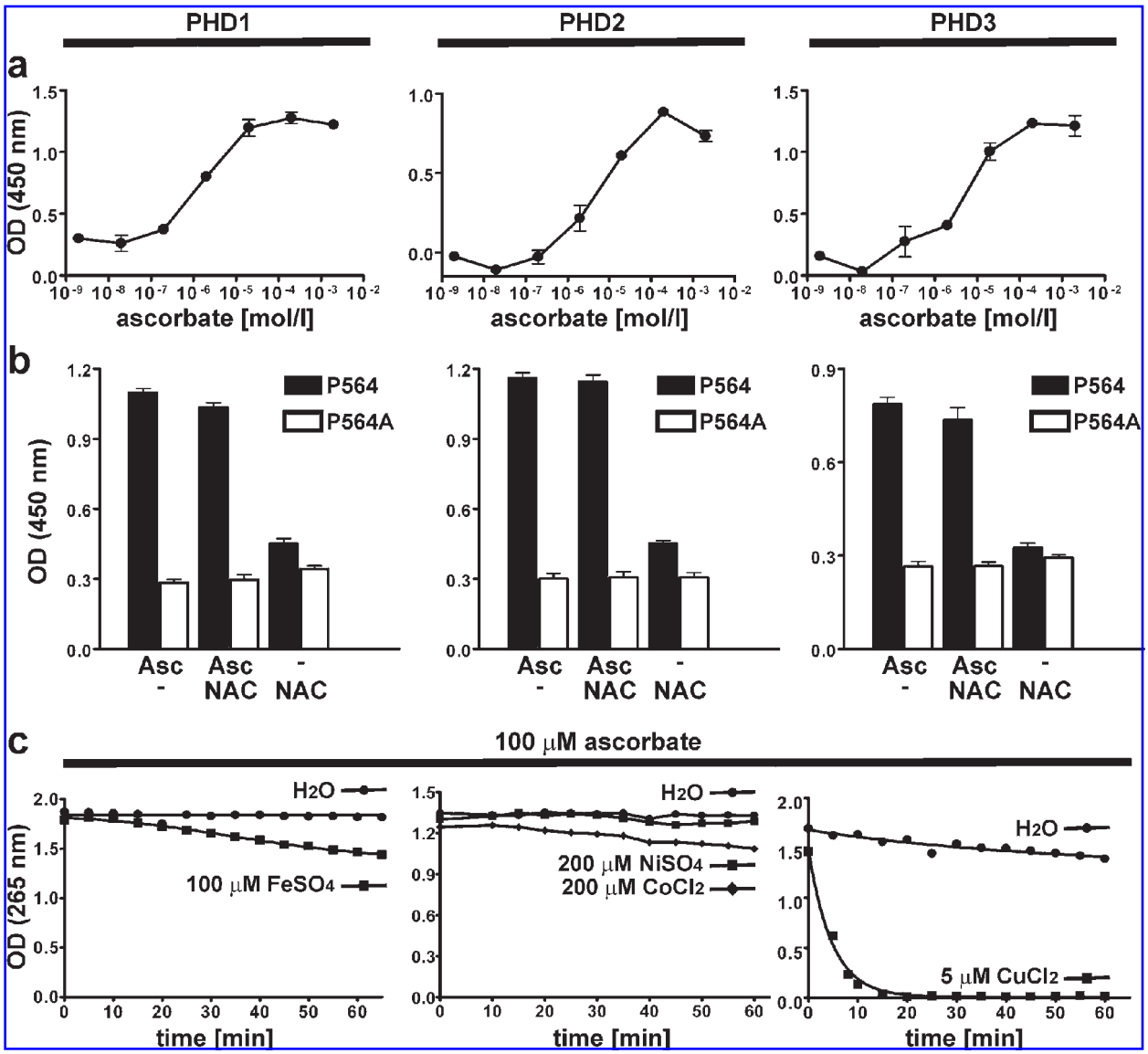

FIG. 3. Regulation of PHD function by vitamin C. PHD1, PHD2, and PHD3 (*from left to right*) hydroxylation activity was estimated in microtiter plate-based VBC binding assays. (**A**) Titration of vitamin C reveals that 0.1 m*M* ascorbate is minimally required for PHD function. (**B**) The antioxidant *N*-acetyl-L-cysteine (250 μM) cannot replace ascorbate (2 m*M*) for PHD function. (**C**) Ascorbate is slowly oxidized by air (dehydroascorbate does not absorb light at 265 nm). Ferrous iron slightly catalyzes this reaction (*left graph*), whereas the effects of the known PHD inhibitors cobalt and nickel are negligible (*middle graph*). However, cupric copper strongly catalyzed ascrobate oxidation (*right graph*). Mean values ± SEM of representative experiments performed in triplicate are shown.

experiments. Antioxidative ascorbate function is specifically required by the PHDs and cannot be replaced by the antioxidant *N*-acetyl-L-cysteine (Fig. 3B).

The absolute requirement for ascorbate might also explain the function of previously reported agents inhibiting PHD activity. Ascorbate oxidation by air can be measured by the drop in absorbance at 265 nm. However, as shown in Fig. 3C, ascorbate oxidation is negligible during the reaction period of 1 h. Even in the presence of equimolar ferrous iron, the ascorbate levels dropped only by 20% after 1 h (Fig. 3C, left graph). The transition metals cobalt and nickel are well-known inducers of HIFα protein stability due to PHD inhibition. Of note, even a twofold molar excess of $CoCl_2$ or $NiSO_4$ did not catalyze more ascorbate oxidation than $FeSO_4$ (Fig. 3C, middle graph). In contrast, copper efficiently catalyzed ascorbate oxidation: 5 μM $CuCl_2$ destroyed >90% of 100 μM ascorbate within 10 min (Fig. 3C, right graph).

The antioxidants gallic acid and n-propyl gallate efficiently inhibit the activity of all three PHDs

A number of natural nutrient compounds has been suggested to induce HIF-1α; among them flavonoid and nonflavonoid polyphenols. Catechins are nonflavonoid polyphenols found in

green tea leaves which have been reported to induce HIF-1α (60). Only catechins containing a 3-gallate moiety activate HIF-1 and have been shown to inhibit PHD2 activity (52, 53). Thus, we tested the sensitivity of PHD-dependent peptide hydroxylation on gallic acid or *n*-propyl gallate. Remarkably, all three PHDs were efficiently inhibited by 33–100 μM gallic acid (Fig. 4A) or by 3.3–10 μM *n*-propyl gallate (Fig. 4B). As shown in Fig. 4C, n-propyl gallate also induced HIF-dependent luciferase reporter gene activity in cultured CHO cells. In contrast to the *in vitro* results, gallic acid treatment of cells only marginally affected luciferase expression (data not shown).

DISCUSSION

Oxygen sensing by protein hydroxylation is a regulated process

Sensing cellular pO_2 is probably not the only function of the PHDs. Otherwise, an excess of a single sensory protein simply regulated by oxygen availability would be all that was required for this task. However, there is ample evidence that the PHD oxygen sensors fulfill additional functions. First, different tissues are differently vascularized, unequally perfused, and show spatially and temporally variable oxygen consumption rates. Thus, the mean pO_2 varies from tissue to tissue, if not from cell to cell. Nevertheless, every cell is capable of sensing a reduction in oxygenation and adequately responds to such a reduction by inducing HIF-dependent gene expression. Therefore, also hypoxia thresholds vary both spatially and temporally, and the PHD oxygen sensors evolved to meet these requirements by showing a variable tissue-specific expression pattern. Second, every successful adaptation to hypoxia eventually results in reoxygenation of the affected tissue. Because following hypoxia there are much higher levels of HIFα in the cell, the oxygen-dependent hydroxylation and degradation machinery must increase its capacity to cope with the degradation of the increased protein mass. Third, HIFα is also induced by a variety of stimuli under normoxic conditions. How can HIFα protein stabilization occur in the presence of active PHDs and sufficient oxygen supply? A plausible explanation lies in the lim-

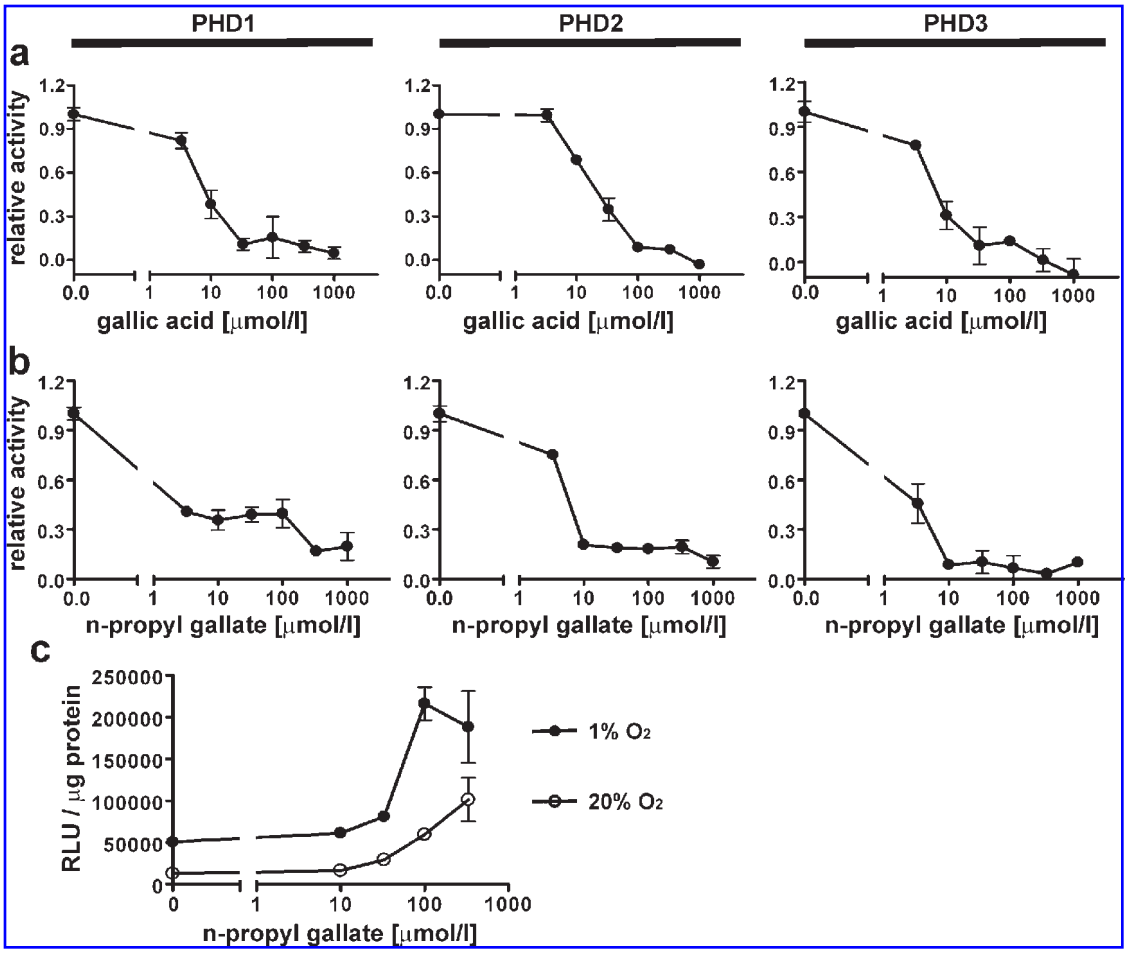

FIG. 4. Regulation of PHD function by gallate. The effects of gallic acid (**A**) and *n*-propyl gallate (**B**) on PHD1, PHD2, and PHD3 (*from left to right*) hydroxylation activity were estimated in microtiter plate-based VBC binding assays. (**C**) CHO cells stably transfected with a HIF-dependent luciferase reporter gene were treated with the indicated concentrations of *n*-propyl gallate for 24 h. Relative light units (RLU) were normalized to the protein content of the lysates. Mean values ± SEM of representative experiments performed in triplicate (quadruplicates for the reporter gene assays) are shown.

ited expression levels of PHDs which are finely counterbalanced by HIFα levels. If either one is upregulated, it overcomes the function of the other. Thus, as well as an increase in PHD synthesis leads to HIFα degradation, an increase in HIFα synthesis leads to its stabilization even under normoxic conditions. We have previously demonstrated that these mechanisms work even under hypoxic conditions, since PHD-dependent HIFα hydroxylation was reduced but still functional in direct proportion to the decrease in available oxygen (50).

Regulation of the three PHD oxygen sensors by small molecules

In this work, we have analyzed the effects of several small molecules on PHD function *in vitro*. Addition of ferrous iron was essential for PHD activity. Hirsilä *et al.* reported iron Km values for PHD1, PHD2, and PHD3 of 30, 30, and 100 n*M*, respectively (18), and McNeill *et al.* reported a ferrous iron binding constant of highly purified PHD2 of $<<1$ μM (36). Our data support an increased iron affinity of PHD3 that reached maximal activity already in the presence of 1 μM $FeSO_4$, whereas PHD1 and PHD2 required 10 μM $FeSO_4$ for full activity. The iron chelator desferrioxamine efficiently inhibited PHD1 and PHD3 activity at concentrations corresponding to the iron concentrations in the reaction buffer. PHD2 inhibition was somewhat less efficient, but a drop in activity was observed with similar desferrioxamine concentrations. Interestingly, it has previously been suggested that desferrioxamine inefficiently inhibited crude PHD enzymes *in vitro*: in the presence of 5 μM Fe^{2+} up to 1 m*M* desferrioxamine inhibited crude PHDs by $<20\%$, whereas inhibition of pure PHDs was much more efficient (18). Thus, the efficiency of inhibition of the PHDs by desferrioxamine might allow us to draw some conclusions on the purity of the enzyme preparation.

We previously determined an iron concentration in the FCS of 148 μM and in the cell culture medium of 16 μM (56). The intracellular concentration of chelatable iron has been reported to be 3–6 μM, depending on the cell type (45). Thus, the estimated concentration of "free" iron for optimal PHD activity *in vitro* corresponds to the iron concentrations *in vivo*, suggesting that iron availability is indeed able to regulate the HIF system by influencing PHD activity. This assumption has been confirmed by experimentally changing the "labile iron pool" in cancer cells (22, 24, 25). Interestingly, important iron uptake and transport proteins are among the targets of HIF-1 that might function also as a regulator of iron homeostasis in addition to oxygen homeostasis (58). These values also explain why ~100 μM of the hexadentate extracellular iron chelator desferrioxamine but only ~10 μM of the bidentate intracellular iron chelator cyclopirox olamine were required to induce the HIF system by inhibiting PHD activity (29).

Krebs cycle intermediates such as succinate and fumarate are known to induce the HIF system by inhibiting PHD activity (20, 43, 48). Moreover, 2-oxoacids such as pyruvate and oxaloacetate also induce HIFα (12, 30). These findings link mitochondrial function and cellular metabolism with the PHD/HIF oxygen sensing system. Mutation of the tumor suppressor *succinate dehydrogenase* has been reported to increase cellular succinate concentration from 120 to ~440 μM (48). The IC_{50} values for succinate have been reported to be in the range of 510–830 μM for purified PHDs (26). We obtained similar IC_{50} values (~600 μM) for succinate when the assays were performed in the presence of 50 μM 2-oxoglutarate. Succinate competes for 2-oxoglutarate binding to the PHDs (16, 26), explaining why we observed only weak succinate inhibition of PHD activity in the presence of high (500 μM) 2-oxoglutarate concentrations.

We demonstrated that ascorbate is essential for the function of all three PHDs. It has been shown that physiological concentrations (25 μM) of ascorbate suppress HIF-1α protein levels in cancer cells, suggesting that intracellular ascorbate concentrations indeed respresent a major regulator of PHD function (25). Ascorbate depeletion thus could also explain how transition metals induce HIF-1α. Interestingly, Salnikow and colleagues reported that cellular ascorbate depletion causes $NiSO_4$ and $CoCl_2$ induced HIF-1α stabilization (23, 46). However, the authors attributed this effect to inhibition of cellular ascorbate uptake. Moreover, we could not observe any relevant nickel- or cobalt-mediated degradation of ascorbate *in vitro*. In contrast, we observed a rapid copper-mediated ascorbate oxidation which most likely explains the previously reported $CuCl_2$-induced HIF-1α stabilization via PHD inhibition in cultured cells (32).

We have found that the antioxidant compounds gallic acid and *n*-propyl gallate efficiently inhibited all three PHDs *in vitro* and the esterified gallate also induced a HIF-dependent reporter gene in cell culture. Tsukiyama *et al.* hypothesized that two

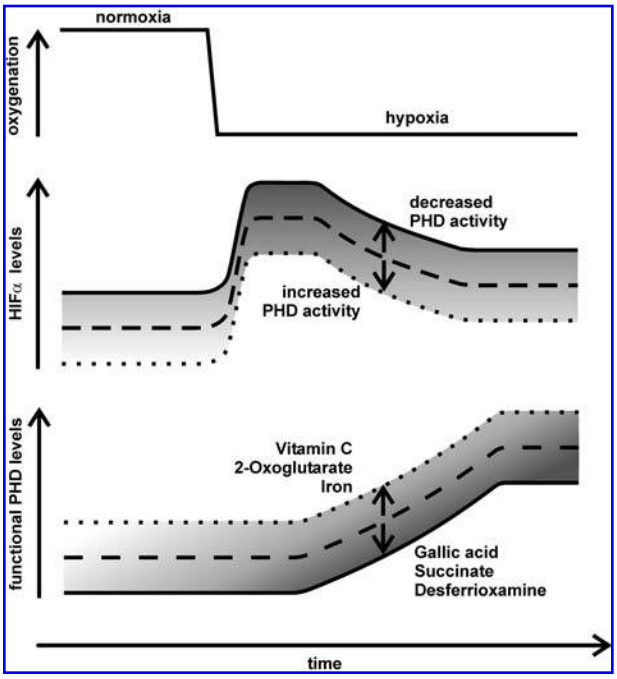

FIG. 5. Regulation of PHD-dependent oxygen sensing. Upon a hypoxic insult, the HIF-dependent transcriptional increase of PHD2 and PHD3 levels provides a negative feedback mechanism that defines a novel, adapted oxygen sensing setpoint capable of reacting to a second hypoxic insult. Small molecules contribute to the regulated oxygen sensing by triggering PHD function, leading to an inverse regulation of the HIF levels (for details see text).

phenolate oxygen atoms of gallate chelate with the active center iron and the carboxyl group of gallate forms a strong ionic/hydrogen bonding with Arg383 of PHD2 (53). In the hands of these authors, *n*-propyl gallate was effective only in cell culture but not in cell-free PHD assays, which was attributed to the esterified carboxyl group of *n*-propyl gallate. However, in our hands *n*-propyl gallate was even more efficient in PHD inhibition *in vitro* than gallic acid. A likely reason for this discrepancy was the use of crude cell lysates in a 2-oxoglutarate conversion assay by these authors. We recently found that 2-oxoglutarate turnover using crude lysates is independent of PHD function (Wirthner R. *et al.*, unpublished observations).

What might be the mechanism of PHD inhibition by gallate-containing antioxidants in comparison to other antioxidants such as *N*-acetyl-L-cysteine? The use of molecular dioxygen for oxidative decarboxylation-coupled protein hydroxylation requires short-lived, highly reactive transition states of oxygen. Antioxidants fitting into the active center of PHDs might scavenge these ROS, thereby blocking the PHD reaction cycle. Similarly, exogenously produced ROS potentially could interfere with these reactive transition states of oxygen. It has been suggested that the increase in ROS content in $junD^{-/-}$ or mucin 1 (MUC1) knock-down cells leads to a decrease in PHD activity and hence to HIF-1α accumulation (15, 59). Thus, antioxidants paradoxically would have the potential to do both: protect PHD function from exogenous ROS and block PHD activity by destruction of PHD-inherent ROS intermediates. The effective function of a given antioxidant would depend on the accessibility to the active center, interference with transition metals and ascorbate, and the scavenging efficiency of mitochondrial and/or NADPH oxidase-derived ROS.

Regulation of the PHD oxygen sensors by other mechanisms

Up to date, only few reports deal with the regulation of PHDs by protein–protein interactions. The E3 ubiquitin ligase Siah2 regulates PHD1 and PHD3, but not PHD2, protein stability (39). PHD3, but not PHD1 or PHD2, appears to be a substrate for the TRiC chaperonin (34). OS-9 apparently is simultaneously interacting with both HIFα and PHD2 or PHD3, but not PHD1, thereby enhancing HIFα hydroxylation and degradation (4). Mitogen-activated protein kinase organizer 1 (MORG 1) might provide the molecular scaffold for HIFα interaction specifically with PHD3 (19). Finally, we recently reported that FKBP38 specifically regulates the stability of PHD2 (5). These examples demonstrate two things: first, abundance and function of PHDs can also be regulated by specific proteins; and second, the three different PHDs are regulated in nonidentical ways, further supporting their nonredundant role in oxygen sensing. While the cell-free *in vitro* assay used in this work provides useful information on the regulated function of each PHD individually, it can of course not take into account the manifold additional modes of PHD regulation, (*e.g.*, by protein–protein interaction), found within a cell.

PHD2 and PHD3, but not PHD1 or FIH, are transcriptionally induced under hypoxic conditions (3, 6, 8, 11, 13, 14, 33). It could be shown that HIF is required for hypoxic induction of PHD2 and PHD3 gene expression, and hypoxia response elements were identified in the regulatory regions of the corresponding genes (37, 42). Because the essential cofactor oxygen is basically lacking under hypoxic conditions, the HIF-dependent hypoxic increase in PHD abundance is somewhat paradoxical. It has been suggested that increased PHD levels accelerate the termination of the HIF response following reoxygenation (2, 3, 14, 33). Indeed, biochemical *in vitro* studies revealed K_m values of purified PHDs for oxygen close to the pO_2 in air, suggesting that the kinetics of specific HIFα hydroxylation under hypoxic conditions are rather slow (17). However, tissues *in situ* have to deal with a great variability of generally very low pO_2 values, even when the inspiratory pO_2 is considered to be "normoxic". Thus, the PHD oxygen sensors need to operate at different pO_2 setpoint values in different tissues. We recently demonstrated that a self-regulatory loop defines a specific threshold for HIFα-activation as a function of the actual pO_2 (50). As schematically outlined in Fig. 5, this negative feedback-loop includes: (a) a HIF-dependent induction of PHD2 and PHD3 upon a reduction in oxygen supply; (b) a PHD-dependent, partial reduction of HIFα even under very low pO_2; (c) the subsequent partial reduction of the PHD2 and PHD3 levels; (d) the definition of a novel setpoint for oxygen sensing by leveling off the HIF/PHD ratio; (e) a secondary response to a further, more severe hypoxic insult; and (f) the triggering by iron, ascorbate, Krebs cycle intermediates, ROS, and antioxidants. Small molecule regulators of PHD activity are thus functionally relevant under normoxic as well as hypoxic conditions.

ACKNOWLEDGMENTS

The authors wish to thank S. Tan for providing the VBC expression vector. This work was supported by individual grants from the Forschungskredit of the University of Zürich (to DPS and GC), Olga Mayenfisch-Stiftung (to GC), Sassella-Stifung (to DPS), Krebsliga des Kantons Zürich (to RHW), 6th Framework Programme of the European Commission/SBF (EUROXY LSHC-CT-2003-502932/SBF Nr. 03.0647-2 to RHW) and Swiss National Science Foundation (3100A0-104219 to RHW and GC).

ABBREVIATIONS

FIH, factor inhibiting HIF; HIF, hypoxia-inducible factor; Hyp, hydroxyproline; ODD, oxygen-dependent degradation; PHD, prolyl-4-hydroxylase domain protein; pO_2, oxygen partial pressure; pVHL, von Hippel–Lindau tumor suppressor protein; ROS, reactive oxygen species; VBC, pVHL/elongin B/elongin C.

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Date of first submission to ARS Central, April 4, 2007; date of final revised submission, April 16, 2007; date of acceptance, April 25, 2007.

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