Inactivation of VHL by Tumorigenic Mutations That Disrupt Dynamic Coupling of the pVHL-Hypoxia-inducible Transcription Factor-1α Complex*

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The von Hippel-Lindau (VHL) gene product, pVHL, targets the α subunit of the hypoxia-inducible transcription factor (HIF-α) for ubiquitin-dependent degradation. This tumor suppressor function is mediated by the α- and β-domains responsible for assembling the pVHL E3 ubiquitin ligase complex and for recognizing the prolyl-hydroxylated HIF-α, respectively. The molecular basis for a large number of tumor-derived mutations can be attributed to alterations that directly compromise the ability of pVHL to assemble the E3 or to contact the substrate. Here we describe a new mechanism of oncogenic inactivation by VHL missense mutations that lie in the L1 and L7 linker regions distal to the HIF-α-binding pocket. Employing molecular dynamics simulations, we show that the tumorigenic L1 loop mutation of Ser65 to Leu, deficient in promoting the degradation of HIF-α, disrupts the coordination of internal motions of the pVHL-HIF-1α complex. Furthermore, we demonstrate that in addition to S65L, five other tumor-derived VHL mutations located within the L1 loop are each defective in mediating proteolysis of HIF-2α. Moreover, dynamic organization of pVHL-HIF-1α recognition is focally centered on Gln145 within the L7 loop, and its tumorigenic mutant Q145H abolishes almost all of the correlated dynamic motions. Intriguingly, Q145H, whereas defective in targeting cellular HIF-α for degradation, had an attenuated hydroxylation dependence in binding to HIF-1α in vitro. Taken together, our results suggest that specific association between pVHL and the hydroxylated HIF-α requires both the L1 and L7 loops to coordinate dynamic coupling among distant pVHL regions, whose mutational disruption inactivates VHL and is hence responsible for tumorigenesis.

Germline mutations in the von Hippel-Lindau (VHL),† tumor suppressor gene lead to a rare familial cancer syndrome that exhibits a dominant pattern of inheritance (1–3). VHL disease is characterized by highly vascularized tumors found in the kidney, retina, cerebellum, spine, pancreas, and adrenal glands (4), including angiomas, hemangioblastomas, pheochromocytomas, and renal clear-cell carcinomas (RCC) (1). Furthermore, the majority of sporadic RCC are associated with a biallelic loss of VHL (3, 4).

The VHL gene product, pVHL, is the targeting subunit of a multiprotein E3 ubiquitin ligase complex, VEC, which is comprised of pVHL, the elonginB/C heterodimeric adaptor, the cullin family member CUL2, and RING finger protein ROC1/Rbx1 (reviewed in Ref. 5), and is activated by NEDD8 conjugation (6–8). The VEC is responsible for polyubiquitination of the α regulatory subunit of the heterodimeric hypoxia-inducible transcription factor, HIF-α, and its subsequent degradation through the proteasomal pathway in an oxygen-dependent manner (9–13). HIF-1α and HIF-2α are the two most extensively studied isoforms and both are found to be regulated in a similar manner (14). pVHL recognizes HIF-α only when the conserved proline residues (Pro564 and Pro402 in HIF-1α) within the oxygen-dependent degradation (ODD) domain of HIF-α are hydroxylated in the presence of oxygen and iron (12, 13, 15–17). Conversely, under hypoxia HIF-α subunits remain unhydroxylated and escape degradation mediated by the VEC. Stabilized HIF-α subunits bind to constitutively stable HIF-β/ARNT subunits forming an active HIF transcription complex that binds to the hypoxia-responsive elements in the promoters of hypoxia-inducible genes, thus activating the cellular responses to hypoxia (12, 18). HIF target genes are involved in a variety of physiological processes such as angiogenesis, iron homeostasis, anaerobic metabolism, and cell growth and survival, including vascular endothelial growth factor, erythropoietin (EPO-1), glycolytic enzymes, glucose transporter 1 (GLUT 1), insulin-like growth factor 2, and cyclin D1 (19, 20).

pVHL is comprised of two domains: the α-domain, which is required for binding ElonginC to assemble the VEC complex, and the β-domain, which recognizes and targets hydroxylated HIF-α subunits for ubiquitination. In the α-domain, the H1 helix plays a major role in establishing the interface with ElonginC and residues in the H2 and H3 helices contribute to the interaction as well (21). In the β-domain, the HIF-1α hydroxylated Pro564 (Hyp564)-binding pocket is formed through a coordinated network of hydrogen bonds between residues located in S2, S3, S4, and S5 β-strands, as well as in the S4–S5 linker. The specificity of the binding pocket for only Hyp564 is mediated through pVHL Ser111 and His115, which form one hydrogen bond each with the Hyp564 4-hydroxyl group (22, 23).

In addition to its direct role in targeting HIF-α for ubiquitin-dependent degradation, pVHL was found to co-fractionate with fibronectin and required for proper assembly of the extracellular fibronectin matrix (24). Moreover, pVHL was identified as a
microtubule-associated protein that can protect microtubules from depolymerization, and therefore was speculated to play a role in the regulation of microtubule dynamics (25).

VHL mutations associated with tumor development have been identified throughout the open reading frame and map to residues on both α- and β-domains. Previous studies have documented that while frequent tumorigenic mutations in the α-domain, such as C162F and R167Q, lead to disassembly of the VEC complex, β-domain mutations that map to the HIF-α binding pocket of pVHL, such as Y98H, disrupt interaction with HIF-α (11, 21–23, 26, 27). In this study, we employed both biochemical and computational studies to characterize oncogenic VHL mutations that occur in the L1 and L7 linker regions, both of which are distant to the HIF-α binding pocket. Our results provide evidence suggesting a significant role for the L1 and L7 loop residues in coordinating dynamic motions that are required for specific interaction between pVHL and prolyl-hydroxylated HIF-α. Dysregulation of such dynamic coordination by VHL mutations in these loops results in stabilization of HIF-α and may hence be responsible for human cancers.

**EXPERIMENTAL PROCEDURES**

**Cells**—Human embryonic kidney cells (293T) and renal clear cell carcinoma cells 786-O (ATCC) were maintained in a humidified tissue culture incubator at 37 °C with 5% CO2. The cells were cultured in Dulbecco’s modified Eagle’s medium, 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids, 40 mM HEPES, 1 mM MgCl2, 1 mM EDTA, 0.1% Nonidet P-40, 1 mg/ml leupeptin and 1 mg/ml aprotinin. Following centrifugation, soluble extracts (50 μg of protein) were separated by SDS-PAGE and visualized by autoradiography.

**In Vitro Ubiquitination of HIF-1α**—To understand the dynamic and thermodynamic coupling between HIF-1α and distant pVHL sites, we described the following procedures. The HIF-1α isoform was expressed in 1 liter of bacteria culture and extracts were made as described previously (15).

**In Vitro Hydroxylation-dependent HIF-1α Degradation**—In vitro hydroxylation of HIF-1α was performed according to the protocol of Bruck and McKnight (18). Briefly, the in vitro translated [35S]-labeled HIF-1α was incubated with MBP-PHD1 (0.25 μg) in a reaction mixture (50 μl) containing 20 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 1 mM DTT, 2 μg/ml leupeptin and aprotinin, and sonicated. Following centrifugation, soluble extracts (50 μg of protein) were separated by SDS-PAGE and visualized with antibodies as indicated.

**Degradation of HIF-2α**—Cells (786-O) were transfected with pcDNA3-Flag-VHL (5 μg) that encodes either the wild type or mutant pVHL using FuGENE 6 Transfectant reagent. Posttranslation was performed by treatment with MG132 (5 μM) for 4–6 h before harvesting. Cells were harvested 24 h after transfection in phosphate-buffered saline and pelleted. Cell pellets were resuspended in buffer E containing 25 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin and aprotinin, and sonicated. Following centrifugation, soluble extracts (50 μg of protein) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane, which was probed with antibodies as indicated.

**In Vitro Hydroxylation-dependent HIF-1α Binding**—HIF-1α was hydroxylated based on a protocol by Bruck and McKnight (18). Briefly, the in vitro translated [35S]-labeled HIF-1α from the rabbit reticulocytes (35S-IPT-HIF-1α, 10 μl) was incubated with MBP-PHD1 (0.25 μg) in a reaction mixture (50 μl) containing 20 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 1 mM DTT, 2 μg/ml leupeptin and aprotinin, and sonicated. Following centrifugation, soluble extracts (50 μg of protein) were separated by SDS-PAGE and visualized as described.

**Transduction Laboratories. Rabbit polyclonal anti-HIF-2α was a gift from N. Pavletich (Memorial Sloan Kettering Cancer Center).** Anti-HIF-1α was obtained from Santa Cruz Biotechnology. Anti-VHL, anti-ElonginB, anti-ElonginC, and anti-FLAG were obtained from Santa Cruz Biotechnology.

**Protein purification**—Protein extracts were dialyzed against buffer B and concentrated (Amicon) to a final concentration of 2 mg/ml. Aliquots were kept at −80 °C. The supernatant was ultracentrifuged in a Beckman Ti45 rotor at 37,000 rpm for 1 h at 4 °C, and the extracts were stored in aliquots at −80 °C.

To immunopurify the VEC complex, extracts (1 mg of protein) were mixed with M2 beads (20 μl) for 16 h at 4 °C. The beads were washed three times with buffer D (25 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA) and phosphate-buffered saline. Bound proteins were released by boiling the beads in SDS loading buffer and separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane and immunoblotted with antibodies as indicated.

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**In Vitro Ubiquitination of HIF-1α**—The in vitro ubiquitination of HIF-1α was carried out using conditions similar to those for the IκBα reaction (29, 28). [35S]-labeled HIF-1α was bound to the VEC E3 on M2 beads, and ubiquitination reactions were performed in buffer C containing 40 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 4 mM ATP, 0.6 mM DTT, ubiquitin (Ub, 500 pmol), E1 (2 pmol), and UbEc5 (30 pmol or otherwise specified), or Cdc34 (115 pmol or otherwise specified) at 30 °C for 30 min or for other times as indicated. The reaction was terminated by the addition of Laemmli loading buffer and boiled for 3 min prior to 6% SDS-PAGE analysis followed by autoradiography.

**In Vitro Hydroxylation-dependent HIF-1α Degradation**—In vitro hydroxylation of HIF-1α was performed according to the protocol of Bruck and McKnight (18). Briefly, the in vitro translated [35S]-labeled HIF-1α from the rabbit reticulocytes ([35S]-IPT-HIF-1α, 10 μl) was incubated with MBP-PHD1 (0.25 μg) in a reaction mixture (50 μl) containing 20 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 1 mM DTT, 2 μg/ml leupeptin and aprotinin, and sonicated. Following centrifugation, soluble extracts (50 μg of protein) were separated by SDS-PAGE and visualized as described.

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Such an analysis is expected to reveal the effects of mutation on the fs-ps atomic motion of the bound pVHL-HIF-1α complex. Thus, whereas it captures the entropic contributions to the bound state, it does not take into account effects on either the unbound or transient states, both of which may contribute to the recognition and binding of HIF-1α in vivo. In this sense, the dynamical basis of the mechanism of S65L and Q145H mutants presented below should be considered as a component of a likely more complex phenomenon.

It should be pointed out that because of the short simulation length (1 ns), we observed only dynamical perturbations but not structural rearrangements. This is consistent with the absence of structural rearrangements in the course of the simulations of the wild type pVHL (root mean structural deviation of backbone Ca < 1.7 Å), and of significant differences between the wild type and pVHL S65L mutant (root mean structural deviation of backbone Ca < 1.8 Å). In addition, as shown in Figs. 3, A and B, 6A, and 8B, the VHL mutations examined in this study are all capable of interacting with ElonginC to form the VHL E3 complex. These observations are in accord with the notion that there are no major structural alterations with VHL S65L or Q145H mutants that may influence the overall stability of the protein.

RESULTS

In Vitro Ubiquitination of HIF-1α Catalyzed by Ubc5c or Cdc34—We reconstituted the in vitro ubiquitination of full-length IVT-HIF-1α as substrate with purified E1, Cdc34, or Ubc5c E2-conjugating enzyme, the VEC E3, Ub, and PHD1 (an HIF-α prolyl-oxidase). In this assay, [35S]-HIF-1α was hydroxylated by PHD1 and subsequently bound to E3, which was immunopurified on M2 beads. Following the addition of Ub, E1, and E2, Ub was covalently ligated to the E3-bound substrate, generating high molecular weight HIF-1α-Ub conjugates, which were measured by autoradiographic analysis.

As shown by the titration experiment, Ubc5c efficiently ubiquitinated HIF-1α in a concentration-dependent manner (Fig. 1A, lanes 1–5). To assess the kinetics of Ubc5c in this reaction, we examined the efficiency of HIF-1α ubiquitination as a function of time. Very high molecular weight substrate-Ub conjugates formed after 9 min, and the level of polyubiquitinated substrate increased over time with a concomitant decrease of the unmodified substrate (Fig. 1A, lanes 6–10). We next tested Cdc34, an E2 of the SCF complexes, for its ability to support VEC-catalyzed ubiquitination of HIF-1α in the reconstituted in vitro system. In comparison to Ubc5c, Cdc34 was able to ubiquitinate HIF-1α but required a 4-fold increase in enzyme concentration (Fig. 1B, lanes 1–4). Graphically, the kinetics of HIF-1α ubiquitination by Cdc34 and Ubc5c show a similar rate (Fig. 1, A and B). Both E2s formed highly conjugated polyubiquitinated chains on the substrate, although Cdc34 showed more heterogeneity in the chains compared with those of Ubc5c (compare Fig. 1, A, lanes 9 and 10, to B, lanes 8 and 9).

The linkage specificity in polyubiquitination of HIF-1α was investigated by substitution of the wild type Ub with UbK48R, which inhibits Lys48-linked Ub chain assembly. The ability of Ubc5c to form polyubiquitin chains on HIF-1α was undeterred with the mutant Ub, suggesting that this E2 can conjugate Ub moieties through lysines other than Lys48 (Fig. 2A, lanes 1–5). We performed a kinetic analysis using Cdc34 with the UbK48R mutant. In contrast to Ubc5c, in the presence of UbK48R, Cdc34 failed to support formation of polyubiquitinated HIF-1α species that migrated above 250 kDa, suggesting a specificity of this enzyme in selectively assembling Lys48-linked polyubiquitin chains onto HIF-1α (compare Figs. 1, B, lane 9, to 2, B, lane 5). Given the heterogeneous migration pattern of HIF-1α displayed on SDS-PAGE (Fig. 2B, lane 1), we were unable to precisely identify the monoubiquitinated HIF-1α species linked with UbK48R. This difficulty precluded us from assessing the rate of the initial ubiquitination reaction that ligated UbK48R to HIF-1α. However, it was evident that Cdc34 synthesized HIF-1α conjugates containing a limited number of UbK48R moieties, as reflected by the formation of discrete HIF-1α species that migrated slower than the unmodified form (in the range of 100–200 kDa) (Fig. 2B, lane 5).

Tumor-derived Mutations at Ser65 in VHL Abolish HIF-1α Binding—Whereas previous studies have documented the structural basis of a large number of tumor-derived mutations within the α- and β-domains (21–23), it remains poorly understood how VHL is inactivated by mutations situated in locations relatively distant from the HIF-α-binding pocket. One such site, Ser65, located in the L1 loop that is N-terminal to the binding pocket of HIF-1α (21–23), is frequently mutated in VHL (S65C, S65P, S65L, and S65W) and displays highly malignant forms in human cancer (Table I and referenced therein).

We investigated two of these tumor-derived mutations, S65W and S65L, as well as an alanine substitution of Ser65 (S65A), and compared the E3 complex formation and the HIF-1α binding activity of these point mutants to that of the wild type pVHL. A glutathione bead pull-down assay was performed to assess the ability of the pVHL mutants to form a pVHL-ElonginC-ElonginB subcomplex assembled in bacteria. As determined by Coomassie stain analysis, all three pVHL mutants retained a comparable level of ElonginC/ElonginB binding activity compared with the wild type pVHL (Fig. 3A). To examine formation of the VEC E3 complex, each FLAG-tagged pVHL mutant and HA-CUL2 constructs were co-transfected in 293T cells and then analyzed for E3 assembly by immunoprecipitation and Western blot. As shown in Fig. 3B, the pVHL point mutants all efficiently associated with VEC components, HA-CUL2 and ElonginC, compared with the wild type. To investigate the ability of the pVHL mutants to bind HIF-1α, we set up an in vitro hydroxylation-dependent binding assay. The VEC E3, immunopurified on M2 beads was incubated with [35S]-IVT-HIF-1α either pretreated or untreated with PHD1, and then washed extensively. Bound proteins were eluted in SDS loading buffer and analyzed by Western blot and autoradiography. Our results revealed that: 1) treatment of HIF-1α by PHD1 stimulated pVHL binding by 3-fold (Fig. 3C, lanes 1 and 2); 2) S65A retained the wild type binding activity (lanes 3 and 4); and 3) both S65L and S65W mutants completely lost their ability to bind hydroxylated HIF-1α (lanes 5–8). S65A also showed efficient ubiquitination activity, and a kinetic analysis of S65A demonstrated a similar rate of HIF-1α ubiquitination to that of the wild type pVHL (data not shown). In all, these studies demonstrate that despite their full capacity to form the VEC E3 complex, tumor-derived Ser65 mutations abolished pVHL function in binding to HIF-1α.

Clifford et al. (27) have previously investigated the molecular defect of the pVHL S65W mutation. Whereas their study agreed with ours in that this mutant is deficient in binding to HIF-1α, Clifford et al. (27) showed that S65W was defective in forming a complex with ElonginC in RCC4 cells. However, as revealed by the above studies using both bacterial and 293T cell transfection systems, S65W was found to interact with ElonginC (Fig. 3, A and B). It remains to be determined whether this discrepancy is because of the use of different cell systems.

The pVHL-HIF-1α Complex Exhibits Coordination of Dynamic Motions among Distant pVHL Regions—The above observation that tumorigenic mutations at the L1 loop-located Ser65 eliminated the interaction with HIF-1α, suggests a role played by regions outside of the HIF-α binding pocket in supporting formation of a stable complex with this substrate. (Ser65 is more than 20 Å away from the HIF-α binding pocket as well as ElonginC.) Importantly, there appear to be no statistically significant structural rearrangements of pVHL upon binding HIF-1α, with the root mean square deviation in Ca positions of 1.7 Å (21, 23). Consequently, we sought to
FIG. 1. In vitro ubiquitination of HIF-1α catalyzed by both Ubc5c and Cdc34. Using protocols described under “Experimental Procedures,” hydroxylated 35S-HIF-1α and the VEC complex immobilized on M2 beads were prepared separately and then mixed to form the substrate-E3 complex followed by addition of ubiquitination agents to initiate the modification reaction catalyzed by Ubc5c (panel A) or Cdc34 (panel B). Aliquots of each reaction were separated by 6% SDS-PAGE followed by autoradiography. Unmodified HIF-1α (110 kDa) and high molecular mass HIF-1α-Ubn reaction products (>250 kDa) were quantitated using a PhosphorImager (Amersham Biosciences). The results are presented in graphs.
determine whether oncogenic mutations of pVHL that lie distant to the HIF-1α binding pocket, such as S65L, can perturb substrate binding by disrupting the dynamics of molecular recognition between pVHL and the substrate. Molecular dynamics make important contributions to the binding and recognition of biomolecules, where dynamics play crucial roles in determining specificity and modulating entropy of binding (reviewed in Ref. 34). Here, we present nanosecond scale molecular dynamics simulations of pVHL/HIF-1α under physiological conditions, using the crystal structure of the pVHL-HIF-1α complex (23) as the starting conformation. The simulated system is a fully solvated model of pVHL/HIF-1α in the presence of over 16,000 water molecules and 80 counterions under periodic boundary conditions. The wild type and mutant systems were energy minimized, heated, and equilibrated to 37 °C and 1 atmosphere pressure in the isothermal-isobaric ensemble, as judged from the stabilization of fluctuations of kinetic energy (data not shown). Systems were then simulated for 1 ns, while being sampled every 0.1 ps. Such an approach allows for an examination of atomic level dynamics of the interaction between HIF-1α and pVHL and its mutants in the bound state in a computationally efficient manner (see “Experimental Procedures”).

To examine the dynamic coupling between the HIF-1α binding site and pVHL regions distant to it, we calculated the covariance of Cα positions, yielding positive and negative correlation coefficients that denote breathing-type (Fig. 4, red) and hinge-like (blue) motions, respectively. The covariance matrix of backbone Cα atom positions of the wild type pVHL/HIF-1α is shown in Fig. 4A. In addition to the structural α- and β-domains and motifs contained therein as revealed by the crystal structure (23), the pVHL-HIF-1α complex also contains a number of dynamic modules, interacting in specific ways. Fig. 4B shows the schematic mapping of breathing (red circles) and hinging (blue diamonds) motions, as derived from the analysis of Cα covariance patterns for the wild type pVHL-HIF-1α. In particular, the structural β-domain of pVHL contains a breathing-type dynamic module constituted by the N-terminal L1 loop containing Ser65, as well as S3, S4, and S6 β-strands that contain Ser111 and largely constitute the HIF-1α binding pocket. The positive coupling of this

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**Table I**

| Mutation | Tumor | Linker | VEC | HIF-1α binding | HIF-2α degradation |
|----------|-------|--------|-----|----------------|-------------------|
| L63P     | PCC   | L1     | Yes | ~80%          | No                |
| R64P     | PCC   | L1     | Yes | ~50%          | No                |
| S65L     | Angioma | L1    | Yes | ~8%          | No                |
| S65W     | Angioma; HAB (43) | L1 | Yes | ~8%          | No                |
| S65P     | RCC (35, 44) | L1 | ND | ND               | ND                |
| S65A     | No    | L1     | Yes | ~90%         | Yes               |
| S68W     | PCC (35, 44) | L1 | Yes | ~20%         | No                |
| R69C     | RCC (45) | L1    | Yes | ~55%         | No                |
| E70K     | PCC (42) | L1 | Yes | ~80%         | No                |
| Q145H    | RCC (35) | L7    | Yes | Loss of specificity | No            |

**a** The abbreviations used are: PCC, pheochromocytoma; carcinoma; HAB, hemangioblastoma. The numbers in parentheses refer to Ref. numbers.

**b** Linker region referred to as described in Ref. 21.

**c** The 5-subunit VEC E3 was assembled as described in the legend to Fig. 3B.

**d** Binding determined as described in the legend to Fig. 3C; expressed as percentage of the wild type activity.

**e** The ability of pVHL or its mutants to degrade HIF-2α was determined as described in the legend to Fig. 6C.

**f** ND, not determined.

**g** Binding to HIF-1α independently of HyP in vitro, does not form a complex with HIF-2α in vivo.
module is accomplished both using through-space (L1 loop to S3–S4 linker, S3–S4 linker to S6, L1 loop to S6) and through-bond (S3–S4 linker to S3 and to S4) interactions (Fig. 4B). Furthermore, this breathing-type dynamic module within the structural β-domain comprises a larger hinge-type dynamic module at the interface of the α- and β-domains, whereby its motions are negatively coupled to the motions of H1 and H3 helices in the α-domain of pVHL (Fig. 4B). Such coordination of dynamic motions play a role in stability of the pVHL-HIF-α recognition, as discussed below.

**Tumor-derived S65L Mutation Disrupts Dynamic Organization of pVHL-HIF-1α Recognition—**We next examined two Ser65 variants, S65A and S65L, using computational analysis of dynamics as described above. To allow for the comparison with the wild type protein, analysis of the effects of pVHL substitutions on the molecular recognition of HIF-1α were performed in the context of the pVHL-HIF-1α complex (see “Experimental Procedures”). Results from the schematic mapping of S65A show minor alterations in the overall dynamics of pVHL-HIF-1α recognition as compared with the wild type (compare Figs. 4, A and B, with 5, A and B), consistent with its ability to bind HIF-1α in vitro (Fig. 3C), and to direct HIF-2α degradation in cells (Fig. 6D). On the other hand, dynamical organization of pVHL-S65L-HIF-1α is perturbed as compared with the wild type (Fig. 5C), as evidenced by a marked disruption of the breathing-type module in the β-domain, and complete absence of the hinge-like module in the α-domain (Fig. 5D). Whereas S65L exhibits positive coupling between the motions of L1 loop and S3–S4 linker, these dynamics fail to be transmitted to the rest of the module in general and to the HIF-1α binding site in particular (Fig. 5D). The wild type behavior of S65A is likely because of the isosteric nature of the S65A substitution that preserves packing of the Ser65-containing L1 loop with the S4 and S6 β-strands and H3 α-helix. In contrast, dynamic disorganization of S65L is likely because of the disruption of these interactions (compare Fig. 5, B to D).

Of prominent note in the dynamical organization of pVHL-HIF-1α recognition is the presence of a focal hinge point between the breathing-type module in the β-domain and hinge-like module in the α-domain, centered around Gln145 (Fig. 4B). The dynamic organization of this focal hinge is prominently destroyed by S65L (Fig. 5, C and D), and suggests that Gln145 may constitute a control node in the pVHL-HIF-1α dynamics, as investigated below. In summary, analysis of molecular dynamics simulations of pVHL-HIF-1α indicates the presence of a unique and non-trivial dynamic organization of the complex, the integrity of which correlates with the loss-of-function of pVHL mutants in vitro and in vivo, with the N-terminal L1 loop playing an important role.

**Tumor-derived Mutations of Residues in the N-terminal L1 Loop of pVHL Lead to Stabilization of HIF-α Subunits—**In further support of a critical contribution by the L1 loop to VHL function, genetic data from familial VHL syndrome and sporadic RCC demonstrate that of the eight L1 loop amino acids, five residues (Ser65 included) are mutated in human cancers (Table I and referenced therein). We therefore analyzed molecular defects of the five tumor-derived pVHL L1 loop mutants that include L63P, R64P, S68W, R69C, and E70K. As shown in Fig. 6A, all five mutants were able to nucleate the VEC complex with efficiency comparable with that of the wild type pVHL, as determined by immunoprecipitation/immunoblot analysis. The effects of these mutations on in vitro binding to HIF-1α are shown in Fig. 6B. Among the five mutants examined, R64P, S68W, and R69C consistently exhibited the significantly re-
duced ability to bind to HIF-1α in comparison to the wild type pVHL (Fig. 6B, compare lanes 2, 6, 8, and 10). On average, R64P, S65W, and R69C had 50, 20, and 55%, respectively, of the wild type activity of pVHL to bind to HIF-1α. Both L63P and E70K had a modest reduction (20%) in HIF-1α binding (Fig. 6B, lanes 4 and 12). Thus, among the six pVHL L1 loop residues that contain tumor-derived mutations, S65L/S65W had the most pronounced effect in abolishing HIF-1α binding (Fig. 3C), followed by S68W, R64P, and R69C with each exhibiting significantly reduced binding activity.

To investigate the ability of the pVHL N-terminal loop mutants to degrade HIF-1α subunits in vitro, we utilized a VHL-null cell line, RCC/786-O, which expresses HIF-2α (10). We first confirmed the ability of the wild type pVHL to degrade HIF-2α in this system in a proteasome-dependent mechanism. FLAG-VHL was transfected into cells, and its effect on the endoge-

uous HIF-2α level was determined by direct Western blot of cell extracts. HIF-2α was efficiently degraded by pVHL, and this degradation was prevented by addition of the proteasome inhibitor, MG132 (Fig. 6C, lanes 1–4). We also tested the S65A mutant in this system and obtained the same results as the wild type pVHL (Fig. 6C, lanes 5 and 6), in keeping with the ability of this variant to interact with HIF-1α (Fig. 3). We next transfected each of the pVHL N-terminal loop mutants into 786-O cells and analyzed their ability to degrade HIF-2α. Our results showed that all of the tumor-derived L1 loop pVHL mutants, while expressed at levels comparable with the wild type, were defective in HIF-2α degradation (Fig. 6D).

These results demonstrate that tumor-derived mutations at six positions within the pVHL L1 loop all have profound defects in targeting HIF-2α for degradation, thus providing compelling biochemical evidence strongly suggesting a critical role for this region in the VHL function. Notably, mutants R64P, S65L/S65W, S68W, and R69C were deficient in both targeting cellular HIF-2α for degradation and binding to HIF-1α in vitro, thus correlating loss of in vitro binding activity with defective degradation of HIF-α subunits. However, future investigation is required to determine how L63P and E70K fail to degrade HIF-2α, which cannot be attributed to a slight reduction of HIF-1α binding activity by either mutant as revealed by the in vitro assay.

Q145H Mutant Disrupts the Dynamic Coupling in pVHL and Is Defective in HIF-α Degradation—Analysis of molecular dynamics simulations of the pVHL-HIF-1α peptide complex revealed that Gln145 may constitute a control node in the dynamics of pVHL-HIF-1α recognition. Gln145 lies in the L7 loop that is close to the interface of the α- and β-domains of pVHL, but is more than 20 Å away from the HIF-α binding pocket as well as ElonginC. It is, therefore, unlikely that this residue is directly involved in binding either ElonginC or HIF-α subunits. However, genetic data indicates that this residue is mutated in the VHL syndrome (35). Using molecular dynamics simulations, we assessed the effect of the tumorigenic mutant of this residue, Q145H, on the dynamic organization of the complex. In agreement with the prediction of the model of pVHL-HIF-1α recognition dynamics above (Fig. 4), Q145H dynamics are grossly disorganized, with an almost complete absence of positively or negatively correlated motions (Fig. 7).

We characterized the effects of the tumor-derived mutant Q145H in pVHL function. When introduced into the 786-O cell by transfection, Q145H was found to be unable to promote the degradation of HIF-2α (Fig. 8A), despite having an ability to form the VEC complex (Fig. 8B). Moreover, as revealed by immunoprecipitation/immunoblot analysis, wild type pVHL interacted with HIF-2α in 786-O cells when the activity of the 26 S proteasome was inhibited by MG132 (Fig. 8C, compare lanes 2 and 3). In contrast, under the same condition pVHLQ145H failed to form a complex with HIF-2α (Fig. 8C, compare lanes 3 and 5). These findings thus suggest that the Q145H mutation rendered pVHL incapable of interacting with HIF-α in vivo, resulting in stabilization of this regulatory subunit.

Unexpectedly, when analyzed by the in vitro binding assay, while the wild type pVHL interacted with HIF-1α in a hydroxylation-dependent manner (Fig. 8D, compare lanes 1 and 2), Q145H efficiently bound to HIF-1α in the absence of PHD1 (Fig. 8D, compare lanes 1 and 3). Subsequent titration experiments showed that at all levels of PHD1 tested, Q145H was able to bind to HIF-1α more efficiently than the wild type (Fig. 8E). As shown by quantitative analysis (Fig. 8E, graph), on average Q145H bound 4–6 times more HIF-1α than wild type pVHL in the absence of PHD1. In the presence of high levels of PHD1, wild type pVHL displayed a 4–7-fold increase in binding to HIF-1α,
whereas Q145H only increased substrate binding by less than 2-fold. Thus, it appears that the Q145H mutation diminished a requirement for prolyl-hydroxylation by pVHL to contact HIF-1α in vitro.

In summary, the above results underscore a critical role for pVHL Gln145 in coordinating the dynamic organization of the pVHL/HIF-1α recognition and in targeting cellular HIF-1α for degradation. Our studies further suggest that this residue may have a unique role for establishing the selective interaction of pVHL with prolyl-hydroxylated HIF-1α. Loss of such coordinating activity by the Q145H mutation leads to stabilization of HIF-1α and may hence be responsible for the malignant RCC phenotype.

DISCUSSION

Ubiquitination of HIF-1α—The ubiquitination of HIF-1α commences with the recognition of ODD by the VEC E3 ligase complex, which recruits an E2 conjugation enzyme to catalyze the transfer of Ub from E2 to the E3-bound substrate. We demonstrate that both Ubc5c and Cdc34 can function as E2 for VEC-dependent ubiquitination of HIF-1α in vitro, albeit substantially more Cdc34 than Ubc5c is required for the reaction (Fig. 1). However, only Cdc34 selectively modifies HIF-1α with Lys48-linked polyubiquitin chains (Fig. 2), which are signaling polypeptides well known for their function to recognize the 26 S proteasome (reviewed in Ref. 36), where substrate degradation takes place. Whether Cdc34 functions in cells to mediate the degradation of HIF-1α requires future investigation.

Distinct to HIF-1α ubiquitination is the presence of two hydroxylated proline-containing motifs (NODD with Pro402 and CODD with Pro564), placed at the N- and C-terminal end of ODD, each capable of interacting with the pVHL substrate-targeting subunit of VEC (14). We have observed in our reconstitution assays that substitution of Pro564 with glycine significantly reduced the binding of HIF-1αP564A to VEC (data not shown). However, ubiquitination still occurred with HIF-1αP564G bound to VEC, via hydroxylated Pro402 (data not shown), suggesting that the Pro402-containing NODD is at least partially functional for mediating Ub ligation. No significant effect was observed when Pro402 in HIF-1α was replaced by alanine (data not shown), suggesting that the Pro564-containing CODD alone was sufficient in supporting ubiquitination in vitro.

Little is known with respect to identity of HIF-1α lysine receptor residue(s) for ubiquitination. Substitution of Lys389, Lys391, or Lys547 with arginine did not affect HIF-1α ubiquitination in vitro (data not shown). Given the presence of two pVHL-interacting motifs (NODD and CODD), it is likely that multiple lysine residues can be used for Ub ligation. However, it is not known whether NODD and CODD can simultaneously engage in contacts with the VEC to initiate ubiquitination. In
this context, it is intriguing to note that Cdc34 formed heterogeneous lower molecular weight HIF-1α UbK48R species (Fig. 2B), which could be attributed to multiple mono-UbK48R conjugates assembled on HIF-1α.

Role of the pVHL N-terminal L1 Loop—Genetic studies from familial VHL syndrome and sporadic RCC underscore a critical role played by the pVHL N-terminal L1 loop in hydroxylation-dependent HIF-1α binding. Previous studies have investigated the L1 loop residues in VHL function. Hoffman et al. (26) and Clifford et al. (27) have shown that R64P and S65W mutations each diminished the ability of pVHL to bind HIF-1α. Crystallographic analysis by Hon et al. (22) suggests that Ser65 provides van der Waals contacts that contribute to the proper orientation of pVHL His115, a residue playing a central role in interacting with hydroxyproline (see below). In addition, the His115-containing, water-mediated hydrogen-bonding network involves the side chain of Asn67 and the main chain amide nitrogen of Arg69 (22).

The results presented in this study confirm and extend the critical contribution by the pVHL N-terminal L1 loop to the binding of HIF-1α. We demonstrate that: 1) all tumor-derived mutations at these six amino acid positions, while preserving

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**Fig. 6.** **Oncogenic pVHL N-terminal loop mutants are defective in HIF-1α binding and degradation.** A, tumorigenic pVHL N-terminal loop mutants are functional in VEC assembly. Cells (293T) were co-transfected with FLAG-VHL or mutants as indicated and hemagglutinin-CUL2, and extracts were immunopurified on M2 beads and analyzed by SDS-PAGE and Western blot as described in the legend to Fig. 3B. Wt, wild type. B, analysis of tumor-derived pVHL N-terminal loop mutants in hydroxylation-dependent HIF-1α binding. Same procedures were performed in Fig. 3B. C, proteasome-dependent degradation of HIF-2α in 786-O cells. 786-O/RCC cells were transfected with FLAG-VHL or variants (5 μg each) as indicated. Cells were treated with MG132 (5 μM) for 6 h and harvested 24 h after transfection (“Experimental Procedures”). Cell extracts were analyzed by centrifugation and 50 μg of total protein was analyzed by SDS-PAGE and Western blot against antibodies as indicated. Western blot of β-actin is shown to ensure equal loading. D, in vivo HIF-2α degradation by pVHL N-terminal loop mutants. 786-O cells were transfected with vectors expressing the wild type or indicated pVHL variants, harvested, and analyzed as in C.
the ability to form the VEC complex (Figs. 3A and 6A), were defective in targeting HIF-1α for degradation in 786-O cells (Fig. 6D); and 2) tumorigenic mutations R64P, S65L, S65W, S68W, and A69C rendered pVHL deficient in interaction with HIF-1α (Figs. 3C and 6B and Table I). We provide further insights by demonstrating that S65L, a mutation causing a malignant type of VHL syndrome, disrupted internal organization of molecular dynamics that are responsible for coordination of dynamic coupling within the β-domain, as well as between the α- and β-domains, of pVHL, respectively (compare Figs. 4 and 5).

Analysis of molecular dynamics simulation revealed extensive dynamic organization of the pVHL molecule (Fig. 4), which is largely disrupted by the S65L mutation (Fig. 5, C and D). Our findings thus suggest that in addition to its localized van der Waals contribution to the positioning of His115, pVHL Ser65 possesses a previously unrecognized dynamic role in vivo.

Role of pVHL Gln145—Our studies revealed intriguing properties of pVHL Gln145, a residue in the proximity of the αβ-domain interface (21) that has been found mutated (to His) in human RCC (Table I and referenced therein). Based on molecular dynamics simulations this residue is crucial for stabilizing the hinge-like motion of the α- and β-domains of pVHL (Fig. 4), and is required for coordinated motions throughout the protein and in the pVHL-HIF-1α interface, as evidenced by a total loss of dynamic coupling observed with the Q145H mutant (Fig. 7). Importantly, the Q145H mutant was unable to direct the degradation of HIF-2α in 786-O cells (Fig. 5A), most likely because of its inability to form a complex with this regulatory subunit in vitro (Fig. 8C). These findings thus provide another example of correlation between loss of dynamic organization and inactivation of the VHL function in targeting HIF-α for degradation.

However, unlike the S65L mutation whose disruption of dynamic coupling is associated with a loss of interaction with HIF-1α (Figs. 3C and 5, C and D), Q145H appears to alter the specificity of binding as evidenced by its association with HIF-1α in vitro with a markedly reduced dependence on hydroxylation (Fig. 8, D and E). Presumably, the formation of productive complex between pVHL and HIF-α in vivo strictly depends on stabilization of the hydroxyproline binding pocket. Contacts to HIF-α by pVHLQ145H are likely provided by 4-hydroxyl group-independent interactions (see below), which may not be sufficiently stable in cells.

Contribution of Dynamic Atomic Motions to the Interaction between pVHL and HIF-α—In recent years, molecular dynamics simulations of biological molecules have provided many insights concerning the importance of internal motions in specific biomolecular function. This approach has been used to elucidate the mechanism of bacterial chaperonin GroEL (37), to elegantly reveal a role for dynamic coupling of protein modules in regulating the activity of Src family tyrosine kinases (38), to advance the understanding of the dynamic gating of acetylcholinesterase (39), and to explore the concerted motions in the DNA-human topoisomerase I complex (40). In this study, we provide evidence suggesting a significant role for dynamic atomic motions in contributing both to the specificity as well as affinity of pVHL-HIF-α binding.

Structural analysis of pVHL bound with the HIF-1α HyP564 peptide (22, 23) has established that the specificity and affinity of the pVHL-HIF-α interaction is primarily provided by the hydroxyproline side chain, which is buried in a deep pocket formed by conserved pVHL residues Trp568, Tyr569, Ser111, His115, and Trp117. Within this pocket, the exposed Ser111 hydroxyl group and His115 imidazole amino group serve as hydrogen-bonding partners to the HyP564 hydroxyl group. Another contributing factor for the specificity at the hydroxyproline-binding site of pVHL is the restriction of the conformational flexibility of the HIF-1α backbone in the vicinity of HyP564, which is fixed through an extensive network of hydrogen bonds involving both backbone and side chain groups from pVHL (23).

Coordination of atomic motions within pVHL and organization of its dynamic modules may be required to establish and/or maintain the recognition of hydroxylated HIF-α through dynamics-mediated entropic effects. Disruption of these coordinated motions and destabilization of pVHL dynamic modules by tumorigenic mutations such as S65L and Q145H leads to loss of efficient and/or specific binding of HIF-α. It is hopeful that future development of simulation techniques will allow an assessment on whether coordinated atomic motions are correlated with proper positioning of pVHL residues involved in the complex network of interactions that support the formation of the hydroxyproline binding pocket and/or the structural rigidity of the HIF-α backbone in the vicinity of hydroxyproline.

Taken together, combined with both computational and biochemical studies, our data points to a previously unrecognized
Total protein (50 μg) was immunoprecipitated with M2 beads and the resulting immunoprecipitates were analyzed by Western blot with the indicated antibodies.

B. Q145H does not form a complex with HIF-2α in vivo. Cells (786-O) were transfected with FLAG-VHL or FLAG-VHLQ145H, treated with MG132, and harvested as described in the legend to Fig. 6C. Bound HIF-1α was quantitated using a PhosphorImager and the results were presented in a bar graph.

C. Q145H mutant does not form a complex with HIF-2α in vitro. Extracts (1 mg of protein) were immunoprecipitated with M2 beads and the resulting immunoprecipitates were analyzed by Western blot with the indicated antibodies. D. Analysis of hydroxylation-dependent HIF-1α binding by Q145H mutant. The same assay was as performed in Fig. 3C. Bound HIF-1α was quantitated using a PhosphorImager and expressed as arbitrary units displayed under each lane. 

E. Titration of PHD1 in the hydroxylation-dependent binding assay. The in vitro HIF-1α binding assay was performed as in D with levels of PHD1 as indicated. Bound HIF-1α was quantitated using a PhosphorImager and the results were presented in a bar graph.

role played by both the L1 and L7 linker regions to coordinate atomic motions that are likely critical for the β-domain to mediate its interaction with HIF-α in a hydroxylation-dependent manner. We have therefore suggested a new mechanism explaining how tumor-derived mutations within these regions, which are distant to the HIF-α binding pocket, profoundly perturb the function of pVHL in targeting HIF-α for degradation.

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Inactivation of VHL by Tumorigenic Mutations That Disrupt Dynamic Coupling of the pVHL·Hypoxia-inducible Transcription Factor-1 α Complex

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