A Conserved Family of Prolyl-4-Hydroxylases That Modify HIF

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Mammalian cells respond to changes in oxygen availability through a conserved pathway that is regulated by the hypoxia-inducible factor (HIF). The alpha subunit of HIF is targeted for degradation under normoxic conditions by a ubiquitin-ligase complex that recognizes a hydroxylated proline residue in HIF. We identified a conserved family of HIF prolyl hydoxylase (HPH) enzymes that appear to be responsible for this posttranslational modification. In cultured mammalian cells, inappropriate accumulation of HIF caused by forced expression of the HIF-1α subunit under normoxic conditions was attenuated by coexpression of HPH. Suppression of HPH in cultured *Drosophila melanogaster* cells by RNA interference resulted in elevated expression of a hypoxia-inducible gene (*LDH*, encoding lactate dehydrogenase) under normoxic conditions. These findings indicate that HPH is an essential component of the pathway through which cells sense oxygen.

The ability of cells to recognize and respond to a low-oxygen environment (hypoxia) is critical in many physiological and pathophysiological conditions (*1*). Almost all mammalian cells express components of a hypoxia response pathway that is conserved in both flies (*2*, *3*) and worms (*4*). The hypoxia-inducible transcription factor HIF lies at the heart of this pathway. HIF is a heterodimer composed of two members of the basic-Helix-Loop-Helix (bHLH)–containing PER-ARNT-SIM (PAS) domain family, HIF-1α and HIF-1β, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) (*5*). Under normoxic conditions, HIF-1α is constitutively expressed. However, this subunit is rapidly targeted for proteasome-mediated degradation (*6*–*8*) through a protein-ubiquitin ligase complex containing the product of the von Hippel Lindau tumor suppressor protein (pVHL) (*9*–*12*). pVHL recognizes the oxygen degradation domain (ODD) of HIF-1α only under normoxic conditions (*13*–*15*). When cells are exposed to a hypoxic environment, this degradation pathway is blocked, thereby allowing HIF-1α to accumulate and migrate to the nucleus, where it activates hypoxia-responsive genes [reviewed in (*16*)].

pVHL recognizes the ODD in HIF-1α through a conserved proline residue that is hydroxylated exclusively under normoxic conditions (*13*–*15*). Cellular extracts prepared under normoxic conditions contain a prolyl-4-hydroxylase activity capable of modifying a proline-containing peptide derived from the ODD, whereas extracts prepared under hypoxic conditions or in the presence of "hypoxia mimics" such as $CoCl_2$ or the iron chelator deferoxamine mesylate are deficient in this activity (*13*–*15*). As is the case for known prolyl-4-hydroxylases, this activity is enhanced by supplementation with Fe^{2+}, ascorbate, and 2-oxoglutarate (*14*, *15*).

The best characterized prolyl-4-hydroxylases modify collagen as it matures along the exocytotic pathway (*17*). These enzymes are typically composed of two α and two β subunits, with the α subunit responsible for the prolyl hydroxylase activity. Three lines of evidence rule out these enzymes as the HIF prolyl hydroxylase. First, the substrate context of the modified proline residues in collagen is different from that surrounding the relevant proline residue in HIF (*13*–*15*). Second, the collagen-modifying enzymes reside within the endoplasmic reticulum rather than the cytoplasm, as expected for the HIF prolyl hydroxylase (*17*). Finally, two recombinant isoforms of the collagen-modifying enzymes expressed from baculovirus were reported to show no activity against the HIF substrate (*14*). To identify prolyl-4-hydroxylases, we queried the GenBank database for sequences related to the catalytic α subunit of the collagen-modifying prolyl-4-hydroxylases. Of the several families of putative prolyl hydroxylases in the database, five contained human homologs. Furthermore, each of these families contained conserved amino acid residues believed to bind Fe^{2+} and 2-oxoglutarate (*17*).

To investigate whether any of these candidate enzymes specify HIF-1α–directed prolyl hydroxylase activity, we cloned a representative member of each family into the pcDNA3.1/V5-His expression vector (*18*). These five polypeptides, designated candidates A, B, C, and D, and one candidate subsequently designated HIF prolyl hydroxylase-1 (HPH-1), were in vitro transcribed/translated in a rabbit reticulocyte lysate system. To measure prolyl hydroxylase activity (*19*), we incubated the translation products in the presence of ascorbate, 2-oxoglutarate, and $FeSO_4$ with a biotinylated peptide derived from the human HIF-1α ODD that contains the target proline residue (biotin-Acp-DLDLEALAP*YIPADDDFQL) (*20*). When this proline residue (P*) is hydroxylated, this peptide binds to human pVHL (*13*–*15*). Streptavidin-coated agarose beads were used to precipitate [^{35}S]-labeled human pVHL associated with the biotinylated peptide and measured by scintillation counting (Fig. 1A). Minimal prolyl hydroxylase activity was observed in rabbit reticulocyte lysate programmed to express a *LacZ* control, demonstrating that the majority of endogenous prolyl hydroxylase activity reported for reticulocyte lysate is inactivated during the 1-hour incubation. Of the five candidate enzymes assayed, only one, HPH-1, enhanced pVHL association with the biotinylated peptide. In addition to HPH-1, the human genome encodes two highly related HPH-1 paralogs, designated HPH-2 and HPH-3 (Fig. 2). Like HPH-1, both of these enzymes catalyzed the hydroxylation of the key proline residue in the HIF-1α peptide substrate as measured by the [^{35}S]-pVHL pull-down assay (Fig. 1B).

Several amino acid residues have been shown through site-directed mutagenesis studies to be involved in coordinating Fe^{2+} within the active site of the collagen-modifying prolyl-4-hydroxylases (*17*). These same residues are also present in the HPH enzymes (indicated by asterisks in Fig. 2). To test the role of these residues in HPH-1–mediated prolyl hydroxylation, we individually changed His^{135}, Asp^{137}, and His^{196} to Ala (H135A, D137A, and H196A, respectively). Each of these mutations eliminated the prolyl hydroxylase activity of the native HPH-1 enzyme (Fig. 1B).

We next expressed the human HPH in bacterial cells and purified soluble protein by affinity chromatography (*21*). As shown in Fig. 1C, purified HPH hydroxylated the HIF-1α peptide substrate, and its activity was greatly stimulated by the addition of ascorbate, 2-oxoglutarate, and $FeSO_4$. Mass spectrometry analysis of the resultant peptide product indicated a mass increase of 16 daltons, consistent with hydroxylation of the proline residue (Fig. 1D). A control peptide in which the target proline residue was replaced by alanine was not modified (*22*). Furthermore, HPH activity was inhibited by an excess of $CoCl_2$, which is known to induce the hypoxic response pathway by stabilizing HIF under normoxic conditions, possibly by

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competing with Fe^{2+} for occupancy within the active site of HPH (Fig. 1C). Together, these data suggest that HPH-1, -2, and -3 are prolyl hydroxylases that modify HIF-1α.

Previous studies have indicated that amino acids in close proximity to the proline residue targeted for hydroxylation (Pro^{564} in human HIF-1α) influence HIF-1α modification and pVHL binding. Specifically, mutation of Leu^{562} to Ala (L562A) (*13*), Ala^{563} to Gly (A563G) (*15*), and Tyr^{565} to Ala (Y565A) (*14*) prevents prolyl hydroxylation by the endogenous HIF prolyl hydroxylase activity in cellular extracts. Conversely, mutation of Pro^{567} to Gly exerts a slight stimulatory effect in pVHL pull-down assays (*14*). To examine the substrate specificity of HPH-1, we synthesized individual biotinylated peptide substrates that contained each of these four point mutations. To distinguish the effects of these mutations on pVHL binding from their effects on proline hydroxylation, we synthesized each peptide with the target proline residue (Pro^{564}) already hydroxylated. Fully hydroxylated peptides corresponding to the wild-type and variant ODD sequences were all recognized by [^{35}S]-pVHL, although the A563G and Y565A mutations did partially compromise pVHL association (Fig. 3A). As reported for the endogenous HIF prolyl hydroxylase, HPH-1 generated by in vitro transcription/translation (Fig. 3B), as well as recombinant HPH-2 purified from *Escherichia coli* (Fig. 3C), did not modify peptides containing the L562A, A563G, or Y565A mutations. However, a peptide containing the Pro^{567} to Gly mutation was an equal, if not better, substrate for the human HPH enzymes.

To test whether HPH enzymes function in the hypoxic response pathway in intact cells, we cotransfected (*23*) human embryonic kidney 293 cells with a hypoxia-responsive luciferase reporter (*24*) and increasing amounts of a vector expressing human HIF-1α under the control of the constitutive human cytomegalovirus (CMV) immediate early promoter. Forced overexpression of HIF-1α can overcome the degradation pathway, resulting in accumulation of HIF under normoxic conditions and subsequent induction of the HRE-containing HIF reporter gene (*24*). Induction of the HIF-dependent luciferase reporter by either 30 ng (Fig. 4A) or 100 ng (Fig. 4B) of HIF-1α expression vector was not affect-

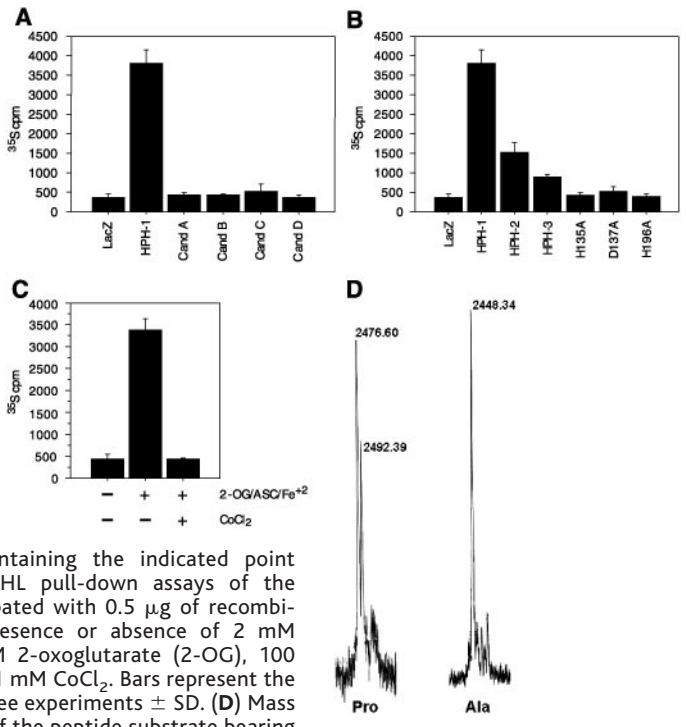

Fig. 1. HPH enzymes hydroxylate a human HIF-1α–derived peptide. (**A**) [^{35}S]-VHL pull-down assays of a biotin-Acp-DLDLEALAPY-IPADDDFQL (*20*) peptide corresponding to residues 556 to 574 of human HIF-1α. Polypeptides encoded by *LacZ* and each candidate gene were synthesized by in vitro transcription/translation in rabbit reticulocyte lysate before incubation with the substrate. (**B**) [^{35}S]-VHL pull-down assays of the peptide substrate incubated with each in vitro transcribed/translated HPH enzyme or HPH-1 containing the indicated point mutations. (**C**) [^{35}S]-VHL pull-down assays of the peptide substrate incubated with 0.5 μg of recombinant HPH-2 in the presence or absence of 2 mM ascorbate (ASC), 2 mM 2-oxoglutarate (2-OG), 100 μM $FeSO_4$ (Fe^{+2}), and 1 mM $CoCl_2$. Bars represent the average values from three experiments ± SD. (**D**) Mass spectrometry analysis of the peptide substrate bearing the target proline residue (left) or the Pro to Ala mutation (right) after incubation in the presence of active recombinant HPH-2.

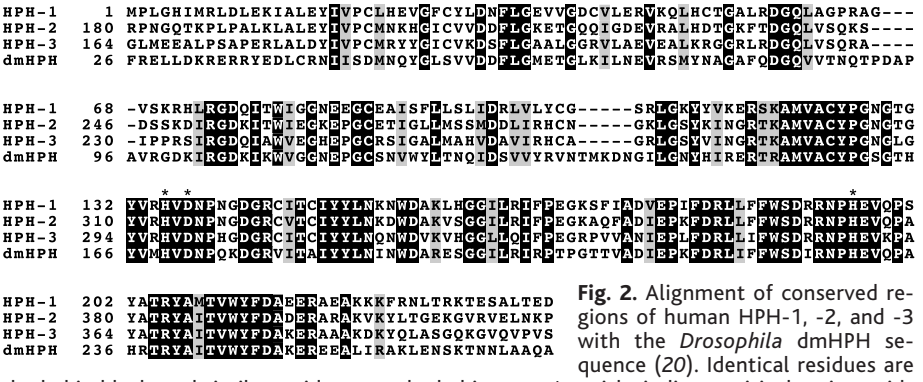

Fig. 2. Alignment of conserved regions of human HPH-1, -2, and -3 with the *Drosophila* dmHPH sequence (*20*). Identical residues are shaded in black, and similar residues are shaded in gray. Asterisks indicate critical amino acids for Fe^{2+} binding in the corresponding prolyl hydroxylase enzymes that modify collagen.

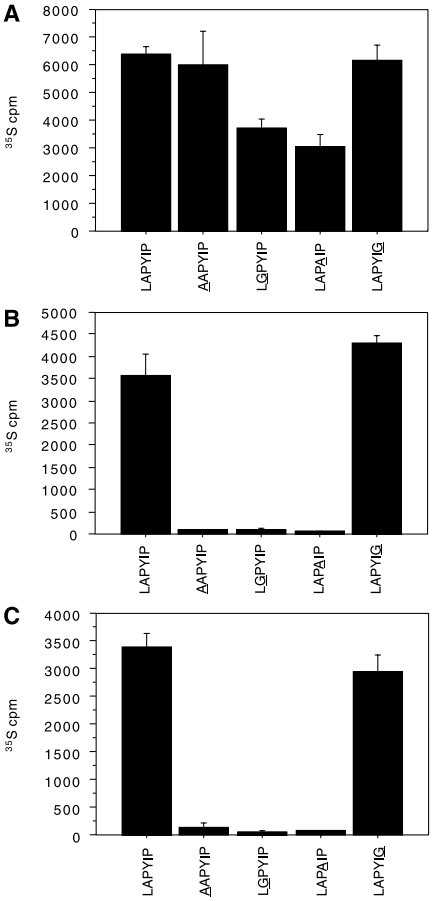

Fig. 3. Substrate specificity of the HPH enzymes. (**A**) Human VHL binds to all hydroxylated peptide substrates. Shown is a [^{35}S]-VHL pull-down assay of the hydroxylated biotin-Acp-DLDLEALAP*YIPADDDFQL peptide (LAPYIP) (*20*) and hydroxylated peptides containing the indicated point mutations (underlined). (**B**) [^{35}S]-VHL pull-down assays of the unmodified peptide substrates incubated with the in vitro transcribed/translated HPH-1 enzyme. (**C**) [^{35}S]-VHL pull-down assay of the unmodified peptide substrates incubated with the recombinant HPH-2 enzyme. Bars represent the average values from three experiments ± SD.

ed by cotransfection of 3 ng of CMV-driven vectors expressing *LacZ* or candidates A, B, C, and D (Fig. 4, A and B). By contrast, HPH-1 attenuated HIF-1α activation of the HRE-driven reporter, as might be expected if the prolyl hydroxylase activity was rate-limiting for HIF-1α degradation under these conditions (Fig. 4, A and B, left panels). Mutation of His^{135}, Asp^{137}, or His^{196} to Ala eliminated the interfering activity of HPH-1 in these transfection assays (Fig. 4, A and B, left panels). Neither HPH-1 nor the other candidate enzymes affected expression of a constitutive, CMV-driven luciferase reporter (Fig. 4C), demonstrating that HPH-1 specifically attenuates HIF-1α–mediated induction of the hypoxia-responsive reporter. Finally, the ability of HPH-1 to diminish induction of the HIF-responsive luciferase reporter was lost when cells were incubated in an atmosphere containing 0.5% O_2. These data suggest that the transiently overexpressed HPH-1 enzyme is inhibited by a low-oxygen environment (Fig. 4, A and B, right panels).

We also examined the role of HPH in the fruit fly *Drosophila melanogaster*. We identified a single *HPH* gene in the *Drosophila* genome (gene CG1114), hereby designated *dmHPH* (Fig. 2). *Drosophila* homologs of HIF-1α (Sima), ARNT (dARNT), and pVHL (dVHL) have been previously identified (*2*, *3*, *25*, *26*), and the activity and stability of Sima have been shown to be regulated by oxygen (*2*). Together, these observations suggest that *Drosophila* has a hypoxic response pathway analogous to that in mammalian cells. We incubated double-stranded RNAs corresponding to Sima or dmHPH with the KC167 cell line derived from *Drosophila* embryos to eliminate expression of these genes by RNA interference (RNAi) (*27*). After incubation under normoxic conditions, we prepared total RNA from the cells and examined it by Northern blotting. These results confirmed that RNAi substantially reduced the levels of mRNAs encoding Sima or dmHPH (Fig. 5A).

To determine the effects of the partial loss of function of these gene products, we examined the expression of the gene encoding lactate dehydrogenase (*dmLDH*), which is a HIF-dependent hypoxia-inducible gene in mammalian cells (*28*, *29*). As shown in Fig. 5B, untreated KC167 cells incubated in 1% O_2 for 15 hours accumulated 7.5 times more dmLDH mRNA than cells maintained under normoxic conditions (20% O_2). KC167 cells treated with double-stranded *Sima* RNA showed a twofold reduction in dmLDH mRNA levels. By contrast, RNAi-mediated reduction of dmHPH mRNA resulted in a 2.5-fold increase in dmLDH mRNA levels under normoxic conditions.

These data suggest that the HPH enzymes are bona fide HIF prolyl hydroxylases and act as integral regulators of the HIF-dependent hypoxia response pathway. In previous studies, a rat protein likely to be homologous to HPH-1, designated SM-20, has been reported to induce apoptosis (*30*). It is noteworthy that the ability of HPH-1 to attenuate HIF-1α induction of the hypoxia-responsive luciferase reporter is not attributable to its effect on cell viability. Cotransfection of HPH-1 with a CMV-driven luciferase reporter did not attenuate luciferase expression (Fig. 4C). Moreover, HPH-1 did not attenuate the HRE-driven luciferase reporter under hypoxic culture conditions (right panels of Fig. 4, A and B). Finally, cell cultures transfected with the HPH-1 expression vector showed no overt evidence of apoptosis.

The three human HPH enzymes identified here may be attractive targets for drug discovery. For example, a recent study with mice has revealed that transgenic expression of a modified form of HIF-1α lacking the ODD in basal keratinocytes results in increased dermal vascularization (*31*). In con-

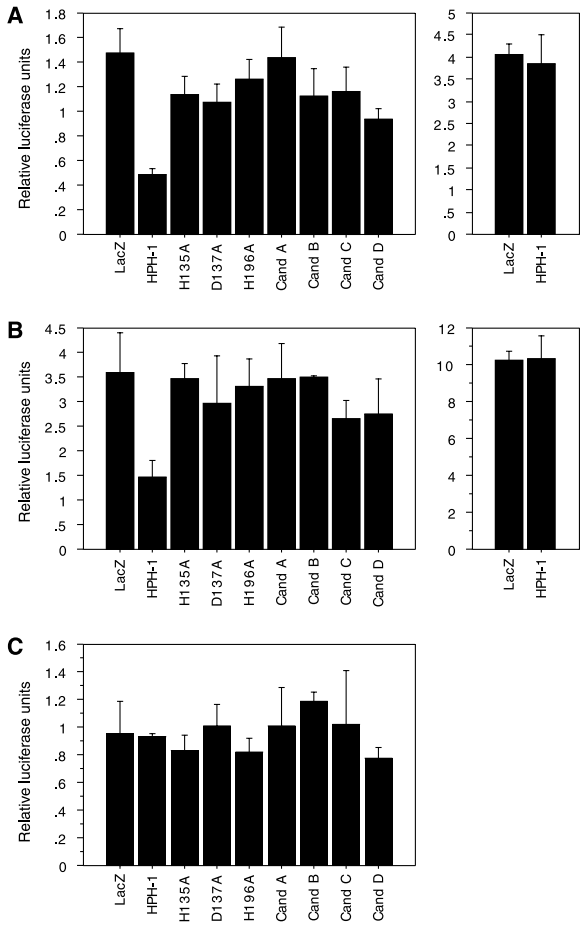

Fig. 4. Cotransfection of HPH-1 specifically attenuates overexpressed HIF-1α activity. Human 293 cells were transfected with 30 ng of a HIF-dependent luciferase reporter and 30 ng (**A**) or 100 ng (**B**) of a constitutive HIF-1α expression vector in the presence of 3 ng of HPH-1, other candidate prolyl hydroxylases, or inactive HPH-1 mutants. Cells cotransfected with the reporter, HIF-1α and Lac Z, or HPH-1 were also incubated in an atmosphere containing 0.5% O_2 for 15 hours starting 5 hours after transfection [(A) and (B), right panels]. (**C**) Three nanograms of each candidate expression vector was cotransfected with 0.5 ng of a constitutive luciferase reporter driven by the CMV promoter. Bars represent the average values from three experiments ± SD.

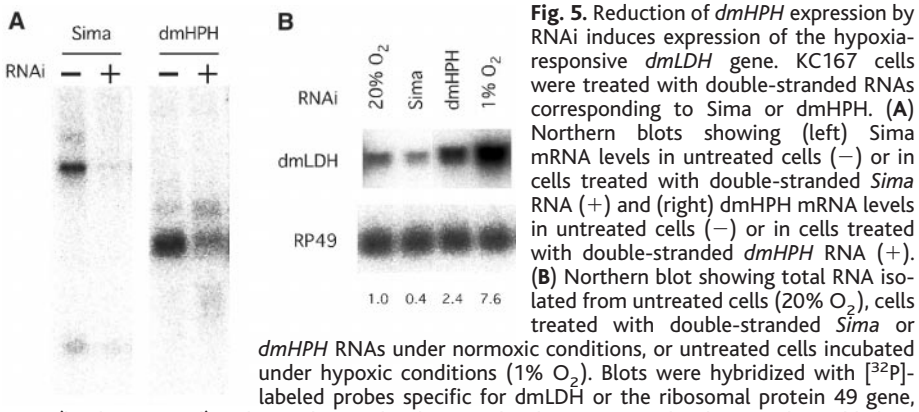

Fig. 5. Reduction of *dmHPH* expression by RNAi induces expression of the hypoxia-responsive *dmLDH* gene. KC167 cells were treated with double-stranded RNAs corresponding to Sima or dmHPH. (**A**) Northern blots showing (left) Sima mRNA levels in untreated cells (−) or in cells treated with double-stranded *Sima* RNA (+) and (right) dmHPH mRNA levels in untreated cells (−) or in cells treated with double-stranded *dmHPH* RNA (+). (**B**) Northern blot showing total RNA isolated from untreated cells (20% O_2), cells treated with double-stranded *Sima* or *dmHPH* RNAs under normoxic conditions, or untreated cells incubated under hypoxic conditions (1% O_2). Blots were hybridized with [^{32}P]-labeled probes specific for dmLDH or the ribosomal protein 49 gene, RP49 (loading control). Relative dmLDH levels normalized to normoxic levels are indicated below.

trast to the leaky vasculature that is induced by overexpression of single vasculogenic growth factors such as VEGF (*32–34*), the HIF-induced vascular bed is stable (*31*). The more substantive neovascularization resulting from constitutive HIF-1α expression may reflect the fact that this transcription factor activates not only VEGF gene expression but also other genes important for the formation of new blood vessels [reviewed in (*35*)]. Selective inhibitors of the HPH enzymes may therefore merit investigation as new drugs for therapeutic angiogenesis.

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18. Coding regions were amplified by reverse transcriptase polymerase chain reaction (RT-PCR) from total RNA prepared from human cell lines with oligonucleotides derived from the following sequences (GenBank accession number): HPH-1 (XM_012332), HPH-2 (AF229245), HPH-3 (BC001723), candidate A (NM_017732), candidate B (AK022130), candidate C (AK001580), and candidate D (AK023553). A splice variant of HPH-2 was used in which residues 76 to 177 were omitted. Point mutations in HPH-1 were generated by PCR. Each cDNA was cloned into the pcDNA3.1/V5-HIS vector (Invitrogen) in frame with the COOH-terminal V5-HIS tag.
19. Candidate polypeptides were synthesized with the TNT Coupled Reticulocyte Lysate System (Promega) for 1 hour at 30°C. Expression of each gene product was confirmed by Western blot analysis with an antibody specific for the COOH-terminal V5 tag. 12.5 μl of each in vitro transcription/translation reaction was incubated for 30 min at 30°C in a reaction buffer containing 20 mM tris-Cl (pH 7.5), 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM 2-oxoglutarate, 2 mM ascorbate, and 250 μM $FeSO_4$ in the presence of 30 μl of ImmunoPure Immobilized Streptavidin beads that had previously been incubated with 1 μg of peptide for 30 min at room temperature and washed three times to remove excess peptide. After incubation, the beads were washed three times with 1 ml of cold NTEN buffer [20 mM tris-Cl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40] and incubated for 10 min at 4°C with about 35 kcpm of [^{35}S]-labeled human VHL in 500 μl of EBC buffer [50 mM tris-Cl (pH 8.0), 120 mM NaCl, and 0.5% NP-40]. The beads were washed three times with cold NTEN buffer, and bound [^{35}S]-VHL was measured by scintillation counting. [^{35}S]-labeled human VHL was synthesized from the human VHL cDNA cloned into the pcDNA3.1/V5-HIS vector (Invitrogen) with the TNT Coupled Reticulocyte Lysate System (Promega) and [^{35}S]-L-Met (Amersham Pharmacia Biotech) and desalted with a PD-10 column (Amersham Pharmacia Biotech).
20. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
21. HPH-2 was cloned into the pMBP-parallel1 vector (*36*) and expressed in the BL21-CodonPlus-RIL *E. coli* strain (Stratagene). Recombinant protein was purified by virtue of the NH_2-terminal MBP protein with amylose resin (New England Biolabs) and eluted in the presence of 10 mM maltose.
22. One microgram of peptide was incubated with 1 μg of recombinant HPH-2 in reaction buffer for 1 hour at 30°C. Peptide mass was determined by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry.
23. Transfection experiments were performed as in (*24*) with DNA amounts indicated in the text. Western blot analysis with an antibody specific for the COOH-terminal V5 tag was used to confirm expression of each polypeptide after transfection of 1 μg of each expression vector.
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27. KC167 cells were maintained at 24°C in CCM 3 media (HyClone). Cells were treated as follows: initially, 4×10^5 KC167 cells were incubated for 7 days in the presence of 25 μg of double-stranded RNAs of about 700 base pairs in length generated with the T7 MEGAscript Kit (Ambion) with double-stranded RNA refreshed daily. Cells were incubated under normoxic (20% O_2) or hypoxic (1% O_2, 99% N_2) conditions for an additional 15 hours, and total RNA was prepared with RNA STAT-60 (Tel-Test). RNAs were resolved by electrophoresis in a 1.2% agarose gel in the presence of 1.8% formaldehyde, transferred to nitrocellulose filters, and hybridized with the indicated ^{32}P-labeled DNA probes.
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Reconstitution of Physiological Microtubule Dynamics Using Purified Components

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Microtubules are dynamically unstable polymers that interconvert stochastically between polymerization and depolymerization. Compared with microtubules assembled from purified tubulin, microtubules in a physiological environment polymerize faster and transit more frequently between polymerization and depolymerization. These dynamic properties are essential for the functions of the microtubule cytoskeleton during diverse cellular processes. Here, we have reconstituted the essential features of physiological microtubule dynamics by mixing three purified components: tubulin; a microtubule-stabilizing protein, XMAP215; and a microtubule-destabilizing kinesin, XKCM1. This represents an essential first step in the reconstitution of complex microtubule dynamics–dependent processes, such as chromosome segregation, from purified components.

Microtubules polymerize and depolymerize by the addition and loss of αβ-tubulin dimer subunits from their ends (*1*). Polymerizing and depolymerizing microtubules coexist and infrequently interconvert between these two states, a behavior known as dynamic instability (*2*). The transition of a polymerizing microtubule to a depolymerizing state is referred to as a catastrophe, and the converse transition is referred to as a rescue (*3*). Microtubules exhibit dynamic instability when assembled from purified tubulin (*3*, *4*) and in a physiological cytoplasmic environment (*5–10*), but there are notable differences between the two. In a physiological environment, microtubules polymerize about fourfold faster than a similar concentration of purified tubulin. At the polymerization rates observed in physiological conditions, purified tubulin has a near-zero rate of catastrophe. In contrast,