Tumor Suppressor von Hippel-Lindau (VHL) Stabilization of Jade-1 Protein Occurs through Plant Homeodomains and Is VHL Mutation Dependent

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ABSTRACT

The von Hippel-Lindau (VHL) gene is the major renal cancer gene in adults. The mechanism of renal tumor suppression by VHL protein is only partly elucidated. VHL loss increases expression of the hypoxia-inducible factor $\alpha$ transcription factors. However, clinical and biochemical data indicate that the hypoxia-inducible factors are necessary but not sufficient for renal tumorigenesis, which suggests other VHL effector pathways are involved. Jade-1 protein interacts strongly with VHL and is most highly expressed in renal proximal tubules, precursor cells of renal cancer. Short-lived Jade-1 protein contains plant homeodomain (PHD) and candidate PEST degradation motifs and is substantially stabilized by VHL. The effect of VHL on Jade-1 protein abundance and relative protein stability was further examined in immunoblots and metabolic labeling experiments using two time points. VHL-Jade-1 binding was tested in coimmunoprecipitations. In cotransfection studies with wild-type VHL, the Jade-1 PHD-extended PHD module, not the candidate PEST domain, was required for full VHL-mediated stabilization. This module is also found in leukemia transcription factors AF10 and AF17, as well as closely related Jade-like proteins, which suggests all might be VHL regulated. Intriguingly, naturally occurring truncations and mutations of VHL affected wild-type Jade-1 binding and stabilization. Although the VHL $\beta$ domain was sufficient for Jade-1 binding, both the $\alpha$ and $\beta$ domains were required for Jade-1 stabilization. Thus, truncating VHL mutations, which are severe and associated with renal cancer development, prevented Jade-1 stabilization. Moreover, well-controlled cotransfection and metabolic labeling experiments revealed that VHL missense mutations that cause VHL disease without renal cancer, such as Tyr98His and Tyr112His, stabilized Jade-1 fully. In contrast, like the VHL truncations, VHL missense mutations commonly associated with renal cancer, such as Leu118Pro or Arg167Trp, did not stabilize Jade-1 fully. Therefore, loss of Jade-1 stability may correlate with renal cancer risk. Endogenous Jade-1 in stable renal cancer lines also exhibited VHL mutation-dependent regulation. As in the cotransfections, VHL truncations did not increase endogenous Jade-1 abundance, whereas the VHL missense mutations tested partially increased Jade-1 expression. Additional studies with non-PHD proteins indicated that Jade-1 stabilization by VHL is highly specific. Fibronectin was not stabilized like Jade-1 by VHL, nor were candidate VHL interactors from a yeast screen. Thus, protein stabilization likely reflects the biological activity of largely intact VHL protein on the PHD-extended PHD module of Jade-1. Disregulation of the VHL protein stabilization pathway or of Jade-1 itself may therefore contribute to VHL renal disease and renal cancer pathogenesis.

INTRODUCTION

von Hippel-Lindau (VHL) disease (OMIM:193300) is a familial cancer syndrome with three major manifestations: (a) pheochromocytoma; (b) hemangioblastoma; and (c) clear cell renal cancer. VHL disease subtypes are distinguished by these features. Type 1 VHL disease, comprising hemangioblastoma and renal cancer, is not associated with pheochromocytoma, whereas type 2 disease is typified by pheochromocytoma and further categorized. Type 2A families get pheochromocytoma and hemangioblastoma but not renal cancer, whereas type 2B patients get all three manifestations and type 2C only pheochromocytoma. With identification of the VHL gene (1) and mutation analysis (2–4), it has become apparent that specific VHL mutations cause predictable VHL disease phenotypes, perhaps by affecting subsets of VHL protein functions. Renal manifestations of VHL disease include premalignant renal cysts that progress to clear cell renal cancer. VHL is also lost in the majority of sporadic clear cell renal cancers (5–8), by far the most common type of renal cancer, making VHL the major renal tumor suppressor gene in adults.

The biochemical functions of the 213 residue VHL tumor suppressor are being elucidated. Substantial cellular VHL protein serves as the receptor component of an ElonginCB/Cul2/Rbx1 E3 ubiquitin ligase complex (9, 10). The VHL COOH-terminal $\alpha$ domain [amino acid (aa) 157–188; Ref. 11] directly binds Elongin C (12–14) and thereby the E3 ligase, whereas the VHL $\beta$ domain (aa 63–154, 193–204; Ref. 11) binds substrates for ubiquitination (see Fig. 3C VHL schematic). Identification of the hypoxia-inducible factor (HIF) $\alpha$ transcription factors as key targets for VHL-mediated ubiquitination has fundamentally advanced understanding of VHL disease and hypoxic control of gene expression (15–19). VHL loss increases expression of hypoxia-inducible angiogenic factors vascular endothelial growth factor, transforming growth factor-$\alpha$, transforming growth factor-$\beta$, and PDGF B chain (20–25). Accordingly, VHL mutations that disrupt normal HIF $\alpha$ processing are precisely those that cause the vascular hemangioblastoma lesions (16, 18, 26, 27). In contrast, clear cell renal cancer pathogenesis is more complex. All tested VHL mutations that cause renal cancer block HIF $\alpha$ degradation (16, 18, 26), and HIF $\alpha$ contributes to renal cancer growth (28, 29), supporting the critical importance of this pathway. However, VHL disease type 2A mutations Tyr98His (Y98H) or Tyr112His (Y112H), which do not typically cause renal cancer, also disrupt HIF $\alpha$ processing (16, 18, 26). This combination of clinical and biochemical data suggests that HIF $\alpha$ overexpression is necessary but not sufficient for renal tumorigenesis. Thus, disruption of a non-HIF pathway is likely required for development of VHL renal disease and sporadic renal cancer (30, 31).

The Jade-1 gene (gene for Apoptosis and Differentiation in Epithelia) and protein were identified using an approach capable of detecting growth inhibitory VHL interactors important in renal cancer (32). Jade-1 protein strongly binds VHL and is most highly expressed in the precursor cells of clear cell renal cancer, renal proximal tubule cells. The VHL-Jade-1 interaction has also revealed a new VHL protein function. Unlike many VHL interactors that are degraded by VHL-mediated ubiquitination (16–19, 33–35), short-lived Jade-1 protein is substantially stabilized by VHL. Jade-1 protein contains an NH$_2$-terminal candidate PEST degradation sequence and 2 plant homeodomains (PHDs; Ref. 36), also known as leukemia-associated protein (LAP; Ref. 37) or trithorax consensus domains (38), which are Cys$^+$HisCys$^+$-type zinc fingers. More than 200 human PHD proteins have been identified (SMART database). The PHD is a well-recognized protein–protein interaction motif (39, 40) and may have E3 ubiquitin ligase activity (41). In support of its importance, the domain...
is mutated or lost as the pathogenic event in several human diseases (42–44), particularly in the acute leukemias (reviewed in Ref. 45).

We have further characterized the VHL-Jade-1 interaction and VHL-mediated Jade-1 stabilization. Surprisingly, the Jade-1 PHD region, not the PEST domain, is critical for both VHL binding and VHL-mediated stabilization, although these VHL activities are distinct. Furthermore, VHL defects alter Jade-1 stability, and Jade-1 destabilization by mutated VHL may even correlate with the risk of renal cancer development. These studies support the potential importance of VHL-mediated protein stabilization and the VHL-Jade-1 relationship in VHL disease and renal cancer pathogenesis.

MATERIALS AND METHODS

Constructs. pFLAG-CMV2 wild-type VHL and VHL truncation and mutation constructs were described previously (46) or generated by PCR cloning and Quik-Change site-directed mutagenesis (Stratagene). Derived from 213 aa full-length VHL, the truncation constructs contain VHL aa 1–175, 1–143, 1–115, or aa 1–213 without aa 96–122 (del96–122), whereas missense mutation constructs contain VHL aa 1–213 with substitutions Gly93Asp (G93D), Y98H, Y112H, Leu118Pro (L118P), or Arg167Trp (R167W), as schematized in Fig. 3C. Jade-1 expression vectors and deletions were described previously (32) and are schematized in Fig. 1C. Jade-1 deletion 1 (del1) contains aa 202–509, deletion 2 (del2) aa 372–509, and double PHD deletion (dd) aa 1–201, 254–311, and 372–509. Jade-1 coding sequence was also subcloned into pcDNA3.1 (Invitrogen) to generate an untagged Jade-1 expression vector. A green fluorescent protein-tagged fibronectin expression vector (pAIPFN-green fluorescent protein) was generously provided by Drs. T. Ohashi and H. Erickson (Duke University; Ref. 47). Clones 6 and 12 and others were obtained from the same human VHL yeast two-hybrid screen of an adult human kidney library (Clontech) that identified Jade-1 (32). These pB42AD library clones (Clontech) were subcloned into pFLAG-CMV2 or pc3.1.unl-hemagglutinin, as described (32).

Cell Lines and Transfections. 293T17 cells grown in DMEM with 10% fetal bovine serum with penicillin and streptomycin were transfected using Lipofectamine 2000 according to manufacturer’s instructions (Life Technologies, Inc.). 786-O renal cancer cells (American Type Culture Collection) were grown in RPMI medium (Life Technologies, Inc.) with the above additives. Clonal 786-O stable lines generated by Lipofectamine 2000 transfection were grown in the above medium supplemented with 0.3 mg/ml G418 (Life Technologies, Inc.). To examine effects of cotransfected VHL on expression of Jade-1 and other expression vectors, 293T17 cells were transfected with pFLAG-CMV2 vector (2 μg) expressing wild-type or mutated VHL or no insert and another expression vector (2 μg), such as pFLAG-CMV2 or pc3.1.unl-hemagglutinin containing Jade-1, library clones, or no insert, as performed previously (32). Experiments were performed in replicate to confirm reproducibility.

Immunoprecipitation and Western Blotting. Immunoprecipitations and Western blots using Jade-1 rabbit antisera were performed as described previously (32). Jade-1 antisera was used at 1:200 dilution in Western blots. Jade-1 antisera (8 μl) was used for each immunoprecipitation. VHL monoclonal antibody was from BD PharMingen. Fibronectin goat antisera was generously provided by Dr. R. Lafyatis (Boston University School of Medicine; Ref. 48). Monoclonal FLAG antibody and hemagglutinin antisera were generously provided by Dr. R. Lafyatis (Boston University School of Medicine; Ref. 48). Monoclonal antibody was from BD PharMingen. Fibronectin goat antiserum was obtained from Cappel (Organics). Jade-1 antiserum (8 μg/ml) was used at 1:200 dilution in Western blots. Jade-1 expression vectors and deletions were described previously (32). Jade-1 antiserum was used at 1:200 dilution in Western blots. Jade-1 major bands are indicated by an asterisk. Corresponding cotransfected VHL expression is shown in the bottom panels. Representative result of three experiments is shown. In B, cotransfected 293T17 cells were metabolically labeled with a 35S-met and -cys pulse and chased for times shown. Jade-1 immunoprecipitations were performed, followed by SDS-PAGE and autoradiography. Jade-1 constructs are described in A legend and schematized in C. Common precipitated VHL is reported as VHL binding in C. Densitometry values for each Jade-1 truncation at the 0- and 2-h chase time points were used to estimate Jade-1 protein half-life and the effect of VHL, as reported in C. Representative result of three experiments is shown. C. Summary of VHL binding and effects on Jade-1 truncation half-life from three metabolic labeling immunoprecipitation experiments, represented in B. Fold stabilization by VHL reflects the Jade-1 protein half-life with cotransfected VHL divided by the half-life without cotransfected VHL. Errors = 1 SD. *P < 0.05 versus full-length Jade-1.

RESULTS

The Jade-1 PHD-Extended PHD Region Is Most Important for VHL-Mediated Stabilization and VHL Binding. Jade-1 protein has several recognizable motifs. The most characteristic Jade-1 domains are the two mid-molecule PHD regions, extending from aa 206 to 250 (PHD1) and from 315 to 368 (PHD2). Moreover, the inter-PHD region and second PHD together are highly homologous to the extended PHD motif described in the AF10 leukemia protein (49) and may therefore fully encompass aa 258–368. Thus, the entire region from aa 206 to 368 may be considered a PHD-extended PHD module (Fig. 2). Jade-1 also has a candidate PEST degradation susceptibility region from aa 5 to 28 (32), as suggested by PESTfind2 (50, 51), but no additional consensus domains.

2 Internet address: http://emb1.bcc.univie.ac.at/embnet/tools/bio/PESTfind.
To determine the Jade-1 regions most important for VHL-depend-ent Jade-1 stabilization, Jade-1 truncation constructs were transiently transfected with VHL, followed by Western blotting for cotransfected Jade-1 abundance. The Jade-1 deletion constructs were described previously (32) and are schematized in Fig. 1C. Three days after cotransfection, VHL strongly increased abundance of both wild-type Jade-1 and Jade-1 del1, which lacks the candidate PEST domain and retains the complete PHD-extended PHD region (Fig. 1A). Jade-1 del1 therefore responds to VHL as well as full-length Jade-1. In contrast, VHL only modestly increased abundance of the Jade-1 double PHD deletion truncation dd. Because VHL typically decreases expression from a cotransfected Sp1-dependent promoter in a plasmid expression vector (23), this result supports a VHL effect on Jade-1 dd abundance. Accordingly, VHL decreased abundance of Jade-1 del2, which lacks the entire PHD-extended PHD region. The correct trans-fected Jade-1 truncation band identified by the arrowheads corre-sponds to the predicted truncated protein size and was also confirmed in side-by-side comparisons with untransfected and vector-transfected cells (data not shown). Thus, an intact Jade-1 PHD-extended PHD region, not the candidate PEST motif, is essential for stabilization by VHL.

To further test the role of the Jade-1 PHD-extended PHD module in protein stabilization by VHL, pulse-chase metabolic labeling studies were performed. 293T17 cell cotransfections were followed by met-abolic labeling and Jade-1 immunoprecipitations (32). The half-life of each Jade-1 protein truncation was estimated by linear regression of Jade-1 densitometry data from the 0- and 2-h chase time points (32). Truncated Jade-1 protein half-life was compared with and without cotransfected VHL. A representative experiment is shown in Fig. 1B. Strong intensity of the immunoprecipitated Jade-1 signal at 2 h with VHL versus without VHL for both full-length Jade-1 and Jade-1 del1 suggests substantial VHL-mediated stabilization. In contrast, the Jade-1 del2 signal shows no increment at 2 h with VHL, suggesting no stabilization occurred. As summarized in Fig. 1C, densitometry and linear regression analysis indicate that VHL does increase the half-lives of full-length Jade-1 and Jade-1 del1 comparably by $\geq$3-fold. These results are similar to values reported previously for full-length Jade-1 (32). In contrast, VHL had little effect or perhaps slightly reduced the half-life of Jade-1 del2, which also did not bind VHL (Fig. 1B). VHL had an intermediate effect on Jade-1 dd, increasing its half-life by only 1.6-fold. These results are consistent with VHL effects on abundance of the Jade-1 truncations shown in Fig. 1A. Moreover, they strongly support the role of the Jade-1 PHD-extended PHD region in VHL-mediated stabilization. Fig. 1C also summarizes VHL-Jade-1 binding data from the labeled coimmunoprecipitation studies, represented in Fig. 1B, which further suggest that the Jade-1 PHD-extended PHD region is also critical for the VHL interaction. The Jade-1 PHD-extended PHD module is compared by ClustalW analysis with several human proteins identified by BLAST search in Fig. 2. Multiple PHD-extended PHD proteins may therefore have the potential to interact functionally with VHL.

VHL-Mediated Jade-1 Stabilization Is Distinct from VHL-Jade-1 Binding. In addition to determining the Jade-1 domain responsible for VHL-mediated stabilization, we also examined whether VHL defects alter Jade-1 stability and whether Jade-1 stabilization is simply a function of VHL binding. To address these questions, several VHL truncations (Fig. 3C) were cotransfected with full-length Jade-1 and tested for binding in coimmunoprecipitation experiments. As shown in Fig. 3A, wild-type VHL, as well as VHL truncations 1–143 and 1–175, showed substantial interaction with endogenous Jade-1. In contrast, a VHL truncation lacking the hydrophobic core region of the substrate-binding $\beta$ domain (VHL del96–122) showed greatly diminished interaction with Jade-1. In the VHL del96–122 construct, the $\alpha$ domain is fully intact. Immunoprecipitations in the reverse direction gave similar results (data not shown). Thus, the VHL $\beta$ domain is most important for Jade-1 binding, whereas the $\alpha$ domain does not substantially contribute.

To test the effect of these VHL truncations on Jade-1 stabilization, the VHL truncation or vector and Jade-1 constructs were cotrans-ferred in 293T17 cells. Three days after cotransfection, Jade-1 abundance was assessed by Jade-1 Western analysis (Fig. 3B). In compar-ison with wild-type VHL and empty vector, none of the VHL truncations substantially increased Jade-1 abundance. Even the VHL 1–175 truncation, which contains an intact $\beta$ domain hydrophobic core and much of the $\alpha$ domain, did not appreciably increase Jade-1 abundance. Thus, Jade-1 stabilization is not the simple consequence of VHL binding but likely reflects a biological activity of largely
VHL mutation-dependent control of Jade-1 through PHDs

intact VHL protein. Jade-1 stabilization was therefore used as a functional assay of more subtle VHL defects.

Control of Jade-1 Stability Depends on the Type of VHL Mutation. As a sensitive, quantitative assay of Jade-1 protein half-life in response to VHL protein harboring truncation or missense mutations, pulse-chase metabolic labeling studies were performed with cotransfected 293T17 cells. As described above, immunoprecipitated Jade-1 was visualized by SDS-PAGE and autoradiography. Jade-1 protein half-life in response to different VHL constructs was estimated by densitometry and linear regression. B, summary of mutated and wild-type VHL. Each experiment was performed in triplicate, and error bars represented 1 SD. *, P < 0.05 versus wild-type VHL. C, equivalent expression of the VHL truncation and mutation construct schematics showing defects relative to the α and β domains and β domain core.

VHL, supporting the notion that the VHL truncations do not augment Jade-1 half-life. This result is consistent with the effect of the VHL truncations on the abundance of cotransfected Jade-1 protein (Fig. 3B). Moreover, considerable coimmunoprecipitated VHL 1–175 is evident at the bottom of the autoradiograph, as observed with the concomitant truncations in Fig. 3A. VHL 1–143 has run off this protein gel. Similarly, VHL del96–122 did not increase immunoprecipitated, labeled Jade-1 at the 1-h chase time. Only a minimal amount of VHL del96–122 was coimmunoprecipitated. Thus, these VHL truncation experiments further support the assertion that VHL binding is not sufficient for Jade-1 stabilization and that both VHL α and β domains are required.

Jade-1 is most highly expressed in renal proximal tubule cells (32), which are precursor cells of clear cell renal cancer. Therefore, sporadic clear cell renal cancer and the renal manifestations of VHL disease might be the most relevant settings to explore the VHL-Jade-1 relationship. A small number of VHL missense mutations has been identified that give rise to VHL disease but confer little risk of renal disease might be the most relevant settings to explore the VHL-Jade-1 relationship. A small number of VHL missense mutations has been identified that give rise to VHL disease but confer little risk of renal cancer.
manifestations, such as type 2A mutations Y98H and Y112H. These may be less severe mutations than those commonly associated with renal cancer, such as VHL truncations or more critical VHL missense mutations. Using stabilization of labeled Jade-1 as an assay, we have compared VHL mutations that either confer (VHL L118P or R167W) or would be expected to confer (VHL truncations) renal cancer risk with VHL mutations that are uncommonly associated with renal cancer, such as Y98H or Y112H (Fig. 3C). As shown in Fig. 4A, the VHL Y98H and Y112H mutations were able to increase immunoprecipitable labeled Jade-1 comparably with wild-type VHL at the 1-h chase time point, whereas VHL L118P or R167W or the VHL truncations were less effective. Each missense-mutated VHL protein coimmunoprecipitated well with Jade-1, indicating again that Jade-1 binding and stabilization are distinct VHL activities. Jade-1 protein half-life in the presence of each VHL construct was quantitated by linear regression. Summary results of four replicate experiments are shown in Fig. 4B. VHL truncations and the R167W missense mutation showed little ability to stabilize Jade-1 more than empty vector, whereas VHL Y98H or Y112H stabilized Jade-1 to the same level as wild-type VHL, and L118P had an intermediate effect that was still significantly different from wild-type VHL or the Y98H and Y112H constructs. Protein expression from each transfected pFLAG-CMV2 VHL construct was comparable (Fig. 4C). Thus, Jade-1 stabilization is altered by VHL defects and further influenced by the specific type of VHL mutation. Loss of Jade-1 stabilization by VHL therefore has the potential to correlate with VHL renal disease risk.

To test the effect of these VHL mutations on endogenous Jade-1 protein, several clonal 786-O renal cancer cell lines stably expressing wild-type or mutated VHL were examined for Jade-1 expression by Western analysis. Cells were harvested at early confluence. As shown in Fig. 5A, Jade-1 expression was comparably low in the vector and VHL 1–143 and 1–175 truncation lines. VHL del96–122 and 1–115 lines had similarly low endogenous Jade-1 levels (data not shown). Jade-1 expression was high in the wild-type VHL lines, as reported previously (32), whereas expression was intermediate and comparable in each of the VHL missense lines G93D, Y98H, L118P, and R167W. Equal protein loading and transfer was confirmed by Ponceau S staining of the membrane (data not shown) and is supported by expression levels of nonspecific protein bands (Fig. 5A). Moreover, the identification of the correct Jade-1 band was confirmed in comparison with transfected, untagged Jade-1 (data not shown). Expression of stably transfected VHL in each of these lines is shown in Fig. 5B. Endogenous Jade-1 expression is not appreciably affected by the amount of VHL expressed, because the high and moderate wild-type VHL-expressing lines have similar Jade-1 protein levels. In summary, Jade-1 expression is substantially affected by type of VHL mutation. These findings are also in large part supportive of the VHL mutation effects on Jade-1 protein half-life in cotransfection studies (Fig. 4, A and B). At the minimum, VHL truncation mutations exert a different activity from VHL missense mutations with regard to Jade-1 stability.

VHL Specifically Augments Expression of Cotransfected Jade-1. Finally, the specificity of VHL for increasing Jade-1 protein abundance was further explored by testing another known VHL interactor, fibronectin (58), and also all confirmed VHL-interacting clones identified in a yeast two-hybrid screen (32). As performed above with Jade-1, expression vectors for the VHL interactors were cotransfected with either VHL or empty pFLAG-CMV2 in 293T17 cells. Three days after cotransfection, expression of the VHL interactor was tested by Western analysis to determine whether it was increased by VHL. All experiments were performed at least four times. Fibronectin was a strong candidate to be regulated similarly to Jade-1. Unlike other VHL interactors, fibronectin expression is augmented after reintroduction of VHL in renal cancer cells (58), as is Jade-1 (32). Moreover, Jade-1 and fibronectin colocalize with VHL in cytoplasmic speckles, suggesting both interactions might occur in endoplasmic reticulum or Golgi (32, 58, 59). Green fluorescent protein-fibronectin (47) was therefore cotransfected with VHL to determine effects on fibronectin protein abundance. VHL does not alter green fluorescent protein-fibronectin expression using this assay (Fig. 6A), suggesting that the mechanism by which VHL affects Jade-1 abundance is different from that of fibronectin. Furthermore, six clones from the VHL yeast two-hybrid screen that also bind VHL are also shown in Fig. 6A. VHL expression causes a significant increase in fibronectin protein abundance (Fig. 6B). Endogenous Jade-1 expression is not appreciably affected by the expression of fibronectin, as shown by Western blotting of fibronectin-immunoprecipitated proteins (Fig. 6C). These data support the hypothesis that VHL specifically augments protein expression of certain interactors, which is consistent with the hypothesis that VHL affects specific interactors but not others.
in mammalian cell cotransfection studies (data not shown) were tested using this same assay. None of these show evidence of VHL-mediated stabilization, as exemplified by clones 6 and 12 (Fig. 6, B and C, respectively). In fact, expression appears slightly diminished by VHL in all cases, as reported previously for VHL interactors Sp1 and protein kinase C ζ (32). In contrast, VHL substantially augments cotransfected Jade-1 expression regardless of the type of expression vector, or the presence, absence, or type of epitope tag at the NH₂ terminus (Fig. 6D). Thus, the VHL effect on Jade-1 protein expression is robust and highly specific.

DISCUSSION

Jade-1 is a short-lived, kidney-enriched candidate transcription factor that binds and is substantially stabilized by the VHL tumor suppressor (32). In addition to an NH₂-terminal candidate PEST degradation domain, M₆, 64,000 Jade-1 protein contains two adjacent mid-molecule zinc-binding regions known as PHD and extended PHD motifs. We have further characterized the VHL-Jade-1 relationship and made several new observations: (a) the Jade-1 PHD-extended PHD module is critical for the VHL-Jade-1 interaction, because it is required for binding and must be intact for full VHL-mediated stabilization; and (b) VHL truncations and mutations alter Jade-1 binding and stabilization. The hydrophobic core region of the VHL β domain is essential for the Jade-1 interaction. However, although VHL truncations with an intact β domain core bind Jade-1 well, they are unable to stabilize Jade-1. Jade-1 binding and stabilization are therefore distinct VHL activities, and stabilization requires both α and β VHL domains. Thus, truncating VHL mutations, which are severe defects associated with renal cancer, cannot stabilize Jade-1. In contrast, VHL missense mutations may retain full, partial, or no ability to stabilize Jade-1, perhaps depending on mutation severity. Therefore, control of Jade-1 stability is VHL mutation dependent. Moreover, on the basis of well-controlled cotransfection studies, VHL-mediated Jade-1 stabilization has the potential to correlate with VHL renal disease risk. Finally, Jade-1 is thus far the only interactor stabilized by the VHL tumor suppressor. Fibronectin, the best candidate to be regulated similarly (58), is not nor is any VHL interactor from a yeast two-hybrid screen. Nevertheless, Jade-1 may represent one example of a group of proteins that are stabilized by VHL. These studies raise the potential importance of VHL-mediated protein stabilization, as well as Jade-1 protein itself in VHL disease and renal cancer pathogenesis.

Intriguingly, the Jade-1 PHD-extended PHD module is most important for VHL-mediated stabilization. The extended leukemia-associated protein/PHD domain was first described in the leukemia protein AF10 as a conventional PHD finger preceded by a 55 aa extension containing 4 Cys/His residues (49). The complete extended PHD domain of AF10 therefore contains 12 canonical Cys/His residues over 115 aa total (see Fig. 2). The extended PHD motif was also found in mixed lineage leukemia protein MLL and in its Drosophila ortholog trithorax. In AF10, AF17, and BR140, as in Jade-1 and the closely related KIAA0239 and E9 polypeptides shown here in Fig. 2, the extended PHD is also immediately preceded by a conventional Cys₄His₃Cys₃-type PHD. Moreover, these regions are lost in the MLL-AF10 protein fusions that occur in acute leukemia chromosomal translocations (60), suggesting they are important and may be growth inhibitory. We have shown here that the Jade-1 PHD-extended PHD motif can bind VHL and must be intact for VHL-dependent protein stabilization. Thus, VHL might act similarly on this interesting group of PHD-extended PHD proteins.

The extended region of the Jade-1-extended PHD may have critical functional properties based on our observations. The extended region of AF10 likely mediates homomultimerization of the recombinant protein (49), suggesting this subdomain may represent a distinct protein interaction motif. Studies with the Jade-1 dd truncation, which has an intact extended region but no conventional PHDs, support and further advance this assertion. The Jade-1 dd truncation was sufficient for VHL binding and contributed to VHL-mediated stabilization. Moreover, the extended region also conferred susceptibility of Jade-1 to degradation. We have assessed the half-life of the Jade-1 truncations at baseline, in the absence of cotransfected VHL. The Jade-1 dd, as well as the del1 truncation, has the same 30–40-min half-life as full-length Jade-1 (data not shown). In contrast, the Jade-1 del2 truncation lacking the entire PHD-extended PHD region has a 54-min baseline half-life. Together, these observations suggest the extended region may destabilize Jade-1, in addition to promoting VHL interaction and stabilization. Thus, these studies support a unique functional role for the extended PHD finger and its extended region in particular.

Jade-1 protein stabilization by VHL is an intriguing phenomenon. Because stabilization is distinct from simple VHL-Jade-1 binding and requires an essentially intact VHL molecule, it likely reflects a biological activity of VHL, such as post-translational modification of Jade-1. VHL has been shown to augment Cul2 NEDDylation (61, 62), which might stabilize Cul2. However, VHL does not induce the M₆ 5,000 mobility shift of Jade-1 expected of NEDDylation, although a smaller modification is possible. Like ubiquitination, NEDDylation by VHL also requires the VHL/ElonginCB/Cul2 complex. The stoichiometry of our radiolabeled Jade-1 immunoprecipitations and VHL coinmunoprecipitations suggests VHL alone might be sufficient for Jade-1 stabilization. Furthermore, coinmunoprecipitation studies do not support a substantial Cul2-Jade-1 interaction (data not shown). Interestingly, VHL promotes microtubule stability, which occurs independently of Cul2 (63). Even a VHL truncation lacking the entire Elongin-binding α domain is able to fully stabilize microtubules (63). Thus, a precedent has been established and is likely extended here for biologically significant VHL function without ElonginCB/Cul2. Whether VHL protection against microtubule dem polymerization alters protein half-life, as occurs with Jade-1, remains unknown. The strict VHL β domain dependence of microtubule stabilization supports a mechanism that is somewhat different from Jade-1 stabilization. As a broad alternative hypothesis, VHL may inactivate a Jade-1 degradative pathway. In either setting, a group of proteins may be stabilized similarly by VHL. Nevertheless, the specificity of VHL for stabilizing Jade-1 is indeed high, because no other VHL interactor tested was so affected. Whether VHL stabilizes other PHD-extended PHD proteins remains to be determined. No clone tested contains these motifs, although several have zinc fingers. In addition, Jade-1 does not substantially stabilize VHL in reciprocal fashion. VHL stabilization does occur in response to ElonginCB binding (64, 65), which likely stabilizes the entire VHL/ElonginCB/Cul2 complex. Although the mechanisms may prove different, VHL-mediated protein stabilization may therefore be important in several contexts.

Surprisingly, the candidate PEST domain plays no role in VHL-mediated stabilization nor in general destabilization of Jade-1. The PESTfind algorithm is a superb tool for identifying candidate protein degradation susceptibility regions (50, 51). The Jade-1 aa 5–28 region has a high PESTfind score of +11.8, with scores > +5 considered noteworthy. No additional candidate PEST motifs were found in the full-length protein. However, as noted above, deletion of the candidate PEST region in the Jade-1 del1 construct did not prolong the baseline half-life in comparison with full-length Jade-1. Thus, the significance of the Jade-1 candidate PEST region is not clear and requires further study.

Major VHL defects have profound effects on Jade-1 stability. Strikingly, the VHL truncations were completely unable to augment...
endogenous Jade-1 expression or to stabilize the protein. These constructs were useful for testing the respective contributions of the VHL α and β domains and, moreover, represent naturally occurring VHL truncating mutations. Specifically, the VHL 1–143 truncation has no α domain but retains the β domain core. The 1–175 truncation includes sufficient α domain to bind the Elongins (12) and β domain core but has no COOH-terminal β domain. The del96–122 truncation has an intact α domain and COOH-terminal β domain region but no β domain core. For completeness, the R167W mutation affects only the α domain, leaving the β domain completely intact. The VHL 1–143 and 1–175 and R167W mutations bound Jade-1 strongly, whereas del96–122 did not. Because none of these defects permitted Jade-1 stabilization, we were able to conclude that both α and β domains are required for Jade-1 stabilization. Naturally occurring truncating VHL defects include frameshifts, stop codons, or genetic deletions, as well as splicing defects, which delete VHL aa 114–154 comprising exon 2 (2–7). These defects typically give rise to type I VHL disease and occur commonly in sporadic renal cancer. Moreover, frameshifts leaving VHL aa 1–143 or 1–175 precisely intact are described in renal cancer (6) and are therefore well represented here. Overall, truncating VHL mutations confer the highest renal cancer risk in VHL disease, which may reach 70% (66). Thus, these high-risk VHL mutations have a clear-cut effect on Jade-1 stability.

VHL missense mutations have more subtle effects on Jade-1 stability, perhaps as expected. Overall, VHL missense mutations confer a lower risk of renal cancer than truncations. We chose the particular missense mutations used here because they all cause VHL disease but differ substantially in their renal disease risk. VHL R167W is one of the commonest VHL disease mutations and has a moderate to high VHL renal disease risk, because 19 of 60 (32%) affected VHL patients were reported with renal cancer (3, 66, 67). The VHL L118P mutation is an uncommon but severe mutation associated with type 1 disease (4) and a high renal cancer risk, because 2 of 2 reported VHL cases had renal cancer (66, 67). Moreover, both VHL L118P and R167W mutations have been found in sporadic renal cancer (6). In sharp contrast, Y98H and Y112H mutations cause largely type 2A disease, which is not associated with renal manifestations. Thus, only 4 of 171 (2.3%) reported Y98H VHL disease patients had renal cancer (3, 68), whereas 0 of 25 Y112H VHL disease patients had renal manifestations (4, 69). Two of 8 reported VHL G93D patients had renal cancer (3, 66). What is particularly intriguing about our results is that the VHL Y98H and VHL Y112H mutations stabilize Jade-1 fully in cotransfections. In contrast, much like the VHL truncations, the VHL L118P and VHL R167W mutations were unable to fully stabilize Jade-1. This well-controlled data set, in conjunction with the truncation result, suggests that loss of Jade-1 stabilizing ability by VHL is associated with an increased renal cancer risk. This observation is important because no VHL protein interaction or function has shown a potential relationship with renal cancer risk, in particular regarding VHL disease type 2A mutations. The effect of the missense mutations on Jade-1 expression in vivo was less clear cut than the truncations but was still interesting. VHL Y98H, L118P, G93D, and R167W all acted similarly and increased endogenous Jade-1 to an intermediate level between the truncations and wild-type VHL. In addition, VHL Y98H increased Jade-1 expression fully only in cotransfection experiments. In comparing approaches, however, one might propose that the cotransfection studies are better controlled, because the starting cellular material is identical with each VHL mutation construct tested. In contrast, the clonal stable lines have arisen from a single cell with unclear baseline Jade-1 levels. This may also explain the absence of a VHL dosage effect on endogenous Jade-1 expression. Alternatively, a small amount of VHL may be adequate for stabilizing endogenous Jade-1, because the amount of Jade-1 and frequency of the VHL-Jade-1 interaction are low (32). We have not tested many more VHL missense mutations because of the challenge of comparing these approaches. At the minimum, the results presented here indicate that control of Jade-1 expression is VHL mutation dependent. Further proof supporting a role for Jade-1 in renal cancer will come from more detailed studies identifying a Jade-1 function or post-translational modification affected by different types of VHL mutations. Moreover, this analysis will also require examining affected tissues from patients with VHL disease and specific familial VHL mutations. Clearly, this work does not support a causal role for Jade-1 in renal cancer. The outlined studies merely support a potential correlation of loss of Jade-1 protein stabilization with VHL renal disease risk.

In summary, the Jade-1 PHD-extended PHD region is required for VHL binding and VHL-mediated stabilization, which suggests homologous proteins may therefore be similarly regulated by VHL. Jade-1 stabilization also requires a largely intact VHL protein, because major disruptions of either VHL α or β domain prevent this activity. Biological activity of VHL is therefore likely required for Jade-1 stabilization. Loss of Jade-1 stabilization is also VHL mutation dependent and may even correlate with renal cancer risk. Thus, these studies support the potential importance of Jade-1 and VHL-mediated protein stabilization in the pathogenesis of VHL renal disease and sporadic clear cell renal cancer. Further study of Jade-1 protein in renal cancer is therefore an essential effort.

ACKNOWLEDGMENTS

We thank Drs. D. Salant and S. Thiagalingam for critical reading of this manuscript and Drs. H. Erickson, R. Lafaytis, and T. Ohashi for reagents.

REFERENCES


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*Cancer Res* 2004;64:1278-1286.

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