VHL Mutations Linked to Type 2C von Hippel-Lindau Disease Cause Extensive Structural Perturbations in pVHL

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pVHL (von Hippel-Lindau tumor suppressor protein) is the substrate recognition subunit of the CBCVHL ubiquitin ligase complex promoting the degradation of hypoxia-inducible factor subunits, HIF-1/2α. Mutational inactivation of pVHL causes the hereditary von Hippel-Lindau tumor syndrome, which predisposes affected individuals to hemangioblastomas, renal cell carcinomas, and pheochromocytomas. Whereas the development of hemangioblastomas and renal cell carcinomas has been attributed to impaired HIF-1/2α down-regulation by pVHL mutant proteins, the molecular defects underlying the development of pheochromocytomas are still unknown. Here, we present a detailed biochemical analysis of pVHL mutant proteins linked to type 2C (pheochromocytoma only) von Hippel-Lindau disease. Type 2C-associated mutations caused extensive structural perturbations of pVHL, as revealed by the reduced stability, increased proteolytic susceptibility, and dramatically altered NMR spectrum of recombinant, mutant pVHL-ElonginC-ElonginB complexes in vitro. In human cell lines, type 2C-linked mutations destabilized the CBCVHL ubiquitin ligase complex and resulted in reduced cellular pVHL levels. Together, our data reveal unexpectedly strong structural defects of type 2C-associated pVHL mutant proteins that are likely to affect both HIF-1/2α-related and -unrelated pVHL functions in the pathogenesis of pheochromocytomas.

von Hippel-Lindau disease (OMIM 193300) is an autosomal dominant, hereditary cancer syndrome caused by germ line mutations in the VHL tumor suppressor gene (1, 2). It predisposes to a variety of malignant and benign tumors, most frequently retinal and cerebellar hemangioblastomas, renal cell carcinomas (RCC), and pheochromocytomas. Based on the incidence of these tumors, the disease is divided into distinct subtypes (1, 2). Type 1 is characterized by RCCs and hemangioblastomas, but not pheochromocytomas, and is associated with disruptive mutations and gross deletions in VHL. Type 2, which is predominantly associated with VHL missense mutations, is defined by the occurrence of pheochromocytomas, either alone (type 2C) or in combination with hemangioblastomas (type 2A) or with hemangioblastomas and RCCs (type 2B). In addition to von Hippel-Lindau disease, VHL is also mutated in the majority of sporadic RCCs and in Chuvash polycthemia (2).

The VHL gene product, pVHL, is the substrate recognition subunit of the CBCVHL E3 ubiquitin ligase complex (3), which in addition to pVHL consists of the scaffold protein Cullin-2, the RING finger protein Rbx1/Roc1, and the adaptors ElonginB and ElonginC that bind and stabilize pVHL. Key cellular targets of CBCVHL are the closely related hypoxia-inducible transcription factor α subunits HIF-1α and HIF-2α, which are ubiquitylated by CBCVHL and rapidly degraded by the 26 S proteasome under normoxic conditions (4–7). Recognition by pVHL depends on the post-translational modification of two HIF-1α/2α prolyl residues by members of the PHD/EglN/PHF prolyl hydroxylase family (8–12). These enzymes turn over molecular oxygen and thus are inactive under hypoxic conditions. Consequently, HIF-1/2α is stabilized under hypoxic conditions and assembles with HIF-1β/ARNT1 to activate transcription of hypoxia-responsive target genes involved in angiogenesis, glucose uptake and metabolism, and erythropoiesis (13).

Most VHL mutations in von Hippel-Lindau disease affect the oxygen-dependent regulation of HIF-1/2α (2). Type 1-associated pVHL mutant proteins fail to assemble into a functional CBCVHL E3 ubiquitin ligase complex (14), whereas type 2A- and type 2B-associated pVHL mutant proteins were shown to be differentially affected in HIF-1/2α binding (15–17). Defective HIF-1α/2α regulation is believed to be sufficient to induce development of hemangioblastomas and to be a key pathogenic event in the development of RCC (reviewed in Ref. 2). This critical role of HIF-1/2α down-regulation for the tumor suppressor function of pVHL is underscored by the finding that inhibition of HIF-2α is necessary and sufficient for pVHL to suppress VHL−/− tumor growth in mouse models (18, 19). Nevertheless, contributions of other, HIF-independent pVHL functions to tumor suppression are possible (20, 21). Most notably, VHL mutations have been shown to affect primary cilia formation in kidney cells (22), microtubule stability (23), and extracellular matrix organization (24).

Although the critical role of HIF-1/2α dysregulation in the development of hemangioblastoma and RCC is widely accepted, the molecular basis of pheochromocytoma in von Hippel-Lindau disease is still under debate. The pathogenesis of pheochromocytoma has been studied by analyzing functional...
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Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml streptomycin. RCC4 VHL−/− renal cell carcinoma cell lines were a gift of P. Maxwell (Imperial College, London, UK). RCC4-derived cell pools expressing wild type or mutant VHL were generated by infection with retroviruses obtained by transfecting LinX-A cells with pBabe-Puro-VHL using FuGENE (Roche Applied Science). Stable cell pools were selected and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml puromycin.

Protein Purification—Expression and purification of recombinant VCB complex were performed exactly as described (15). For isotopically enriched protein preparations, VCB or ElonginBC complexes were co-expressed in minimal medium containing 15NH4Cl and purified as will be published elsewhere.4

Biochemical Analyses—In vitro transcription and translation using pcDNA3-HA-VHL as template and HIF-1α peptide binding measurements were performed exactly as described (15). Partial tryptic proteolysis was essentially performed as described (15), using 800 ng of VCB complex and 8 ng of tryptic. For the analysis of VCB aggregation state by size exclusion chromatography, 30 μg of VCB complex were incubated over-night at 4, 30, or 37 °C and subsequently loaded at 4 °C onto a Superdex S75 HR10/30 column (GE Healthcare) equilibrated in 40 mM Tris/HCl, pH 7.9, 150 mM KCl, 5 mM dithiothreitol. Eluted fractions were concentrated by trichloroacetic acid precipitation and analyzed by SDS-PAGE.

NMR Spectroscopy—1H,15N HSQC spectra of 15N-labeled wild type VCB complex, pVHL188V-containing VCB complex, and ElonginBC complex (all 0.1 mM in 40 mM Hepes, pH 7.2, 150 mM NaCl, 5 mM dithiothreitol, 5% D2O) were recorded at 25 °C on a Bruker AVANCE 700 MHz spectrometer equipped with a cryo probe head. Full assignments of the wild type VCB spectrum will be published elsewhere.4

Immunoprecipitation and Immunoblots—Expression of FLAG-pVHL in 293T cells and immunoprecipitation were performed as described (42). Bound proteins were analyzed by immunoblots using antibodies directed against the FLAG epitope (M2; Sigma), Cullin-2 (Zymed Laboratories Inc.), Rbx1 (Ab-1; NeoMarkers), and ElonginC (R-20; Santa Cruz). Protein levels in RCC4-derived cell pools were analyzed by immuno-blotting using antibodies directed against pVHL (VHL-C7; gift of W. Krek), HIF-1α (clone 54; BD Transduction), and tubulin (Sigma).

Quantitative PCR—To quantify mRNA levels in RCC4-derived cell pools, total RNA from 106 cells was purified using a High Pure RNA isolation kit (Roche Applied Science). cDNA was synthesized from 1.5 μg of total RNA using a Transcriptor First Strand cDNA synthesis kit (Roche Applied Science) with a combination of oligo(dT)18 primer (final concentration, 2.5 μM) and primer pBabe3 (5’-CTG ACA CAC ATT CCA CAG GG-3’; final concentration, 2 μM). Real time PCR was performed on a LightCycler 480 (Roche Applied Science) using a SYBR Green I Master kit (Roche Applied Science) according to

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**TABLE 1**
Substrate binding of wild type and mutant VCB complexes

|            | $K_d$ (nm) | $k_{on}/k_{off}$ | $K_{d}$ (nm) | $k_{on}/k_{off}$ |
|------------|------------|------------------|--------------|------------------|
| Wild type  | 5.7 ± 0.5  | 0.018 ± 0.00012  | 3.2 × 10^6   | 0.00018          |
| P81S       | 15.8 ± 1.8 | 0.023 ± 0.00016  | 1.4 × 10^6   | 0.00059          |
| L188V      | 18.1 ± 1.6 | 0.018 ± 0.00019  | 0.99 × 10^6  | 0.00019          |
| P81S/L188V | 21.6 ± 3.6 | 0.021 ± 0.00059  | 0.97 × 10^6  | 0.00019          |
| V84L       | ND         | 0.014 ± 0.00018  | ND           | ND               |

$^a$ The $k_{on}/k_{off}$ values were calculated using the equation $k_{on} = k_{off}/K_d$.

$^b$ ND, not determined. $K_d$ could not be determined because limiting yields of recombinant pVHL indicated VCB complex precluded the preparation of meaningful titration series.

RESULTS

Type 2C-associated pVHL Mutant Proteins Are Proficient in Substrate Binding-Type 2C-associated mutations do not affect pVHL residues in close proximity to the HIF-1α-binding site (14, 43, 44) (supplemental Fig. S1). Consistently, no significant defects in HIF-1α binding were detected in previous, qualitative analyses of type 2C-associated mutant pVHL proteins (25, 26). To test this more rigorously, we used recombinant wild type and mutant VCB complexes to quantify the binding of a fluorescent, HIF-1α-derived substrate peptide (15). In kinetic measurements of substrate peptide release, all of the mutant VCB complexes were found to possess dissociation rate constants very similar to the wild type complex (Table 1). In equilibrium titration experiments, the pVHL$^{P81S}$-containing VCB complex and the type 2C-associated pVHL$^{L188V}$- and pVHL$^{P81S/L188V}$-containing VCB complexes bound substrate peptide with slightly (~3-fold) lower affinity than the wild type complex (Table 1). Thus, type 2C-associated mutations affect substrate binding of pVHL only moderately. In particular, the affinities for the HIF-1α-derived peptide are still in the low nanomolar range and thus 1–2 orders of magnitude higher than for prototypical type 2A- and type 2B-associated mutant pVHL proteins (15).

Type 2C-associated pVHL Mutant Proteins Assemble into Unstable VCB Complexes—Next, the stability of the wild type and mutant VCB complexes was analyzed. To that end, VHL was transcribed and translated in vitro at 30 °C in reticulocyte lysate providing endogenous ElonginB and ElonginC, and the formation of stable VCB complex was monitored by nondenaturing polyacrylamide gel electrophoresis (15, 45, 46). When the electrophoresis was performed at 4 °C, wild type and type 2C-associated pVHL mutant proteins migrated in a defined VCB complex, unlike the assembly-deficient pVHL$^{C162F}$ mutant protein that served as a negative control (Fig. 1). At 30 °C, in contrast, pVHL$^{P81S/L188V}$ and pVHL$^{V84L}$ failed to migrate in a stable VCB complex, and the level of stably assembled pVHL$^{L188V}$ was significantly reduced. At 37 °C, the differences between wild type and mutant pVHL proteins were even more pronounced, because pVHL$^{L188V}$ completely failed to migrate in a stable complex, and the amount of pVHL$^{P81S}$ incorporated into VCB was strongly reduced. These data show that type 2C-associated mutant pVHL proteins are impaired in their ability to form or maintain stable VCB complexes at physiologically relevant temperature. Moreover, the destabilization caused by the L188V and P81S amino acid substitutions was additive in this assay, because the pVHL$^{P81S/L188V}$ double mutant protein was significantly more affected than pVHL$^{L188V}$. It is important to note, however, that all type 2C-associated mutant proteins were fully proficient in forming...
proteins do not cause gross structural perturbations or able elution profiles consistent with their molecular masses of overnight incubation at 4, 30, or 37 °C (Fig. 2). After incubation exclusion chromatography of recombinant VCB at 4 °C after co-expression of the subunits recombinant mutant VCB complexes to homogeneity over several defects. This conclusion is also supported by our ability to purify pVHLC162F mutant protein, do not possess a general assembly lated at 30 °C, indicating that they, unlike the type 1-associated type 2C-associated pVHL mutant proteins form unstable recombinant VCB complexes. Recombinant VCB complexes containing the indicated wild type (WT) and mutant pVHL proteins were incubated overnight at the indicated temperature and subsequently analyzed by size exclusion chromatography on a Superdex S75 HR10/30 column at 4 °C. Left panels, chromatographic fractions were analyzed for the presence of pVHL, ElonginB, and ElonginC by SDS-PAGE followed by Coomassie staining. l.c., loading control. a stable VCB complex at 4 °C after they had been in vitro translated at 30 °C, indicating that they, unlike the type 1-associated pVHL C162F mutant protein, do not possess a general assembly defect. This conclusion is also supported by our ability to purify recombinant mutant VCB complexes to homogeneity over several chromatographic steps at 4 °C after co-expression of the subunits in Escherichia coli at room temperature. To further analyze the temperature-dependent stability of the wild type and mutant VCB complexes, we performed size exclusion chromatography of recombinant VCB at 4 °C after overnight incubation at 4, 30, or 37 °C (Fig. 2). After incubation at 4 °C, wild type and mutant complexes showed indistinguishable elution profiles consistent with their molecular masses of 43 kDa, confirming that type 2C-associated pVHL mutant proteins do not cause gross structural perturbations or destabilization of the VCB complex at this temperature. After incubation at 30 °C, however, significant amounts of pVHL L188V- and pVHL P81S/L188V-containing VCB complex eluted in the void volume of the column, indicating a higher oligomerization/aggregation state and/or altered shape at this temperature. Preincubation at 37 °C aggravated this effect and resulted in the accumulation of ~50% of pVHL L188V-containing and more than 50% of pVHL P81S/L188V-containing VCB complex in the void volume, as judged by the relative peak areas of the chromatograms. At this temperature, some pVHL P81S-containing VCB complex also eluted in the void volume, whereas wild type VCB complex was exclusively detected as a 43-kDa species. Together, these results indicate that type 2C-associated mutations cause temperature-dependent aggregation or structural alterations of the VCB complex, consistent with the results obtained with the reticulo- \textit{cyte system.} Type 2C-associated pVHL Mutant Proteins Are Partially Unfolded—To specifically analyze the conformation of pVHL at 37 °C, the susceptibility of wild type and type 2C-associated mutant recombinant VCB complexes to tryptic degradation was tested (Fig. 3). While the ElonginB and ElonginC subunits were protease resistant during the time course of the experiment, wild type pVHL was gradually degraded and barely detectable after 2 h. The pVHL P81S mutant protein was degraded only slightly faster than the wild type. In contrast, pVHL L188V, pVHL P81S/L188V, and pVHL V84L were extremely susceptible to tryptic degradation and undetectable after 10–20 min. Of note, the protease concentration in these experiments was 10-fold lower compared with our previous analysis of type 2A/B-associated mutant pVHL proteins (15) in order to detect type 2C-associated pVHL mutant proteins at all. These results suggest that type 2C-associated mutations induce significant conformational changes in pVHL, either within the VCB complex or upon dissociation from ElonginB and ElonginC. To analyze the folding state of wild type pVHL and the type 2C-associated pVHL L188V mutant protein within the VCB complex in more detail in a noninvasive manner, we employed two-dimensional, heteronuclear NMR spectroscopy. The $^1H,^15N$ HSQC spectrum of the wild type complex at 25 °C
shows the expected number of predominantly well dispersed cross-peaks, with some spectral overlap in the central part and the side chain region (Fig. 4a, blue spectrum). The spectrum of the heterodimeric ElonginB-ElonginC complex overlaps extensively with the wild type VCB spectrum (Fig. 4a, yellow spectrum), illustrating that the overall structure of ElonginB and C is retained in the VCB complex. While the $^{1}H,^{15}N$ HSQC spectrum of the pVHL$^{P81S}$-containing VCB complex is almost indistinguishable from the wild type complex (data not shown), the spectrum of the pVHL$^{L188V}$-containing complex is dramatically different (Fig. 4b, red spectrum). The most obvious difference is the almost complete lack of native pVHL resonances, highlighting the loss of structure of the pVHL subunit of the mutant VCB complex. Although pVHL$^{L188V}$ shows extensive structural disorder, as evident from the intense, overlapping resonances in the central region of the spectrum, most ElonginB and C signals are retained, demonstrating the individuality of the Elongins within the VCB complex. Of note, however, some weak residual native pVHL resonances could be identified, including the $\beta$ domain residues Gly$^{123}$, Asn$^{131}$, Gln$^{132}$, and Gly$^{144}$ (Fig. 4, c and d). These signals indicate conformational exchange between the folded and unfolded states of pVHL$^{L188V}$, with an emphasis on the unfolded state. In addition, they show that at least a subpopulation of pVHL$^{L188V}$ is bound to ElonginB and C at equilibrium. Together, the NMR spectra provide direct proof that the fold of type 2C-associated pVHL protein is globally destabilized at room temperature even in the presence of ElonginB and C.

**Type 2C-associated pVHL Mutant Proteins Assemble into Unstable CBC$^{VHL}$ Complexes in Vivo**—The pronounced alterations in pVHL conformation and VCB stability observed at physiologically relevant temperature in vitro suggested that type 2C-associated VHL mutations should also destabilize the full CBC$^{VHL}$ E3 ubiquitin ligase complex in vivo. To test this prediction, we transfected human 293T cells to transiently express FLAG epitope-tagged wild type or mutant pVHL, immunoprecipitated FLAG-pVHL from cell lysates, and compared the amounts of co-precipitated, endogenous CBC$^{VHL}$ subunits (Fig. 5). Sim-
ilar amounts of Cullin-2, Rbx1, and ElonginC were co-pre-
cipitated with wild type pVHL and pVHL<sup>P81S</sup>, but not with
type 1-associated, assembly-deficient pVHL<sup>C162F</sup>. Import-
rantly, reduced amounts of ElonginC were co-precipitated
with all type 2C-associated pVHL mutant proteins, indicat-
ing that type 2C-associated mutations indeed affect the sta-
dility of the CBC<sup>VHL</sup> E3 ubiquitin ligase complex <i>in vivo</i>. Interestingly, pVHL<sup>L188V</sup> and pVHL<sup>P81S/L188V</sup>, but not
pVHL<sup>V84L</sup>, also bound significantly less Cullin-2 and Rbx1, a
distal CBC<sup>VHL</sup> subunit binding via Cullin-2. These results
suggest that the V84L mutation primarily affects ElonginC
binding, whereas the L188V mutation affects both ElonginC
and Cullin-2 binding.

**Type 2C-associated pVHL Mutant Proteins Are Mildly Defective in HIF-1α Regulation**—To analyze potential physiological
consequences of the observed CBC<sup>VHL</sup> destabilization, we sta-
ably reintroduced wild type and mutant <i>VHL</i> into the renal cell
carcinoma-derived RCC4 <i>VHL</i><sup>−/−</sup> cell line. A low copy num-
er, episomal expression vector was used to avoid masking of
potential subtle defects by overexpression. Surprisingly,
the steady-state levels of all three type 2C-associated pVHL mutant
proteins, but not of pVHL<sup>P81S</sup>, were strongly reduced com-
pared with wild type pVHL, and the level of pVHL<sup>C162F</sup> was
below detection (Fig. 6a). These differences were reproducibly
observed in independently selected cell pools that had been
infected with independently generated retroviral inocula.
The differences in pVHL levels are much more pronounced than
those observed in transiently transfected 293T cells (cf. input in
Fig. 5), presumably because of the weaker, more physiological
expression from the episomal constructs. Importantly, the
reduced pVHL mutant protein levels in the RCC4-derived cell
pools are not the consequence of reduced <i>VHL</i> expression,
because <i>VHL</i> mRNA levels were comparable between wild type
and mutant cell pools and did not correlate with pVHL protein
levels (Fig. 6b). Rather, the reduced pVHL levels suggest that
type 2C-associated pVHL mutant proteins are short-lived <i>in vivo</i>,
presumably because they associate less stably with the
other subunits of the CBC<sup>VHL</sup> complex.

Next, the impact of reduced pVHL protein levels on the reg-
ulation of the key cellular CBC<sup>VHL</sup> target, HIF-1α, was ana-
yzed. Under normoxic conditions, HIF-1α was hardly detect-
able in cell pools expressing wild type pVHL or pVHL<sup>P81S</sup>,
whereas it strongly accumulated in pools carrying empty vector
or expressing pVHL<sup>C162F</sup> (Fig. 6a). Of note, cell pools express-
ing type 2C-associated pVHL mutant proteins showed slightly
higher HIF-1α levels under normoxic conditions, indicating
that the reduced pVHL levels in these cell pools lead to an
incomplete down-regulation of HIF-1α. The residual HIF-1α
levels observed in cell pools expressing type 2C-associated
pVHL mutant proteins were, however, insufficient to drive
expression of the HIF-1α target gene, GLUT1 (Fig. 6b). Thus,
at least in the setting of a renal cell carcinoma-derived cell line,
the mild defects in HIF-1α down-regulation exhibited by type
2C-associated pVHL mutant proteins do not result in a meas-
urable up-regulation of HIF-1α target genes.

**DISCUSSION**

This is the first study investigating defects of type 2C-associ-
pated pVHL mutant proteins at the molecular level. We show
that pVHL<sup>L188V</sup>, pVHL<sup>P81S/L188V</sup>, and pVHL<sup>V84L</sup> are structu-
urally perturbed and impaired in forming stable VCB and
CBC<sup>VHL</sup> complexes.

Using biochemical and structural approaches, we were able
to directly analyze pVHL folding and stability in the context of
the purified, recombinant VCB complex. We found a signifi-
cant destabilization at physiologically relevant temperature of
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VCB containing type 2C-associated pVHL mutant proteins consistent with the extensive structural alterations observed by NMR spectroscopy. Our results are compatible with two slightly different interpretations regarding the integrity of the VCB complex. In one scenario, the equilibrium between free pVHL and the VCB and CBCVHL complexes, respectively, is shifted toward free pVHL by type 2C-associated mutations. Because free pVHL is unable to adopt its native conformation in vivo, it is highly susceptible to degradation both by trypsin in vitro and by cellular proteases in vivo. Indeed, pVHL has been shown to be stabilized in vivo by incorporation into the CBCVHL complex (48, 49), providing a straightforward explanation for the reduced levels of type 2C-associated pVHL mutant proteins in vivo (Fig. 6a). Alternatively, the mutant pVHL proteins may remain associated with VCB and CBCVHL while having lost most of their native three-dimensional structure. Again, this would lead to high susceptibility toward proteolysis. In addition, this interpretation could explain the co-elution of ElonginB and C with pVHL in high molecular weight size exclusion chromatography fractions upon incubation at elevated temperature (Fig. 2), and the presence of residual, native cross-peaks in the NMR spectrum (Fig. 4, c and d).

The destabilizing effect of type 2C-associated VHL mutations can at least partially be rationalized based on the known three-dimensional structure of the VCB complex (14, 43, 44) (see supplemental Fig. S1). Residue Leu188 is located in one of the three helices of the pVHL α domain, which are complemented by a helix from ElonginC to form an intermolecular four-helix cluster (14). Consequently, the L188V mutation is expected to affect the packing of these helices and hence ElonginC binding. In addition to local effects on the α domain, our NMR data also reveal gross structural alterations of the β domain (Fig. 4b), most likely caused by disorder in the carboxyl-terminal region of pVHL and/or the α/β domain interface. According to a recently presented structural model of VCB in complex with Cullin-2 (50), a perturbation of the four-helix cluster would also be expected to affect critical contacts with Cullin-2. Indeed, we found that the interaction between pVHL L188V and Cullin-2 is significantly weakened in vivo (Fig. 5), indicating that the proper orientation of the helices comprising the pVHL α domain is crucial for the ordered assembly of the entire CBCVHL complex.

Residue Pro81 is located at the interface between the pVHL β domain and ElonginC. Although the P81S substitution does not cause significant structural perturbations on its own, it might affect the orientation of the neighboring residue Arg82, whose side chain forms the center of a hydrogen bond network stabilizing both the α/β domain interface of pVHL and the pVHL/ElonginC interface (14). Suboptimal hydrogen bonding might further destabilize the interaction with ElonginC upon mutation of Leu188, consistent with the additive defects we observed in some, but not all, of our assays for the pVHL P81S/L188V double mutant protein. We speculate that the P81S substitution might also aggravate molecular defects caused by concomitant VHL mutations in trichloroethylene-induced renal cell carcinomas (37, 38). In contrast, the reported link of the P81S substitution to type 1 VHL disease (36) remains enigmatic, given the lack of significant functional defects of the pVHL P81S mutant protein in vivo.

The side chain of residue Val84 contributes to the hydrophobic core of the pVHL β domain. Consequently, the V84L substitution destabilizes pVHL, presumably by weakening hydrophobic interactions within the β domain. In contrast to many type 2B-associated pVHL proteins mutated in neighboring residues, however, the binding of pVHL V84L to HIF-1α is not severely affected (25, 26) (Table 1 and supplemental Fig. S2). Rather, this mutation affects binding to ElonginC, either by altering the interface of the β domain with loop 5 of ElonginC (14) or more indirectly by altering the intermolecular four-helix cluster via the Arg82 hydrogen bond network. Interestingly, the binding of pVHL V84L to Cullin-2 is not significantly compromised (Fig. 5), suggesting that it is separable from ElonginC binding despite the requirement for an intact α domain (see above).

Previous studies did not report defects of type 2C-associated pVHL mutant proteins in the down-regulation of HIF-1/2α in vivo (25, 26). It is possible that the low VHL expression levels in our stable cell lines facilitated the detection of relatively subtle effects on HIF-1α regulation that otherwise might be masked by VHL overexpression. Nevertheless, a careful examination of published data reveals that defects of type 2C-associated pVHL mutant proteins in HIF-1/2α regulation were detectable in several studies. Examples include slightly elevated HIF-1α and/or HIF-2α levels in stable 786–0/pVHL V84L (25), RCC10/pVHL V84A (51), RCC4/pVHL V84L (52), RCC4/pVHL L188V (52), and 786–0/pVHL L188V (27, 53) cell lines. Together, these pieces of evidence support our data demonstrating small, but detectable defects in HIF-1α regulation for pVHL V84L, pVHL P81S/L188V, and pVHL V84L (Fig. 6). The risk of renal cell carcinomas in type 2 von Hippel-Lindau disease has been shown to strongly correlate with the ability to bind, ubiquitylate, and down-regulate HIF-1/2α (15–17). The residual down-regulation of HIF-1α by type 2C-associated pVHL mutant proteins is more efficient than that by type 2A-associated pVHL mutant proteins (26), consistent with the absence of renal cell carcinomas in type 2C von Hippel-Lindau disease. Consequently, no significant up-regulation of classical HIF-1/2α target genes is observed in renal cell carcinoma-derived cell lines expressing type 2C-associated pVHL mutant proteins (Fig. 6b; Refs. 25, 27, 52, and 53). This does not, however, exclude the possibility that mild defects in HIF-1α regulation lead to an up-regulation of target genes requiring relatively low HIF levels for expression in other cell types, thereby potentially contributing to the pathogenesis of pheochromocytomas in type 2 von Hippel-Lindau disease.

Irrespective of the role of HIF-1/2α in pheochromocytoma, the reason for the lack of pheochromocytoma in type 1 von Hippel-Lindau disease remains enigmatic. The most obvious difference between type 1- and all type 2-associated pVHL mutant proteins studied so far is the ability of the latter to assemble at least to some extent into CBCVHL complexes (this study and Refs. 15 and 26), which protects them from rapid intracellular degradation (48, 49). Thus, development of pheochromocytoma appears to require some minimal cellular pVHL level and to be incompatible with total loss of pVHL function. In type 2B von Hippel-Lindau disease, pheochromocytomas are found in the context of complete loss of HIF-1/2α down-regulation, suggesting that some HIF-independent function of pVHL is critical for the pathogenesis of pheochromocytoma.
Recently, the down-regulation of atypical PKC isoforms (aPKCs) and junB, which is required for apoptosis of neuronal progenitor cells upon nerve growth factor withdrawal, was reported to be pVHL-dependent but partially HIF-independent (27). In a model taking into account the dual role of pVHL in HIF-1/2α and aPKC/junB regulation, it was proposed that the balance of defects in the HIF-1/2α versus aPKC/junB pathways determines whether VHL mutations are associated with pheochromocytoma (2). However, this model appears to be inconsistent with the finding that HIF-2α, its target gene product GLUT1, and junB accumulate to very similar levels in VHL−/− renal cell carcinoma cell lines stably expressing type 1 and type 2B mutant proteins, respectively (27). Another HIF-independent function of pVHL that has been implicated in pheochromocytoma pathogenesis is its involvement in extracellular matrix assembly by virtue of interactions with fibronectin (25) and collagen IV (54, 55). Again, however, type 1- and type 2-associated pVHL mutant proteins exhibit similar defects (25), making it unlikely that extracellular matrix assembly is critical with respect to pheochromocytoma.

Although further efforts are needed to clarify the as yet elusive HIF-independent pVHL function required for the development of pheochromocytoma, our study suggests the possibility that partially defective HIF-1/2α regulation contributes to the pathogenesis of pheochromocytoma in von Hippel-Lindau disease.

Acknowledgments—We thank Nicola Pavletich, Song Tan, Wilhelm Krek, and Patrick Maxwell for materials; Wilhelm Krek and Claudio Thoma for generous help in generating RCC4-derived stable cell pools and for sharing reagents; Stefan Müller for help with real time PCR; Stefan Müller and Martin Beck for critical reading of the manuscript; and Stefan Jentsch for continued support.

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