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The HIF prolyl hydroxylase PHD3 is a potential substrate of the TRiC chaperonin ☆

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Abstract Hypoxia-inducible factor-1 (HIF) is regulated by oxygen-dependent prolyl hydroxylation. Of the three HIF prolyl hydroxylases (PHD1, 2 and 3) identified, PHD3 exhibits restricted substrate specificity in vitro and is induced in different cell types by diverse stimuli. PHD3 may therefore provide an interface between oxygen sensing and other signalling pathways. We have used co-purification and mass spectrometry to identify proteins that interact with PHD3. The cytosolic chaperonin TRiC was found to copurify with PHD3 in extracts from several cell types. Our results indicate that PHD3 is a TRiC substrate, providing another step at which PHD3 activity may be regulated.

Keywords: Prolyl hydroxylase; Protein folding; TRiC chaperonin

1. Introduction

Hypoxia-inducible factor (HIF) plays a key role in mediating cellular responses to oxygen [1]. HIF itself is regulated by oxygen-dependent hydroxylation of specific amino acids within HIF-α subunits [2]. Asparaginyl hydroxylation mediated by factor inhibiting HIF-1 (FIH-1) [3] regulates HIF transactivation, while prolyl hydroxylation regulates degradation of HIF-α chains. HIF-1α and HIF-2α each possess two oxygen-dependent degradation domains (ODD), denoted NODD and CODD (for N- and C-terminal ODD [4]), containing the prolyl residues essential for oxygen-dependent regulation (residues 402 and 564 in human HIF-1α). In mammals three HIF-prolyl hydroxylases have been identified (denoted PHD1, 2 and 3) that oxidise these prolines to the corresponding *trans*-4-hydroxyproline residues [5,6], leading to ubiquitylation by the von Hippel–Lindau (VHL) E3 ubiquitin ligase and degradation. Like FIH, the PHD enzymes are members of the Fe(II)- and 2-oxoglutarate-dependent dioxygenase superfamily. The relative importance of each PHD isoform in the physiological regulation of HIF is unclear, although one study suggests that PHD2 is critical for achieving the normally low steady state levels of HIF-1α in normoxia [7]. Studies of the PHDs in vitro indicate differences in substrate specificity with PHD3 unable to hydroxylate the NODD [6,8]. The PHD's also differ in their tissue distributions [9,10] and, at least under conditions of overexpression, have distinct patterns of subcellular localisation [11,12]. Inducibility of the PHD genes also varies. For instance, both PHD2 and PHD3 mRNAs are induced by hypoxia [6,13,14], PHD1 mRNA has been reported to be oestrogen-inducible [15] and PHD3 has been identified as a gene induced by p53 [16], by stimuli inducing smooth muscle differentiation [17,18] and by nerve growth factor withdrawal [19].

Given the inducibility of PHD3 by various stimuli (including hypoxia) and its restricted substrate specificity for the HIF-α CODD, we have sought to understand more about the cellular regulation of PHD3. As an initial approach, we have identified a set of 50–60 kDa polypeptides that copurify with PHD3 from cell extracts. These proteins correspond to subunits of the cytosolic chaperonin TRiC (TCP-1 ring complex, also called CCT, for chaperonin containing TCP-1). The interaction between PHD3 and TRiC appears to be specific to this particular HIF hydroxylase and was stabilised by EDTA, suggesting that PHD3 is a TRiC substrate.

2. Materials and methods

2.1. Plasmids and in vitro translation

PHD1PK, PHD2PK, PHD3PK and FIHPK expression vectors were constructed in pcDNA3. pUHD10 [20] was used to make the tet-operator-dependent PHD3PK expression plasmid. [^{35}S]Methionine-labelled proteins were generated in TNT reticulocyte lysate (Promega). For the EDTA pulse chase experiment, PHD3PK was translated for 15 min at 30 °C, cycloheximide was then added (2 mM final concentration) and the mixture incubated for a further 40 min with or without EDTA (5 mM final concentration).

2.2. Cell culture, transient and stable transfections

HeLa and HEK 293T cells were maintained in Dulbecco's modified Eagle's medium with 10% foetal calf serum, glutamine (2 mM), penicillin (50 IU/ml) and streptomycin sulfate (50 μg/ml). For metabolic labelling, cells were pre-incubated for 1 h in serum-free medium, lacking methionine and cysteine followed by incubation for 5 h in medium containing 250 μCi/ml [^{35}S]methionine/cysteine (Pro-mix, Amersham Biosciences). Transient transfections were performed using

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FuGene 6 (Roche Molecular Biochemicals). To generate the PHD3PK stable cell line, U20S cells bearing the reverse tetracycline responsive transactivator [20] and a tetKRAB silencer construct [21] were transfected with pUHD10-PHD3PK plasmid and colonies picked after selection in hygromycin B (200 μg/ml). The cell line was maintained in medium containing 10% Tet System approved FBS (BD Biosciences), hygromycin B (200 μg/ml), and blasticidin S (5 μg/ml). PHD3PK expression was induced by administration of doxycycline (0.1 μg/ml). Hypoxic incubations were performed in a Napco 7001 incubator (Jouan).

2.3. Antibodies, immunoprecipitation and immunoblotting

Anti PK-tag (sv5-pk) (Serotec), anti-TCP-1α (91a) (Calbiochem) and anti-HIF-1α antibody (Transduction Laboratories clone 54) were used. Monoclonal antibodies to PHD2 and PHD3 produced in this laboratory will be described elsewhere (Y. Tian unpublished). Cell extracts were prepared under denaturing conditions (8 M urea, 10% glycerol, 1% SDS, 1 mM DTT, and 10 mM Tris, pH 6.8), or in NP-40 lysis buffer [10 mM Tris, pH 7.5, 0.25 M NaCl, and 0.5% NP-40 with "Complete" Protease inhibitor (Roche Molecular Biochemicals)]. For immunoprecipitation, cell extract was pre-cleared for 1 h at 4 °C with Protein G–Sepharose beads. Samples were incubated with antibody at 4 °C for 1 h followed by 2 h incubation with Protein G–Sepharose. Beads were washed five times in Wash Buffer (125 mM NaCl, 25 mM Tris, pH 7.5, and 0.1% NP-40). Following SDS–PAGE, proteins were Coomassie stained, or transferred onto Immobilon-P membrane (Millipore) and immunoblotted. [^{35}S]methionine-labelled proteins were first diluted (10 μl reticulocyte lysate diluted to 500 μl with NP-40 lysis buffer) prior to addition of antibody and immunoprecipitation as above.

2.4. MALDI-MS protein identification

Protein bands were digested in-gel using 5 μl of 12 ng/mL porcine trypsin (Promega). Tryptic peptides were then desalted and concentrated on chromatographic beads [22] consisting of Poros R2 material (Boehringer Mannheim) prior to analysis by MALDI-TOF mass spectrometry using a REFLEX MALDI-TOF mass spectrometer (Bruker–Daltonik) operating in reflectron mode. Peptide mass fingerprints were analysed using the Mascot algorithm (http://www.matrixscience.com), using a peptide mass tolerance of 0.3 Da. Unidentified peptides were subjected to MALDI-PSD analysis and fragmentation spectra analysed with the Mascot algorithm using a fragment mass tolerance of 0.8 Da.

3. Results and discussion

To identify proteins that interact with PHD enzymes, a co-immunopurification approach was adopted (Fig. 1A). A set of 50–60 kDa polypeptides were found to copurify with PHD3PK but not with PHD1PK following transient transfection of expression plasmids into HeLa cells.

To maximise the potential for successful identification of the PHD3-associated proteins, a doxycycline-inducible stable PHD3PK U20S cell line was made. Anti-PK immunoblottting confirmed doxycycline-inducible expression of PHD3PK (Fig. 1B). Parallel immunoblottting with anti-HIF-1α antibody established that the overexpressed PHD3PK enzyme was functional, significantly reducing HIF-1α protein level induced by hypoxia and even the basal level in normoxia (Fig. 1B). Finally, [^{35}S]methionine-labelling and anti-PK immunoprecipitation confirmed that association between PHD3PK and the set of 50–60 kDa polypeptides also occurred in this cell line (Fig. 1C).

To identify the copurifying proteins, large-scale purifications were performed from the PHD3PK inducible stable cell line. Coomassie-staining (Fig. 2A) was sufficient to reveal a set of 50–60 kDa polypeptides copurifying with PHD3PK (consistent with ^{35}S-labelling, Fig. 1A and C). The identity of these

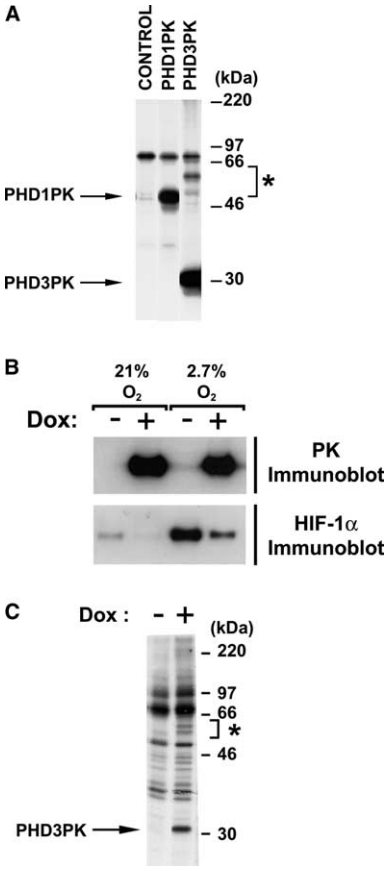

Fig. 1. PHD3PK associates with a set of 50–60 kDa proteins in vivo. (A) HeLa cells were transiently transfected with empty vector (control), PHD1PK or PHD3PK as indicated. Cells were labelled with [^{35}S]methionine, lysed and analysed by immunoprecipitation with anti-PK antibody. Captured proteins were resolved by 10% SDS–PAGE and detected by fluorography. The 50–60 kDa PHD3PK-associated proteins are indicated by an asterisk. (B) PHD3PK cells were either untreated (−) or induced (+) with doxycycline (Dox) for 20 h, followed by exposure to normoxic (21% O_2) or moderate hypoxic (2.7% O_2) conditions for 4 h. Extracts were prepared under denaturing conditions and analysed by immunoblotting. (C) PHD3PK cells were either untreated (−) or induced (+) with doxycycline (Dox) for 24 h as indicated, followed by [^{35}S]methionine-labelling, harvest and immunoprecipitation with anti-PK antibody. The position of the PHD3PK protein is shown and the copurifying 50–60 kDa proteins marked with an asterisk.

proteins was determined by mass spectrometry. A match to the TRiC subunit, TCP-1γ, was obtained with the 55 kDa band (Fig. 2B). The 60 kDa band appeared to be a mixture of proteins with the Mascot algorithm unable to clearly identify any protein (Supplementary Data). However, the analysis did yield some hits to the TRiC subunits TCP-1α, θ, ε, and β. To gain further clarification on the 60 kDa band, the major peptide 1150.57 was subjected to MALDI-PSD analysis (Fig. 2C) confirming the presence of TCP-1θ subunit in the protein mixture of the 60 kDa band. Immunoblotting was then used to demonstrate association of PHD3 with the TCP-1α TRiC subunit (Fig. 2D). These results demonstrate that PHD3 interacts with at least three subunits of TRiC in U20S cell extracts (TCP-1γ and θ by mass spectrometric analyses and TCP-1α by immunoblotting). To ensure that association of TRiC with PHD3PK was not an artefact of the PK-tag, native PHD3 was transiently overexpressed in HEK 293T cells and immunoprecipitated with a PHD3 specific antibody (Fig. 2D).

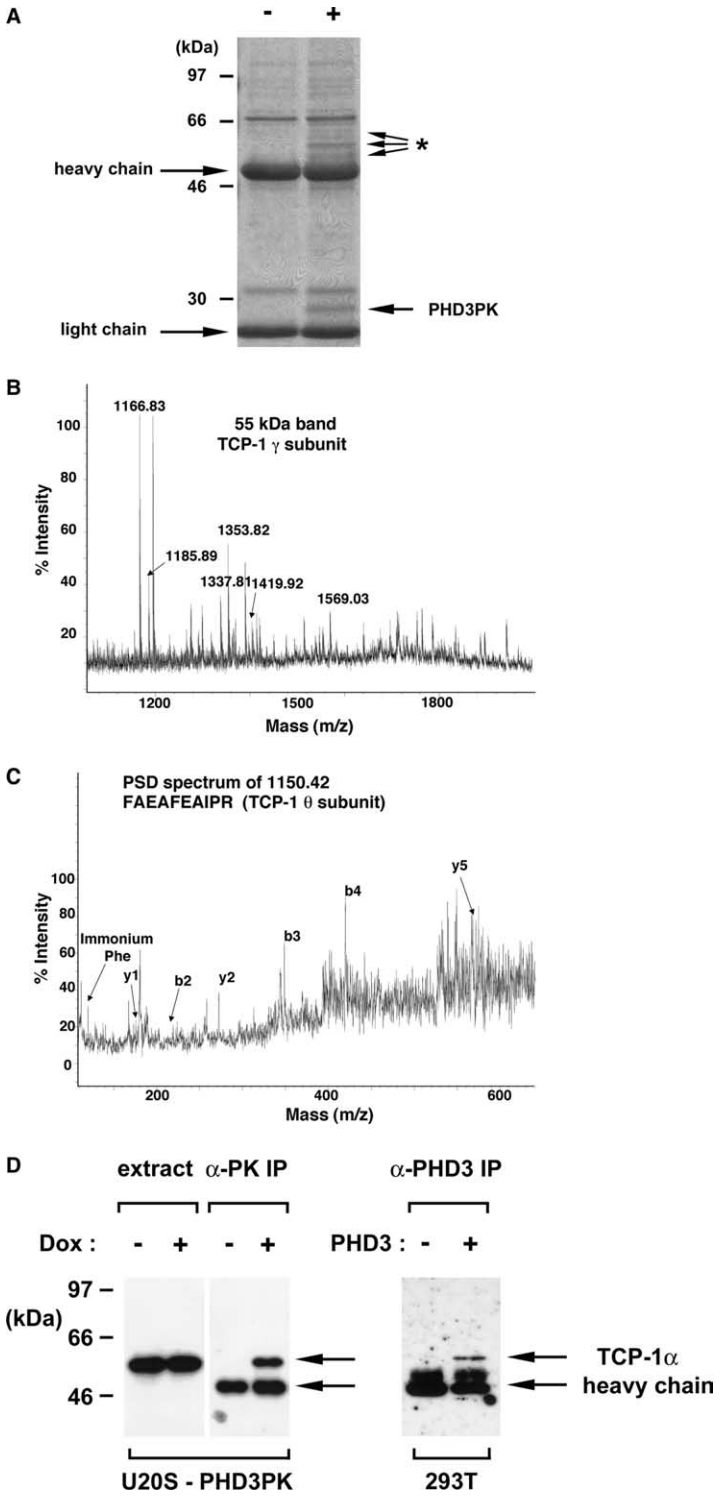

Fig. 2. Identification of the TRiC chaperonin as a PHD3 associated complex in U20S cells. (A) The PHD3PK inducible stable cell line was either mock treated (−) or induced with doxycycline (+). Cell lysates were immunoprecipitated with anti-PK antibody, resolved by 10% SDS–PAGE and retrieved proteins stained with Coomassie blue. The anti-PK immunoprecipitates revealed a doxycycline-inducible band with the expected mobility for PHD3PK. This assignment was confirmed by immunoblotting and mass spectrometric analysis (data not shown). The positions of the heavy and light chain (resulting from the anti-PK antibody) are shown. PHD3PK co-precipitating proteins are indicated by the asterisk and include a major band at 60 kDa. (B) The 55 kDa PHD3PK-associated band from U20S extracts (Fig. 2A) was excised, digested in-gel with trypsin and analysed by MALDI-TOF. Using the Mascot algorithm, the labelled peptides from the 55 kDa band were identified as tryptic peptides from (P49368) TCP-1γ subunit with a score of 34 and 8% sequence coverage. C, The PSD analysis of peptide 1150.42 in the 60 kDa band. The fragmentation pattern was identified by Mascot as originating from the peptide FAEAFEAIPR with a score of 21. D, the PHD3PK inducible stable cell line was either mock treated (−) or induced with doxycycline (''Dox'', +). Cell extracts were either analysed directly, or immunoprecipitated with anti-PK antibody, prior to 10% SDS–PAGE and immunoblotting with TCP-1α antibody. 293T cells were either mock transfected (−) or transfected (+) with PHD3 plasmid. 40h after transfection, extracts were immunoprecipitated using anti-PHD3 antibody. The position of the interacting TCP-1α protein is shown.

Immunoblotting for TCP-1α confirmed association between native PHD3 and TRiC. To address whether the PHD3–TRiC interaction observed in transfected cells occurred because of PHD3 overexpression, association was analysed following synthesis of PHD3 in reticulocyte lysate. In vitro translation produces very low concentrations of protein (fM-pM) that are vastly lower than the concentration of TRiC in the lysate (μM range, [23]). All four HIF hydroxylases were synthesised in reticulocyte lysate and assayed for association with TRiC by immunoblotting with anti-TCP-1α antibody (Fig. 3A). Association with TRiC appears to be specific to the PHD3 enzyme, since no interaction was seen with PHD1, PHD2 or FIH in this system.

Since the immunoprecipitation conditions used would not be expected to disrupt the TRiC chaperonin, it appears that PHD3 is binding to the holo-TRiC complex. TRiC is a large (900 kDa) cylindrical double ring complex, with each ring consisting of 8 homologous subunits. It binds non-native polypeptides in its central cavity and mediates folding and release in a Mg^{2+}/ATP-dependent manner [24,25]. The ability to purify Coomassie-stainable levels of TRiC with PHD3 (Fig. 2A) indicates that TRiC is likely to be a regulator rather than a substrate of PHD3. To determine whether TRiC may be required for PHD3 folding, protein association was studied in a pulse chase experiment in the presence or absence of EDTA (Fig. 3B). Newly translated PHD3 was found associated with TRiC but was released during the subsequent chase. In contrast, addition of EDTA to the chase mixture stabilised the PHD3–TRiC association, suggesting that as with other TRiC substrates, Mg^{2+}/ATP is required. TRiC-bound proteins include binding partners [26] as well as substrates. A limited number of TRiC substrates have been identified which include cytoskeletal proteins such as actin, α- and β-tubulin, and signalling proteins such as G α-transducin and cyclin E [27]. Known TRiC substrates appear to have either aggregation-prone folding intermediates or a requirement for binding to an oligomeric partner(s) to complete folding [27]. It is likely that PHD3 conforms to the former class of substrates, since overexpression of PHD3 (and not the other HIF hydroxylases) in mammalian cells is known to result in aggregates [12] and immunoprecipitations from ^{35}S-labelled cell lysates did not reveal any evidence of a PHD3 multiprotein enzyme complex, only the association with TRiC (Fig. 1A and C).

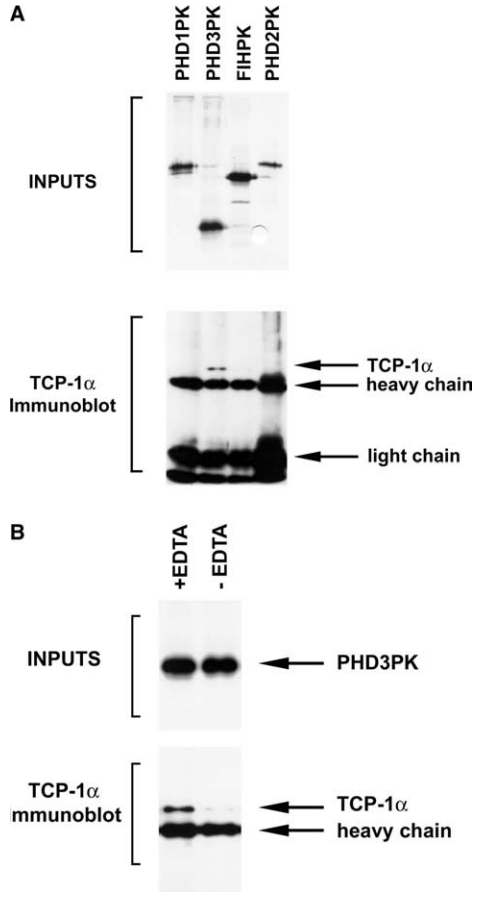

Fig. 3. PHD3 is a specific substrate of TRiC in reticulocyte lysate. (A) PHD1PK, PHD2PK, PHD3PK and FIHPK proteins were synthesised in reticulocyte lysate and immunopurified using either anti-PK antibody (PHD1PK, PHD3PK and FIHPK) or anti-PHD2 antibody (PHD2PK). The immunopurified proteins were analysed by 15% SDS–PAGE (INPUTS) to check recovery. Immunoprecipitates normalised for PHD/FIH protein level were then resolved on 10% SDS–PAGE and immunoblotted with TCP-1α antibody. The position of the TCP-1α protein is shown. (B) The effect of EDTA on PHD3-TCP-1α association in reticulocyte lysate was studied. ^{35}S-labelled PHD3PK was translated for 15 min at 30 °C, cycloheximide was added and the mixture further incubated in the presence or absence of 5 mM EDTA as indicated. PHD3PK was then immunopurified using anti-PK antibody and recovery checked by autoradiography (INPUTS). Immunoprecipitates normalised for PHD3PK protein were then immunoblotted with anti-TCP-1α antibody.

TRiC has been proposed to play a specific role in the formation of enzymatically active multiprotein complexes [28,29], including the VHL/elongin B/C complex [30–32]. The identification of PHD3 as a novel TRiC substrate raises questions as to whether interplay may exist between PHD3–TRiC and VHL–TRiC complexes in vivo, which may impact upon HIF regulation. Endogenous PHD3 protein is expressed at low levels under normoxic conditions but is induced by hypoxia (Y. Tian et al., unpublished) and PHD3 mRNA is also known to be induced in different cell types by a range of other stimuli [16–19]. In the future, it will be of interest to determine whether the induction of PHD3 protein in vivo has any effect on VHL–TRiC association and the extent of cross-talk between these interactions in direct responses to hypoxia.

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