

## Leu-574 of HIF-1 $\alpha$ Is Essential for the von Hippel-Lindau (VHL)-mediated Degradation Pathway\*

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Oxygen homeostasis is crucial for a myriad of developmental, physiological, and pathophysiological processes. Hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) plays a pivotal role in response to hypoxia by transcriptionally activating target genes involving oxygen uptake, transport, delivery, and consumption. HIF-1 $\alpha$  activity is regulated primarily through the ubiquitin-proteasome degradation pathway, which targets the oxygen-dependent degradation domain (ODD) of HIF-1 $\alpha$ . In particular, the von Hippel-Lindau (VHL) protein complex, an E3 ubiquitin ligase, binds to the ODD upon hydroxylation of HIF-1 $\alpha$  Pro-564. Here, we show that *in vivo* VHL interacts with the N-terminal as well as the C-terminal ODD independently, supporting the notion of functional redundancy within the ODD. Moreover, we demonstrate that Leu-574 of HIF-1 $\alpha$  is essential for VHL binding to the C-terminal ODD. Despite the presence of Pro-564, deletion or mutation of Leu-574 resulted in a loss of VHL binding and a gain of protein stability. Furthermore, the identification of Leu-574 redefines the N-terminal activation domain of HIF-1 $\alpha$  to be constitutively active. Taken together, this study provides new insight into the mechanisms underlying VHL-mediated HIF-1 $\alpha$  degradation and transcriptional activation, and a molecular basis for drug targeting.

Hypoxia-inducible factor 1 (HIF-1)<sup>1</sup> is a heterodimeric transcription factor that plays a critical role in regulating mammalian oxygen homeostasis (1–3). Adaptation to changes in oxygen tension is involved in a variety of developmental, physiological, and pathophysiological processes including embryonic development, angiogenesis, cerebral and myocardial ischemia, and tumorigenesis (4, 5). HIF-1 consists of HIF-1 $\alpha$  and HIF-1 $\beta$  subunits, both of which belong to the basic helix-loop-helix Per-AhR-Sim family (6, 7). Under hypoxia, HIF-1

becomes activated and up-regulates target genes such as erythropoietin, vascular endothelial growth factor, glucose transporter, and glycolytic enzymes (2).

HIF-1 activation is regulated primarily by the accumulation of HIF-1 $\alpha$  protein (8). Both *HIF-1 $\alpha$*  and *HIF-1 $\beta$*  genes are constitutively expressed in many cell lines examined (8–11), whereas HIF-1 $\alpha$  protein is constantly degraded under normoxia by the ubiquitin-proteasome pathway (12, 13). The degradation is controlled by a unique oxygen-dependent degradation domain (ODD) consisting of ~200 amino acids within HIF-1 $\alpha$  (12). Deletion of the entire ODD gave rise to a stable HIF-1 $\alpha$ , capable of heterodimerization, DNA binding, and transactivation in cell culture systems. Consistently, the ODD-deleted HIF-1 $\alpha$  (but not the full-length), when transgenically expressed in the mouse epidermis, activated HIF-1 target genes, thereby resulting in epidermal hypervascularity (14), providing compelling evidence that a stable HIF-1 $\alpha$ , irrespective of hypoxic signal, is sufficient for transcriptional activation in animal models.

In search of the mechanisms underlying HIF-1 $\alpha$  degradation, Maxwell *et al.* (15) first reported that the tumor suppressor protein, von Hippel-Lindau (VHL), targets HIF-1 $\alpha$  for oxygen-dependent proteolysis in an iron-dependent way. Inactivation of the *VHL* gene is linked to the development of the VHL disease, a hereditary human cancer syndrome characterized by the predisposition to develop highly angiogenic tumors (16). VHL protein is in a multiprotein complex with elongin B, elongin C, and Cul2, which share sequence similarity with the Skp1 and Cdc53 components of the SCF ubiquitin ligase (17–20). Furthermore, VHL is associated with Rbx1 or ROC1 (21), a potent SCF ubiquitin ligase activator that facilitates degradation of substrate proteins by recruiting a ubiquitin-conjugating enzyme to the complex (22, 23). All these observations have led to the hypothesis that the VHL complex functions as an E3 ubiquitin ligase for HIF-1 $\alpha$  polyubiquitination (24) by specifically targeting the ODD of HIF-1 $\alpha$  (25–27).

VHL binding requires specific recognition of hydroxylated Pro-564 of HIF-1 $\alpha$  (28–30). The proline hydroxylation, catalyzed by a conserved family of prolyl-4-hydroxylases, relies on molecular oxygen and iron (31, 32), indicating these enzymes as oxygen sensors. Moreover, recent studies of HIF1 $\alpha$ -VHL complexes provide a structural basis for VHL recognition of hydroxyproline in HIF-1 $\alpha$  (33, 34), suggesting a central role for proline hydroxylation in oxygen signaling. Interestingly, Pro-402 of HIF-1 $\alpha$  is also subjected to hydroxylation and, in turn, targeted by the VHL E3 ubiquitin ligase for HIF-1 $\alpha$  ubiquitination (35).

Despite the identification of a crucial role for Pro-564 in controlling HIF-1 $\alpha$  degradation, our earlier study showed that partial deletion of the ODD containing Pro-564 failed to completely stabilize HIF-1 $\alpha$  (12), indicating functional redundancy within the ODD. In this study, we show that VHL interacts

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<sup>1</sup> The abbreviations used are: HIF-1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ ; ODD, oxygen-dependent degradation domain; VHL, von Hippel-Lindau; HA, hemagglutinin; NAD, N-terminal activation domain; CAD, C-terminal activation domain.

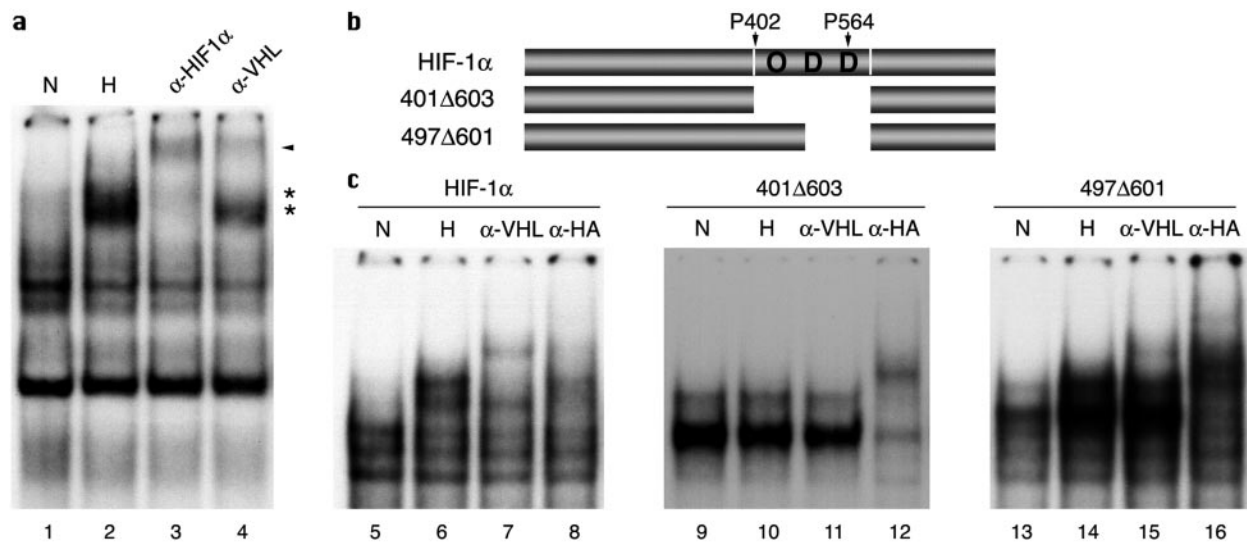


FIG. 1. **VHL binds to both N-terminal and C-terminal ODD.** As shown in *a*, Hep3B cell extracts prepared from normoxic (N) or hypoxic (H) treatment were subjected to an electrophoretic mobility shift assay. Hypoxic cell extracts were also incubated with anti-HIF-1 $\alpha$  ( $\alpha$ -HIF1 $\alpha$ ) or anti-VHL ( $\alpha$ -VHL) antibodies. The supershifted complexes were indicated with an arrowhead, and HIF-1 DNA binding activity was marked with asterisks. *b*, a schematic representation of constructs expressing wild-type HIF-1 $\alpha$  and deletion mutants 401 $\Delta$ 603 and 497 $\Delta$ 601. Pro-402, Pro-564, and ODD are denoted. As shown in *c*, extracts from 293 cells transfected with the expression aforementioned plasmids were subjected to supershift assays with the addition of anti-VHL and anti-hemagglutinin ( $\alpha$ -HA) antibodies.

independently with the N-terminal as well as the C-terminal ODD, that Pro-564 is necessary but not sufficient for VHL binding, and that Leu-574 is required for VHL binding and VHL-mediated degradation.

#### EXPERIMENTAL PROCEDURES

**Plasmids**—HIF-1 $\alpha$  expression plasmids p(HA)HIF1 $\alpha$ , p(HA)HIF1 $\alpha$ (401 $\Delta$ 603), and p(HA)HIF1 $\alpha$ (497 $\Delta$ 601) were described previously (12). Gal4-HIF1 $\alpha$  fusions were constructed by the standard PCR cloning method using specifically designed PCR primers that contain appropriate codons. Site-directed mutagenesis was done using the QuikChange site-directed mutagenesis kit (Stratagene). p(HA)VHL was a gift of William G. Kaelin, Jr. (Dana-Farber Cancer Institute), and pEYFP-Nuc was purchased from Clontech.

**Transfection, Luciferase Assay, and Immunoprecipitation with Western Blot Analysis**—Hep3B and 293 cells were cultured in 12- and 6-well plates, respectively, and transfected with Eugene 6 (Roche Molecular Biochemicals) as described previously (36). A typical transfection for luciferase assays contains 0.25  $\mu$ g of pGal4-luc, 0.1  $\mu$ g of effector plasmid, and 0.1  $\mu$ g of pEYFP-Nuc for normalizing transfection efficiency. Fluorescence and luciferase activities were sequentially scanned through a microplate fluorescence reader (FLx800, Bio-Tek) in which the Bright-Glo luciferase substrate (Promega) was used for luciferase reaction. For the determination of protein levels, 1  $\mu$ g of Gal4-HIF1 $\alpha$  fusions were transfected into 293 cells. At 24–40 h after transfection, cells were lysed in radioimmune precipitation buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). Gal4 fusions were precipitated with polyclonal anti-Gal4 antibody (Santa Cruz Biotechnology) and were further probed with monoclonal anti-Gal4 antibody (Santa Cruz Biotechnology) in Western blot. All of the experiments were repeated three to four times.

**Supershift Assay**—Electrophoretic mobility shift assays were performed essentially as described previously (12). A specific antibody (0.5–1  $\mu$ l) was added to the DNA binding reaction. Anti-VHL antibody was a gift of William G. Kaelin, Jr., and anti-Gal4 and anti-hemagglutinin antibodies were acquired as described previously (12).

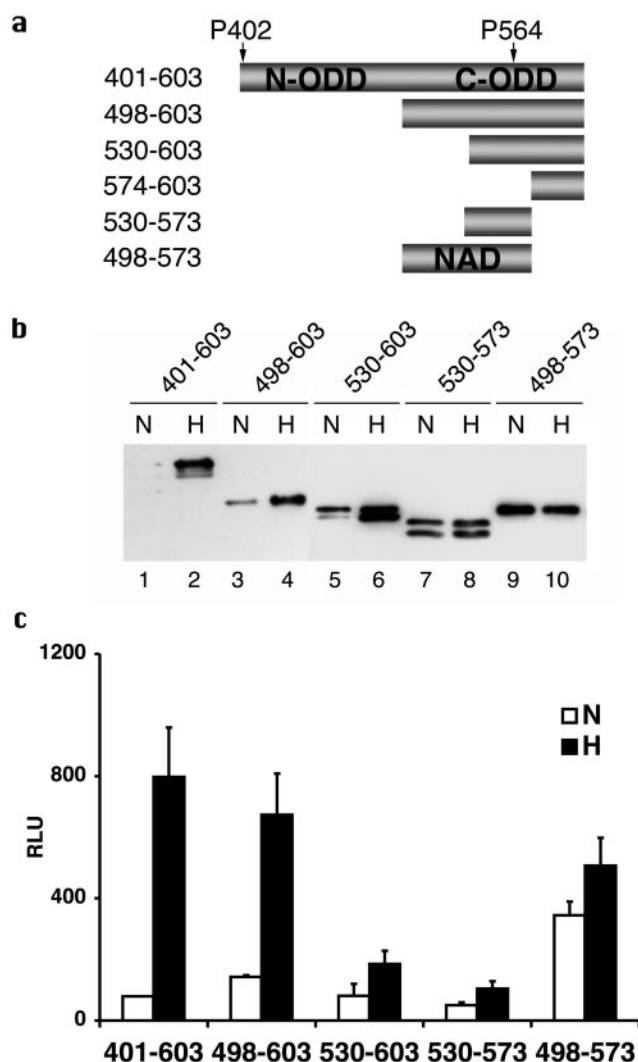
**In Vitro Co-immunoprecipitation**—Both VHL and Gal4 fusions were *in vitro*-translated as described previously (36). [ $^{35}$ S]methionine-labeled Gal4 fusions were mixed with unlabeled VHL in NETN buffer (36) containing anti-VHL antibody. The reaction mixture was washed five times with NETN buffer before SDS-PAGE.

#### RESULTS

**VHL Binds to Both N-terminal and C-terminal ODD Independently**—Direct involvement of the VHL protein in HIF-1 $\alpha$  degradation was first demonstrated by the addition of anti-VHL antibody (15), which caused a supershift of the slower (but

not the faster) migrating HIF-1 DNA binding activity (Fig. 1*a*, lane 4, asterisks), whereas addition of anti-HIF1 $\alpha$  antibody resulted in a complete shift of both HIF-1 binding activities (lane 3). Previously, we proposed functional redundancy within the ODD because each part of the ODD independently confers degradation (12). In particular, removal of the C-terminal ODD that harbors Pro-564 led only to a more stable but not completely stable HIF-1 $\alpha$ , suggesting additional mechanisms controlling HIF-1 $\alpha$  degradation. To test whether VHL interacts with the N-terminal ODD, we transfected 293 cells with a plasmid expressing hemagglutinin (HA)-tagged HIF-1 $\alpha$  that was wild-type, ODD entirely deleted from amino acids 401–603 (401 $\Delta$ 603), or ODD C-terminally deleted from amino acids 497–601 (497 $\Delta$ 601), as illustrated in Fig. 1*b*. VHL binding activity of the transfected HIF-1 $\alpha$  was analyzed in supershift assays. As expected, the addition of anti-VHL antibody shifted the slower migrating wild-type HIF-1 DNA binding activity (Fig. 1*c*, lane 7). To differentiate the transfected HIF-1 $\alpha$  from the endogenous, we also included in the assay an anti-HA antibody, which only supershifted the DNA binding complex resulting from transfected HIF-1 $\alpha$  but retained the weak endogenous binding activity (compare lane 6 and lane 8). When ODD-deleted HIF-1 $\alpha$  was used, a constitutive HIF-1 binding pattern was observed (lanes 9 and 10), as shown in our previous reports (12, 37). However, no supershift was observed with anti-VHL antibody (lane 11), whereas anti-HA antibody slowed the binding mobility (lane 12). This result is in agreement with the observation that VHL targets the ODD for HIF-1 $\alpha$  degradation. In contrast, deletion of the C-terminal ODD retained a weaker yet noticeable supershift by anti-VHL (lane 15), indicative of VHL binding to the N-terminal ODD. In keeping with this notion, HIF-1 $\alpha$ (497 $\Delta$ 601) gave rise to an inducible rather than a constitutive binding pattern (compare lane 13 and lane 14). This finding is also in good agreement with a recent report that hydroxylation of HIF-1 $\alpha$  Pro-402 independently confers VHL binding (35). Thus, our results provide *in vivo* evidence that VHL interacts with the N-terminal as well as the C-terminal ODD for HIF-1 $\alpha$  degradation.

**The ODD Contains a Core Region That Is Stable and Transcriptionally Active**—To understand the structure and function of the ODD, we fused a Gal4 DNA-binding domain to various



**FIG. 2. Characterization of the ODD.** *a*, a schematic representation of Gal4-ODD fusions that are serially deleted from the N-terminal and the C-terminal. Both Pro-402 and Pro-564 were indicated, and the N-terminal ODD (N-ODD), the C-terminal ODD (C-ODD), and the NAD are denoted. *b*, immunoprecipitation-Western blot analysis of the Gal4-ODD deletion mutants expressed in 293 cells under normoxic (N) and hypoxic (H) conditions. As shown in *c*, Gal4-ODD deletion mutants were transfected into Hep3B cells along with pGal4-luc reporter plasmid and examined for transcriptional activity under both normoxia (N) and hypoxia (H). Transcriptional activity is registered as relative luciferase units (RLU), which were plotted as means plus standard errors from four experiments.

ODD mutants derived from serial N-terminal and C-terminal deletions, as schematized in Fig. 2*a*. Transfection of these mutants into 293 cells revealed that deletion of the N-terminal ODD resulted in increasing expression of Gal4-HIF1 $\alpha$ -(498–603) and Gal4-HIF1 $\alpha$ -(530–603) under normoxia (Fig. 2*b*, lanes 3 and 5), consistent with the notion of VHL binding to the N terminus. However, deletion of amino acids 574–603 from the C terminus significantly elevated normoxic expression levels of Gal4-HIF1 $\alpha$ -(530–573) and Gal4-HIF1 $\alpha$ -(498–573) (lanes 7 and 9), thereby abrogating hypoxic induction. In addition, Gal4-HIF1 $\alpha$ -(574–603) exhibited strong constitutive expression (data not shown). Interestingly, both Gal4-HIF1 $\alpha$ -(530–573) and Gal4-HIF1 $\alpha$ -(498–573) contain Pro-564, which is critical for VHL binding through hydroxylation (28–30). Thus, our results suggest that in addition to Pro-564, the downstream sequence also contributes to oxygen-dependent proteolysis.

Because part of the ODD overlaps an N-terminal activation

domain (NAD) of HIF-1 $\alpha$  (38–40), we examined the functional role of these Gal4-HIF1 $\alpha$  fusions in a Gal4 luciferase reporter system to establish a link between HIF-1 $\alpha$  degradation and its transcriptional activity. Consistent with the protein levels mentioned previously, removal of the N-terminal ODD (Gal4-HIF1 $\alpha$ -(498–603)) slightly increased normoxic but modestly decreased hypoxic transcriptional activity (Fig. 2*c*). Further deletion from the N terminus (Gal4-HIF1 $\alpha$ -(530–603)) resulted in significant loss of luciferase reporter activity, although the protein was more stable, implying the requirement of amino acids 498–530 for transcriptional activity. Interestingly, Gal4-HIF1 $\alpha$ -(498–573), which was missing amino acids 574–603 but stable, exhibited a striking 2.4-fold increase in transcription under normoxia in comparison with Gal4-HIF1 $\alpha$ -(498–603), indicative of inherent transcriptional activity. However, both Gal4-HIF1 $\alpha$ -(498–530) (data not shown) and Gal4-HIF1 $\alpha$ -(530–573) alone showed much weaker activity. Therefore, we conclude that the core NAD lies within amino acids 498–573 of HIF-1 $\alpha$  and is constitutively active.

*Pro-564 Is Necessary but Insufficient for VHL Binding*—To investigate the molecular basis for the constitutive activity of HIF-1 $\alpha$ , we performed supershift assays to analyze *in vivo* VHL binding activity. As expected, transfection with Gal4-HIF1 $\alpha$ -(401–603) exhibited hypoxic induction of Gal4 DNA binding activity (Fig. 3, lanes 1 and 2) and VHL binding activity (lane 5). The Gal4 binding activity was confirmed by the addition of excessive unlabeled Gal4 oligonucleotides and anti-Gal4 antibody, respectively, resulting in abrogation or supershift of the binding activity (lanes 3 and 4). Likewise, a supershift by anti-VHL antibody was observed with Gal4-HIF1 $\alpha$ -(498–603) and Gal4-HIF1 $\alpha$ -(530–603) (lanes 8 and 12). However, we could not detect such a mobility shift with Gal4-HIF1 $\alpha$ -(498–573) and Gal4-HIF1 $\alpha$ -(530–573) (lanes 10 and 14) when the C terminus of ODD (amino acids 574–603) was deleted. Thus, these results suggest that stabilization of Gal4-HIF1 $\alpha$ -(498–573) and Gal4-HIF1 $\alpha$ -(530–573) was due to the loss of VHL binding, providing further evidence that, in addition to Pro-564, the downstream sequence is required for *in vivo* VHL binding.

To further test this hypothesis, we performed co-immunoprecipitation of *in vitro*-translated Gal4-ODD fusions with VHL (26). Consistently, Gal4-HIF1 $\alpha$ -(401–603), Gal4-HIF1 $\alpha$ -(498–603), and Gal4-HIF1 $\alpha$ -(530–603) were co-precipitated by anti-VHL antibody (Fig. 4*a*, lower panel, lanes 1–3). By contrast, Gal4 fusions lacking amino acids 574–603 (Gal4-HIF1 $\alpha$ -(498–573) and Gal4-HIF1 $\alpha$ -(530–573)) showed no interaction with VHL (lanes 5 and 6). In addition, Gal4-HIF1 $\alpha$ -(574–603) alone did not bind to VHL (lane 4), although all the constructs were translated *in vitro* at similar levels (upper panel). Taken together, these results indicate the requirement of HIF-1 $\alpha$  amino acids 530–603 for VHL binding.

The interaction between VHL and Gal4-HIF1 $\alpha$ -(530–603) was further validated in Fig. 4*b*, in which no interaction was observed when *in vitro*-translated VHL or anti-VHL antibody was omitted (Fig. 4*b*, lanes 8 and 9). Furthermore, because the VHL protein used contains an HA tag, the addition of anti-HA antibody also co-precipitated Gal4-HIF1 $\alpha$ -(530–603) (lane 10). To ensure the critical role of HIF-1 $\alpha$  Pro-564 for VHL binding in our systems, we made an alanine substitution and, as expected, observed no VHL interaction (Fig. 4*c*, lane 13). In contrast, alanine substitutions of other proline residues within the ODD (Pro-513, Pro-516, and Pro-567) produced no effect, suggesting the specific requirement of Pro-564 for VHL binding, consistent with previous reports (28–30). Therefore, we conclude that VHL binding requires not only Pro-564 of HIF-1 $\alpha$  but also the downstream sequence.



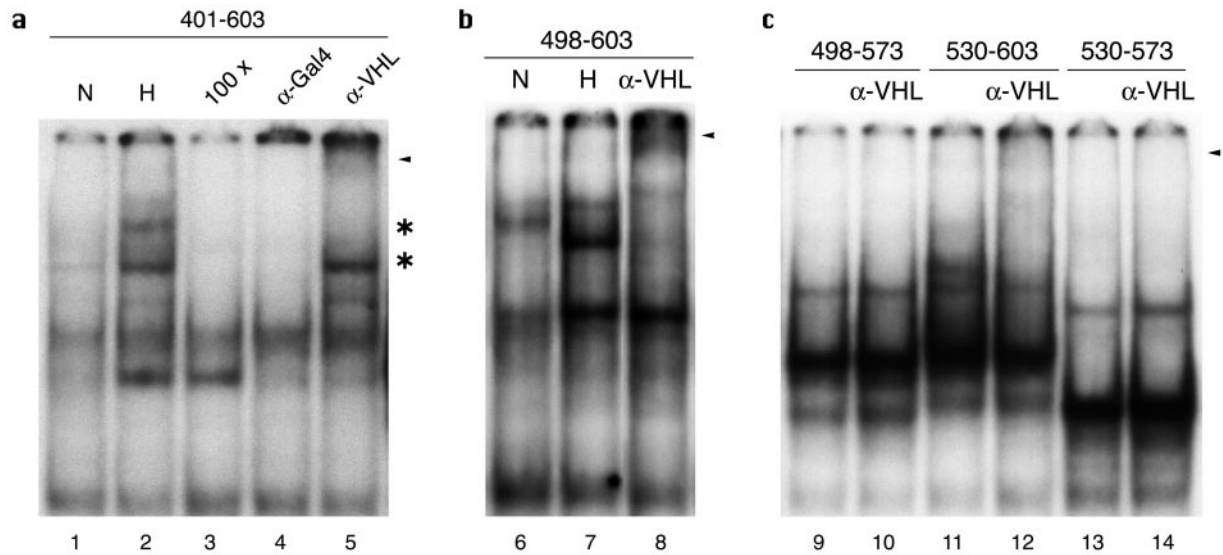


FIG. 3. **VHL binding *in vivo* requires amino acids 574–603 of HIF-1 $\alpha$ .** As shown in *a*, Gal4-HIF1 $\alpha$ (401 $\Delta$ 603) expressed in 293 cells was analyzed in an electrophoretic mobility shift assay. Gal4 DNA binding activity (indicated with asterisks) was confirmed by the addition of excessive unlabeled oligonucleotides (100 $\times$ ) or anti-Gal4 antibody ( $\alpha$ -Gal4). The supershifted DNA complexes by anti-Gal4 and anti-VHL antibodies were marked with an arrowhead. *N*, normoxia; *H*, hypoxia. As shown in *b* and *c*, Gal4-ODD deletion mutants as described in Fig. 2*a* were transfected into 293 cells. *In vivo* VHL binding activity was examined by supershift assays.

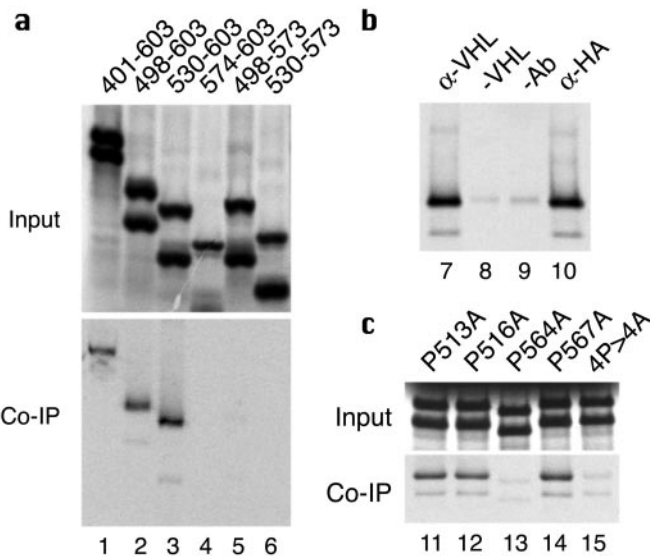


FIG. 4. **Pro-564 of HIF-1 $\alpha$  is necessary but not sufficient for VHL binding *in vitro*.** As shown in *a*, the Gal4-ODD deletion mutants and VHL were *in vitro*-translated in the presence and absence of [ $^{35}$ S]methionine, respectively. The translated products were mixed together for co-immunoprecipitation (*Co-IP*) with anti-VHL antibody. Input (10%) was shown in the upper panel. As shown in *b*, *in vitro*-translated Gal4-HIF1 $\alpha$ (530–603) was tested for the specificity of VHL binding by respective addition of anti-VHL ( $\alpha$ -VHL) and anti-HA ( $\alpha$ -HA) antibodies or by respective omission of the VHL protein ( $-VHL$ ) and antibody ( $-Ab$ ). As shown in *c*, Pro-513, Pro-516, Pro-564, and Pro-567 in Gal4-HIF1 $\alpha$ (401–603) were mutated to alanine individually or simultaneously (4 Pro  $\rightarrow$  4 Ala). The mutants were examined subsequently for VHL binding. Input (10%) was shown in the upper panel.

**Leu-574 Is Required for VHL-mediated Degradation**—To further examine the molecular determinants of VHL binding, we asked whether Leu-574 could be involved because this residue is highly conserved and included in all the studies of HIF-1 $\alpha$  oligopeptide binding to VHL (28, 29). To that end, Gal4-HIF1 $\alpha$ (498–574) was constructed and analyzed for *in vitro* VHL binding. Remarkably, addition of a single leucine residue to Gal4-HIF1 $\alpha$ (498–573) gained VHL binding (Fig. 5*a*, lanes 1 and 2), whereas mutation of Leu-574 to serine abolished the binding (lane 3), indicating an essential role for Leu-574 in VHL binding.

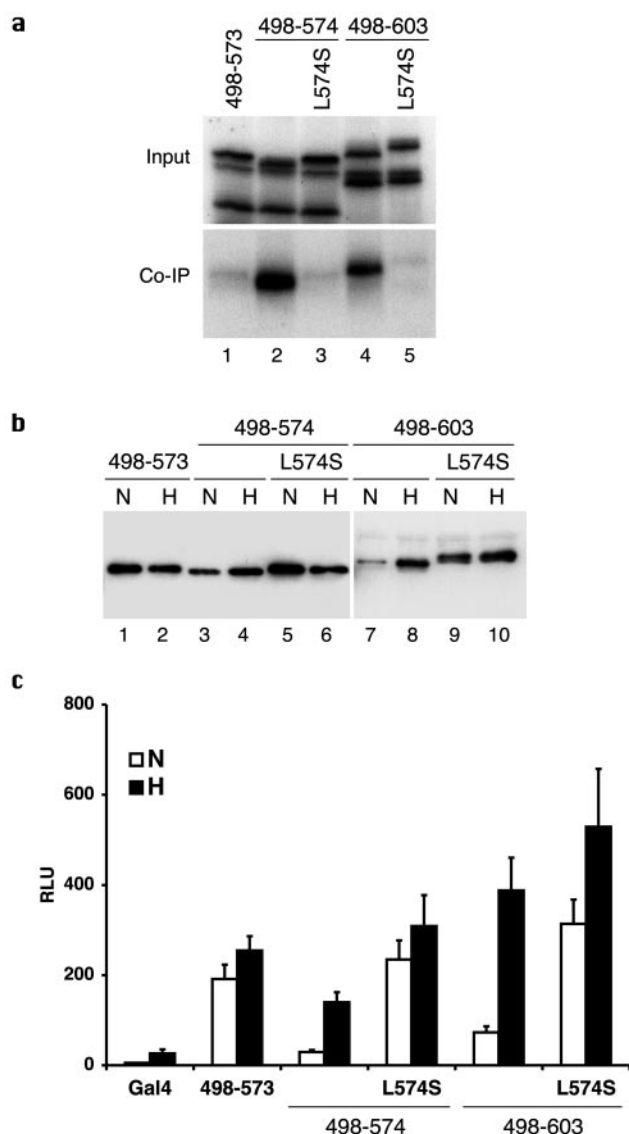
To corroborate the importance of Leu-574, we also mutated Leu-574 in Gal4-HIF1 $\alpha$ (498–603) and asked whether substitution with serine would affect VHL binding. Consistently, the mutation resulted in the loss of VHL binding (compare lane 4 and lane 5). Thus, these results provide compelling evidence that Leu-574 is critically involved in VHL binding.

To address the role of Leu-574 in protein stability, we examined expression levels of these constructs by Western blot analysis. In comparison with Gal4-HIF1 $\alpha$ (498–573), Gal4-HIF1 $\alpha$ (498–574) exhibited an  $\sim$ 3-fold decrease in protein levels under normoxia (Fig. 5*b*, compare lane 1 and lane 3), supporting the role of Leu-574 in VHL-mediated degradation. Consistently, mutation of Leu-574 prevented the degradation process, resulting in a stable Gal4-HIF1 $\alpha$ (498–574) (compare lane 3 and lane 5). Furthermore, mutation of Leu-574 gave rise to a 7-fold increase in the expression level of Gal4-HIF1 $\alpha$ (498–603) (compare lane 7 and lane 9). Taken together, these results demonstrate that Leu-574 plays a critical role in the VHL degradation pathway, although additional residues between amino acids 575–603 may also contribute oxygen-dependent degradation of the C-terminal ODD.

As shown above, the core NAD lies within amino acids 498–573 of HIF-1 $\alpha$ . To examine the effect of Leu-574 on transcriptional activity, we employed the aforementioned Gal4 luciferase reporter system. As expected, the addition of Leu-574 significantly lowered the luciferase reporter activity (Fig. 5*c*) by 6.5-fold under normoxia. In contrast, Gal4-HIF1 $\alpha$ (498–574)L574S gave rise to a similar reporter activity as that of Gal4-HIF1 $\alpha$ (498–573), supporting the involvement of Leu-574 in ODD instability. Furthermore, under oxygenated conditions, Gal4-HIF1 $\alpha$ (498–603)L574S exhibited a 4.3-fold increase in luciferase activity in comparison with the wild-type. Therefore, these results provide further evidence that Leu-574 plays a critical role in VHL-mediated degradation of HIF-1 $\alpha$ , thereby regulating HIF-1 $\alpha$  transcriptional activity.

#### DISCUSSION

Significant progress has been made in the last few years in the understanding of the mechanisms underlying HIF-1 $\alpha$  degradation, which has led to the identification and characterization of the oxygen sensing and signaling pathway (28, 29, 31, 32). Much of the focus has been on the C-terminal ODD of



**FIG. 5. Inclusion of Leu-574 renders HIF-1 $\alpha$  unstable under normoxia.** As shown in *a*, *in vitro*-translated Gal4-HIF1 $\alpha$ -(498–573), Gal4-HIF1 $\alpha$ -(498–574), Gal4-HIF1 $\alpha$ -(498–573)L574S, Gal4-HIF1 $\alpha$ -(498–603), and Gal4-HIF1 $\alpha$ -(498–603)L574S were mixed with VHL and co-immunoprecipitated (Co-IP) by anti-VHL antibody for VHL binding activity. Input (10%) was shown in the upper panel. N, normoxia; H, hypoxia. As shown in *b* and *c*, these Gal4 fusion constructs were transfected into 293 cells for analyzing their protein levels by Western blot or transfected into Hep3B cells for examining their transcriptional activity in a Gal4 reporter system. Transcriptional activity is registered as relative luciferase units (RLU), which were plotted as means plus standard errors from four experiments.

HIF-1 $\alpha$  that binds to VHL, although Pro-402 has also been shown to confer independent VHL binding through hydroxylation (37). In this study, we provided *in vivo* evidence that VHL interacts with the N-terminal ODD as well, which is consistent with and supported by the notion of functional redundancy within the ODD (12). Although VHL binding to the N-terminal ODD appears to be weaker, it is capable of rendering HIF-1 $\alpha$  unstable in normoxia, as demonstrated in the ODD partial deletion studies (12) (Fig. 1c). Moreover, simultaneous mutation of both Pro-402 and Pro-564 in full-length HIF-1 $\alpha$  resulted in a far more stable HIF-1 $\alpha$  than Pro-564 mutation alone (35) (data not shown). The notion of multiple mechanisms controlling HIF-1 $\alpha$  stability is further supported by the involvement of p53-MDM2 and Hsp90 in a VHL-independent fashion, although the molecular determinants have not yet been identi-

fied (41–43). In addition, we have identified recently another destruction motif within the ODD, which is also distinct from the VHL-mediated degradation pathway.<sup>2</sup> Thus, degradation of HIF-1 $\alpha$  seems to be mediated by multiple pathways, among which the VHL pathway is arguably responsible for targeting HIF-1 $\alpha$  for oxygen-dependent degradation.

Pro-564 of HIF-1 $\alpha$  plays a pivotal role in VHL binding to the C-terminal ODD (28–30, 32) (Fig. 4c). In this study, we demonstrate that Leu-574 is indispensable to VHL binding *in vitro* and *in vivo* and to VHL-mediated degradation. This finding is based on the evidence that (i) unlike Gal4-HIF1 $\alpha$ -(498–603), Gal4-HIF1 $\alpha$ -(498–573) was unable to bind to VHL, and therefore, constitutively expressed; (ii) the addition of Leu-574 to Gal4-HIF1 $\alpha$ -(498–573) resulted in a gain of VHL binding and a loss of protein stability; (iii) the mutation of Leu-574 in Gal4-HIF1 $\alpha$ -(498–603) led to a decrease in VHL binding and an increase in protein stability; and (iv) Leu-574 is highly conserved in HIF-1 $\alpha$ , HIF-2 $\alpha$ , and HIF-3 $\alpha$ . Furthermore, our finding is consistent and corroborated by the fact that Leu-574 was required in all of the VHL-HIF binding studies (28–30). On the other hand, all other Pro-564 neighboring residues were found to be mostly nonessential with the exception of Leu-562 (28), Ala-563 (30, 32), and Tyr-565 (29, 32), although the exact contribution for each of these residues remains to be determined. Moreover, Ivan *et al.* (28) also showed that Gal4-HIF1 $\alpha$ -(555–575) but not Gal4-HIF1 $\alpha$ -(555–573) interacted with VHL. Therefore, we conclude that Leu-574 plays a specific and crucial role in the VHL-dependent degradation pathway.

It is noteworthy, however, that the biological function of Leu-574 may go beyond VHL binding *per se*. It is equally possible that this residue participates in the interaction of the C-terminal ODD with HIF-1 $\alpha$  prolyl hydroxylases. Consequently, lack or mutation of Leu-574 would prevent Pro-564 hydroxylation, thereby resulting in protein stabilization and loss of VHL binding. In fact, this hypothesis is consistent with recent structural studies of VHL-HIF1 $\alpha$  interaction, which concluded that hydroxyproline Pro-564 provides strict specificity for VHL recognition, whereas the neighboring amino acid residues only contribute to the complex stability through a  $\beta$  sheet-like contact (33, 34). In particular, these studies showed that amino acids 571–574 (Asp-Phe-Gln-Leu) of HIF-1 $\alpha$  are not as important for VHL binding because amino acids 560–567 (Glu-Met-Leu-Ala-Pro-Tyr-Ile-Pro), when synthetically hydroxylated, exhibited similar VHL affinity as the peptide containing both regions. Therefore, it would be interesting to use synthetic peptides to test whether Leu-574 is required for hydroxylation of Pro-564, thus providing insight into the event(s) upstream of VHL binding.

The identification of Leu-574 has enabled us to redefine the core NAD. HIF-1 $\alpha$  contains an NAD in addition to its C-terminal activation domain (CAD) (38–40). The CAD specifically interacts with p300 (40, 44–47) through a leucine-rich interface (36, 48, 49) to confer HIF-1 transcriptional activity. HIF1 $\alpha$ -p300 interaction appears to be regulated by a hydroxylated asparagine (Asn-803), which prevents p300 binding under normoxia (50). Furthermore, the asparaginyl hydroxylase of HIF-1 $\alpha$  has been identified recently (51, 52) as FIH-1, a factor that mediates repression of HIF-1 transcriptional activity (53). On the other hand, NAD lacks consensus with respect to its location, primary sequence, and protein stability (38–40). Moreover, its role in HIF-1 $\alpha$  function is elusive. Both small NADs (amino acids 531–582) (38, 39) and large NADs (amino acids 481–603) (40) were defined, and their transcriptional activity either dependent (39, 40) or independent (38) of protein

<sup>2</sup> L. E. Huang, E. A. Pete, and J. Gu, manuscript in preparation.

stability was proposed. All of these studies, however, showed that NAD transcriptional activity was hypoxia-dependent. In this report, we demonstrated that the core NAD lies between amino acids 498–573 and confers constitutive transcriptional activity irrespective of changes in oxygen tension. Our finding suggests that the NAD possesses an intrinsic (or hypoxia-independent) transcriptional activity. In keeping with this notion, addition of Leu-574 to Gal4-HIF1 $\alpha$ -(498–573) regained VHL binding, normoxic degradation, and therefore normoxic repression (or hypoxic induction) of transcription. Conversely, Leu-574 mutation in Gal4-HIF1 $\alpha$ -(498–603) produced the opposite effects. Thus, NAD transcriptional activity is constitutive and dependent upon protein stability.

In summary, degradation of HIF-1 $\alpha$  is a complex process, at least involving VHL binding to the N-terminal and C-terminal ODD independently. Leu-574 of HIF-1 $\alpha$  is crucial for *in vivo* VHL binding and VHL-mediated degradation, possibly through direct contact with VHL or with HIF prolyl hydroxylases. Regardless of the precise mechanism, our finding may provide a molecular basis for the development of therapeutic agents to alter HIF-1 $\alpha$  function.

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## **Leu-574 of HIF-1 $\alpha$ Is Essential for the von Hippel-Lindau (VHL)-mediated Degradation Pathway**

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