The von Hippel-Lindau Tumor Suppressor Stabilizes Novel Plant Homeodomain Protein Jade-1*

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The von Hippel-Lindau disease gene (VHL) is the causative gene for most adult renal cancers. However, the mechanism by which VHL protein functions as a renal tumor suppressor remains largely unknown. To identify low occupancy VHL protein partners with potential relevance to renal cancer, we screened a human kidney library against human VHL p30 using a yeast two-hybrid approach. Jade-1 (gene for Apoptosis and Differentiation in Epithelia) encodes a previously uncharacterized 64-kDa protein that interacts strongly with VHL protein and is most highly expressed in kidney. Jade-1 protein is short-lived and contains a candidate destabilizing (PEST) motif and plant homeodomains that are not required for the VHL interaction. Jade-1 is abundant in proximal tubule cells, which are clear-cell renal cancer precursors, and expression increases with differentiation. Jade-1 is expressed in cytoplasm and the nucleus diffusely and in speckles, where it partly colocalizes with VHL. VHL reintroduction into renal cancer cells increases endogenous Jade-1 protein abundance up to 10-fold. Furthermore, VHL increases Jade-1 protein half-life up to 3-fold. Thus, direct protein stabilization is identified as a new VHL function. Moreover, Jade-1 protein represents a novel candidate regulatory factor in VHL-mediated renal tumor suppression.

VHL gene defects are responsible for both von Hippel-Lindau disease (1) and most sporadic clear-cell renal cancers (2–5). VHL is therefore the most commonly affected renal cancer gene. Clear-cell renal cancer is also the most malignant VHL disease lesion, which suggests VHL protein exerts strongest tumor suppressor activity in renal proximal tubules, which are the precursor cells of this common malignancy.

VHL disease manifestations, which include retinal angiomata; central nervous system hemangioblastomas; renal, pancreatic, and epididymal cysts; and pheochromocytomas, pancreatic neuroendocrine tumors, and clear-cell renal cancers, suggest VHL protein has multiple functions (6). VHL binds and promotes ubiquitination of hypoxia-inducible transcription factors HIF-1α and HIF-2α (7), protein kinase C (PKC) lambda (8), heterogeneous nuclear ribonucleoprotein A2 (9), and VHL-interacting deubiquitinating enzyme-1 (VDU1) (10). VHL inhibits transcription elongation (11–14), mRNA stability (9, 15–17), Sp1-related promoter activity (18, 19), and PKC activity (8, 20, 21). VHL also increases abundance of the directly interacting protein fibronectin and promotes its incorporation into extracellular matrix (22). VHL induces morphogenesis, cellular differentiation, and contact inhibition of renal cancer cells or proximal tubule cells (23–27). Like the retinoblastoma tumor suppressor, VHL inhibits apoptosis, particularly in response to cell stresses, such as serum depletion (28), glucose depletion, endoplasmic reticulum (ER) stress (29), or UV irradiation (30). VHL functional heterogeneity is further supported by its residence in cytoplasm (31), the nucleus (32), mitochondria (33), ER (34), and perhaps Golgi (32).

VHL functions most important for renal tumor suppression remain unclear. Based on the well-recognized association of specific VHL mutations with partial VHL disease phenotypes, a likely hypothesis is that VHL missense mutations may disrupt some, but not necessarily all, VHL functional pathways. For example, VHL mutations that prevent HIF ubiquitination and therefore promote HIF and vascular endothelial growth factor overexpression are precisely those that correlate with hemangioblastoma development (35–38). Although HIF overexpression contributes importantly to renal cancer pathogenesis (39, 40), no VHL biochemical function or protein interaction has been found that correlates with renal cancer risk, leaving unresolved the full role of VHL in renal tumor suppression (41).

To identify molecules potentially important in the pathogenesis of clear-cell renal cancer, we screened an adult human kidney library with human VHL p30 using a yeast two-hybrid approach. We have named the gene encoding a novel, strong VHL-interactor as Jade-1 (gene for Apoptosis and Differentiation in Epithelia). Jade-1 protein is short-lived and most highly expressed in kidney. It contains a candidate PEST degradation domain and plant homeodomain (PHD) motifs, which are not required for the VHL interaction. Jade-1 protein is directly stabilized by VHL protein, which is a new VHL function. Preliminary results suggest Jade-1 is also growth suppressive. Jade-1 may therefore participate in VHL-mediated renal tumor suppression.
**EXPERIMENTAL PROCEDURES**

**Constructs—** Plasmids pFLAG-cytomegalovirus (CMV)-2 VHL and derivatives have been described previously (18, 19). pRc-hemagglutinin (HA)-VHL (42), pFLAG-p53 (43), and pFLAG-FKC ζ were generously provided by Drs. W. Kaelin (Dana-Farber Cancer Institute), U. Moll (Stony Brook), and A. Toker (Boston Biomedical Research Institute), respectively. VHL nucleotide (nt) sequence encoding amino acids (aa) 2–213 and 55–143 was PCR-cloned into pGilda (CLONTECH) using EcoRI and SacI sites. A mouse VHL cDNA clone was generously provided by Dr. M. Lerman (NCI, National Institutes of Health, Frederick, MD) and subcloned similarly into pGilda. The Jade-1 5'-untranslated region and coding sequence were cut from pB42AD with NotI and XhoI and subcloned into NotI and SacI cut pFLAG-CMV2. The Jade-1 complete coding sequence (aa 1–509) and truncations (deletion 1 (del1), aa 220–213 and 55–143 was PCR-cloned into pGilda (CLONTECH) using aa 1–201, 254–311, 372–509 (see Fig. 1, A and B) were PCR-amplified and inserted using NotI and XhoI into pcDNA3.1 (Invitrogen) as an untagged expression vector and into pcRS3.1 uni (Invitrogen) that had been modified to contain an HA tag. Deletion of the Jade-1 PHD region was performed by recombinant PCR. Additional details about plasmid constructs can be obtained from the authors.

** Yeast Two-hybrid Analysis—** An adult human kidney cDNA library in pB42AD (CLONTECH) was screened against human VHL aa 2–213 in the LexA-expressing, inducible yeast expression vector pGilda (CLONTECH), according to the manufacturer’s instructions (19). pB42AD library clones initially positive by growth on deficient medium and by X-gal staining were rescued and individually screened against both human and mouse VHL in pGilda in yeast before sequencing. Interaction of VHL with a candidate interactor peptide was confirmed by the amount of redirected co-transformation required for yeast colonies to appear blue on X-gal plates. Clones positive at 24 h were designated as the strongest (3+) interactors. Those positive at 48 or 72 h were designated as 2+ or 1+ interactors, respectively.

**Cell Lines and Transfection—** 293T17 human embryonic kidney cells and HT1080 fibrosarcoma cells were generously provided by Drs. Z. Luo (Boston University School of Medicine) and R. Widom (Boston University School of Medicine), respectively. 786-O renal cancer cells and HeLa cells were obtained from American Type Culture Collection (Manassas, VA). These lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, glutamine, and penicillin-streptomycin (Invitrogen). HA-VHL 786-O (42) and A498 renal cancer lines (44) were generously provided by Drs. W. Kaelin (Dana-Farber Cancer Institute) and W. Krek (Friedrich Miescher Institut, Basel), respectively. 786-O (18, 19) and UMRMC 45 (46) stably-transfected renal cancer lines were described previously. Stable renal cancer lines were maintained as above in 0.2–0.4 mg/ml G418 (Invitrogen). SV40 T antigen-transformed mouse proximal tubule (MPT) cells, generously provided by Dr. M. Loomis-Adham (University of Utah) (46), were grown in the above medium supplemented with 5 units/ml interferon-gamma at 33 °C. MPT cells were differentiated without interferon-gamma at 37 °C for 10 days. Primary culture mouse proximal tubule cells were generously provided by Dr. W. Lieberthal (Boston University School of Medicine) (47). Cells were transfected using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions or by calcium phosphate precipitation (48).

**Antibodies—** To generate Jade-1 antisera, 2 New Zealand White rabbits were injected with the same carboxyl-terminal 20 residue Jade-1 peptide amino terminally linked to keyhole limpet hemocyanin. Anti-serum 1 was affinity-purified using the same peptide coupled to Sepha-rose (Alpha Diagnostics International, San Antonio, TX). Human VHL antisera was generously provided by Dr. R. Burk (Albert Einstein College of Medicine, Bronx, NY) (34). Human VHL monoclonal antibody and VHL M5 monoclonal antibody were from Pharmingen and Sigma, respectively. Sp1, glutathione S-transferase (GST), and HA antisera were from Santa Cruz Biotechnology, as were fluorescein isothiocya- nate (FITC)- and cyamine-3 (cy3)-tagged anti-rabbit and anti-mouse secondary antibodies. Horseradish peroxidase-linked anti-rabbit and anti-mouse IgGs were from Bio-Rad.

**Immunoprecipitation and Western Blotting—** Cultured cells were lysed in lysis buffer (pH 7.4, NaCl 150 mM, EDTA 30 mM, Triton X-100 0.5%), with Complete protease inhibitor (Roche Molecular Biochemicals), precleared with protein A-agarose bead (Santa Cruz Biotechnology), and mixed with antibody at excess (15, 19). Complexes were pelleted with protein A-agarose, washed with lysis buffer, eluted with sample buffer, and analyzed by SDS-PAGE. Immunoblotting was performed as previously described using the antibodies above. For multiissue Western analysis, tissues were minced and homogenized with a Teflon pestle in lysis or KETN buffers (100 mM KCl, 1 mM EDTA, 10 mM Tris, pH 7.5, 0.1% Nonidet P-40) containing protease inhibitor. Human tissue lysates were obtained from Genotech (St. Louis, MO). Relative intensities of positive bands were assessed by densitometry using Image 1.62 (National Institutes of Health). Backgrounds were subtracted to assign densitometric values.

**Northern Analysis—** Total RNA was prepared using RNeasy (Tel-Test) according to the manufacturer’s instructions. A multitissue Northern blot was probed as recommended (CLONTECH) using a 1-kb 5’ Jade-1 coding sequence fragment. Northern analysis was otherwise performed as described earlier (19).

**Metabolic Labeling—** Cells were grown on Chamber slides (Lab-Tek) and fixed with 1:1 methanol and acetone for 2 min at room temperature. Immunodetection was performed with affinity-purified anti-Jade-1 serum or VHL monoclonal antibody followed by FITC- or cy3-conjugated anti-rabbit or anti-mouse antibodies. Images were obtained by fluorescence microscopy (Nikon Optiphot) and digital imaging (RT Color camera, Diagnostic Instruments).

**RESULTS**

The **Jade-1 Gene and Protein**—The human Jade-1 gene was identified as a particularly strong VHL-interacting clone in a yeast two-hybrid screen of human VHL p30. VHL is a low abundance intracellular protein that resides in multiple subcellular compartments (22, 31–34). Thus, the yeast screen was performed to identify potentially rare or short-lived VHL protein interactions. An adult human kidney library was chosen because half of the mass of an adult human kidney is composed of proximal tubule cells. One million library clones in pB42AD yeast expression vector were screened against human VHL aa 2–213 in LexA-expressing, inducible yeast expression vector pGilda. Positive yeast colonies were confirmed by restreaking under double-selection conditions. Library clones rescued from confirmed positive colonies were tested individually for interaction with human VHL aa 2–213 and 55–143 as well as full-length mouse VHL in pGilda. VHL aa 55–143 were chosen as a minimum substrate-binding VHL beta domain. Jade-1 was one of 14 strongest interactors (3+) in yeast of 40 different genes identified, because VHL-Jade-1 cotransformed colonies appeared blue at 24 h of incubation. Jade-1 also interacted convincingly with human VHL aa 55–143 (3+) as well as mouse VHL (2+) in yeast. VDU1 (10) was also recovered in the screen, supporting the validity of this approach for identifying bona fide VHL interactors. Additional confirmed positive interactors in yeast include four other novel genes, 17 known genes encoding a wide range of proteins, and 14 known genes encoding proteins of unknown function, all of which are common false-positives. As reported in another screen, VHL-binding protein-1 (3+) and filamin (2+) were also recovered (49), whereas other known VHL-interacting proteins were not.

GenBank™ BLAST searches indicate that the Jade-1 library clone contains the complete coding sequence of a novel, single gene (Fig. 1A). The 3570-nt clone was found in-frame with a Teflon pestle in lysis or KETN buffers (100 mM KCl, 1 mM EDTA, 10 mM Tris, pH 7.5, 0.1% Nonidet P-40) containing protease inhibitor. Human tissue lysates were obtained from Genotech (St. Louis, MO). Relative intensities of positive bands were assessed by densitometry using Image 1.62 (National Institutes of Health). Backgrounds were subtracted to assign densitometric values.
FIG. 1. Jade-1 cDNA clone and protein sequence (A) and schematic (B). A and B, the 3570-nucleotide library clone contains the complete Jade-1 coding sequence as well as the complete 3'-end and additional 5'-untranslated sequence. The 509-aa Jade-1 protein has two mid-molecule CadHC3 PHD domains (underlined, aa 202–253 and 312–371). An amino-terminal candidate PEST domain includes aa 5–28 (bold) (PESTfind). Candidate post-translational modifications are represented by enclosed aa and include N-glycosylation (□) and N-myristoylation (◦), as well as phosphorylation by kinases cAMP-dependent protein kinase (circle), PKC (horizontal oval), and CK2 (vertical oval), as identified by Prosite scanning. Jade-1 deletion 1 (del1) contains aa 202–509 and deletion 2 (del2) aa 371–509. Jade-1 double-PHD deletion (dd) contains aa 1–202, 254–311, and 372–509. The nucleotide and amino acid sequences have been given GenBank™ accession number AF520952.
with the B42 activation domain and has 178-nt 5’ untransla-
ted, 1527-nt coding, and 1831-nt 3’ untranslated sequence, as
well as a polyadenylation signal and poly(A)+ tail. The coding
sequence is followed by several stop codons (Fig. 1A).

The deduced Jade-1 509 aa sequence (Fig. 1A) has a pre-
dicted 58.4-kDa mass and 5.25 isoelectric pH. It has two con-
sensus mid-molecule PHDs (50), also known as leukemia-assoc-
ated protein (51) or trithorax consensus domains (52), which are
50- to 70-aa C4HC3 zinc-binding motifs (Fig. 1, A and B). Alter-
natively, the second PHD may represent an extended
PHD (53) and include aa 257–371. Jade-1 residues 5–28 com-
prise a strong candidate PEST domain (PESTfind score of +11)
(Fig. 1, A and B), which is a charged, unstructured region that
promotes susceptibility to degradation (54). Jade-1 has no signal
or transmembrane sequences. Candidate sites for N-glycosyla-
tion, myristoylation, and serine or threonine phosphorylation are
shown, based on Prosite analysis, although the patterns found
are short and not highly specific. No closely homologous proteins
have been characterized. However, a transcript called E9 was
identified in a differential screen of genes induced with apoposis
in a breast cancer line and is predicted to encode a closely related
PER- and PHD-containing protein (55).

To determine whether transfected Jade-1 encodes a protein of
the anticipated size, the protein coding sequence was sub-
cloned into CMV promoter-driven tagged and untagged expres-
sion vectors. A 64-kDa expressed protein is consistently seen in
Western blots of Jade-1 transfected but not untransfected or
control transfected cells (see Figs. 2A, 3A, and 3E), indicating
production of Jade-1 protein that is just larger than the pre-
dicted 58.4-kDa molecular mass. Jade-1 may therefore be post-
translationally modified.

To confirm the identity of endogenous Jade-1 protein, immu-
noprecipitability and electrophoretic mobility of vector-ex-
pressed and endogenous human Jade-1 proteins were com-
pared. Antisera were generated against a 20-aa peptide
corresponding to the Jade-1 carboxyl terminus in two rabbits.

By enzyme-linked immunosorbent assay, both antisera detect
the immunizing peptide to 1:100,000 dilution. Both Jade-1
immune sera immunoprecipitate from 2 mg of cell lysates
a similar prominent 64-kDa band, whereas neither preimmune
serum does so, as assessed by SDS-PAGE and Jade-1 Western
analysis (Fig. 2A). 293T17 cells were also transiently trans-
ferred with an untagged human Jade-1 expression vector. An
band identical in appearance was immunoprecipitable from
only 250 μg of transfected cell lysate (Fig. 2A, far right lane),
consistent with vector-expressed Jade-1. Similarly, tagged,
transfected Jade-1 was identical in immunoprecipitability and
appearance, although it was of higher molecular weight (data
not shown). Endogenous Jade-1 (Fig. 2B) and transfected
Jade-1 immunoprecipitation (data not shown) could also be
completely blocked with the immunizing peptide. Thus, both
endogenous and transfected Jade-1 protein can be immunode-
tected and immunoprecipitated in a highly specific manner.
Moreover, these observations support the notion that the en-
dogenous and transfected Jade-1 aa sequences are identical.
Jade-1 antiseraum 1 was used for subsequent experiments.

To examine Jade-1 protein distribution, Western analysis
was performed on several tissues (Fig. 2C). The human and
mouse Jade-1 proteins, 64 and 61 kDa, respectively, were by far
most highly expressed in the kidney. Lower Jade-1 expression
was also seen in human pancreas, liver, and heart and in
mouse liver. Jade-1 was not readily detectable in human brain.
In human cell lines, Jade-1 has been observed in HeLa, 293,
and in multiple renal cancer cell lines, although at low levels
(data not shown). Thus, although some difference in tissue
distribution was found between human and mouse, the kidney
was the major site of Jade-1 protein expression.

Jade-1 message and expression pattern were characterized
with a human multitissue Northern blot (Fig. 2D). A 3.6-kb
Jade-1 transcript, identical to the expected size, was most
prominent in kidney. This message was also highly expressed
in pancreas and skeletal muscle, but was found in all tissues
 tested with longer exposure (data not shown). The absence
of Jade-1 protein in tissues, such as brain and skeletal muscle,
where message was clearly expressed suggests that the pri-
mary level of Jade-1 control may not be at the RNA level.
Another major 6-kb transcript was also seen with a distribution
similar to Jade-1 (Fig. 2D). Thus, as observed for Jade-1 pro-
tein, Jade-1 message was most highly expressed in kidney, and
a major band corresponds to the library clone.

To confirm that Jade-1 is expressed in renal proximal tubule
cells, Western blots of kidney cortex and cultured mouse prox-
imal tubule cells were performed. Kidney cortex is roughly 80%
proximal tubule cells by mass. Jade-1 was prominently ex-
pressed in renal cortex (Fig. 2E, right lane) and had lower
expression in renal medulla (data not shown). However, when
mouse proximal tubule cells proliferated in primary culture,
Jade-1 expression was greatly reduced, and the protein exhib-
ited a slight reduction in molecular mass (Fig. 2E, left lane).

These cells are at least 95% mouse renal proximal tubule cells
(47). Jade-1 expression was also high in a temperature-sensi-
tive SV40 T antigen transformed mouse proximal tubule (MPT)
line and increases with differentiation (Fig. 2F). Together these
observations indicate that Jade-1 is expressed in renal cancer
precursors and that differentiation or perhaps quiescence in-
creases Jade-1 expression.

Jade-1 and VHL Proteins Interact—To verify the strong
VHL-Jade-1 interaction observed in yeast, binding was tested
in transiently transfected mammalian cells. Coding sequence
for Jade-1 and the other positive library clones was inserted
into mammalian expression vector pFLAG-CMV2. HA-tagged
VHL was cotransfected into 293T17 cells with FLAG-Jade-1 or
other FLAG-tagged library clones, several of which are in-
cluded for comparison. In these experiments, immunoprecipi-
tation of HA-VHL coimmunoprecipitated much FLAG-Jade-1,
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cluded for comparison. In these experiments, immunoprecipi-
tation of HA-VHL coimmunoprecipitated much FLAG-Jade-1,
enous Jade-1 was detectable by Western blotting, whereas Jade-1 was not visible following control immunoprecipitation (Fig. 3C). Conversely, immunoprecipitation with Jade-1 serum, but not preimmune serum, allowed detection of transiently transfected VHL by Western analysis (Fig. 3D). VHL stably transfected renal cancer cell lines provide similar results (data

Figure 2: Identification of the Jade-1 gene and protein. A, confirmation that the Jade-1 library clone encodes the same protein as endogenous (endog.) Jade-1. Immunoprecipitations (IPs) were performed on whole cell lysates (2 mg of protein total) from VHL stably transfected 786-O renal cancer cells or untransfected 293T17 cells, or on lysates (250 µg of protein total) from 293T17 cells transfected with untagged Jade-1 (trfd. Jade). Preimmune (pre) or corresponding immune serum (post) from two different rabbits was used for immunoprecipitations, followed by Western analysis for Jade-1 with antiserum 1. B, the Jade-1 antiserum is specific for Jade-1 protein. Endogenous Jade-1 protein was immunoprecipitated (IP) from untransfected 293T17 cell lysates using Jade-1 antiserum in the absence or presence of the immunizing peptide (+ pep). Western analysis was performed with Jade-1 antiserum. C, human (above) and mouse (below) multitissue Western analysis was performed using Jade-1 antiserum. Human (Hs) Jade-1 is 64 kDa, whereas the mouse (Mm) protein is 61 kDa (arrows). Skeletal muscle (sk. mm.), placenta (placnt.), pancreas (pancr.). D, Jade-1 human multitissue Northern analysis reveals the Jade-1 transcript at 3.6 kb, and possibly an alternatively spliced form at 6 kb. Abbreviations are as in C. E and F, high Jade-1 expression is associated with proximal tubule cell differentiation. E, mouse (Mm) kidney cortical tissue was finely minced and either lysed and tested immediately for Jade-1 expression by Western analysis (fresh) or subjected to 10 days primary culture (prim. cult.) (47) and tested. The upper band present in both lanes but more prominent in primary culture cells is nonspecific. F, Jade-1 Western analysis of a temperature-sensitive T antigen-transformed MPT cell line (46) grown under permissive, proliferating conditions (prolif.) or with partial differentiation (diff.) at 37 °C for 10 days.
FIG. 3. Jade-1 and VHL proteins interact. A and B, cotransfected Jade-1 and VHL interact strongly. A, left panel: FLAG (FL)-tagged Jade-1 and other library clones were cotransfected with hemagglutinin (HA)-tagged VHL in 293T17 cells. HA antibody immunoprecipitations from 1.5 mg of cotransfected cell lysates were followed by FLAG antibody Western blotting. Right panel: FLAG Western blot of transfected 293T17 whole cell lysates (60 μg per lane), the same as those used in immunoprecipitations in A and B. Other FLAG-tagged library clones, FL-4, FL-5, and FL-10, or empty vector (FL-vec), are shown for comparison. B, cotransfections were performed as in A but were followed by FLAG immunoprecipitation and VHL Western blotting. C and D, endogenous Jade-1 binds transfected VHL. C, 293T17 cells were transiently transfected with VHL, and whole cell lysates were immunoprecipitated using a VHL antiserum or control (ctrl) GST antiserum and assayed for the presence of Jade-1 by Western blotting. D, lysates from VHL-transfected 293T17 cells were immunoprecipitated using preimmune serum (pre) or Jade-1 immune serum (post). VHL was detected using a VHL monoclonal antibody. E–G, the Jade-1 PHD regions are not required for interaction with VHL. E, expression levels of cotransfected Jade-1 (J) (upper panels) or VHL protein (V) (lower panels) as measured by Western blotting of the same whole cell lysates used for immunoprecipitations in F and G. In 293T17 cells, VHL or empty pFLAG-CMV2 (ev) was cotransfected with FLAG- or HA-tagged Jade-1 or truncations (see Fig. 1B for construct schematics), or with empty pCR3.1 uni HA (ev). Lower panels are independent immunodetections of the same blots probed in the upper panels. F, Jade-1 immunoprecipitations of cell lysates from E were followed by VHL immunoblotting. G, VHL immunoprecipitations of the same cell lysates were followed by Jade-1 immunodetection.
not shown). These observations indicate that endogenous Jade-1 binds VHL.

To determine the Jade-1 regions responsible for the VHL interaction, HA-tagged Jade-1 truncations were generated lacking the amino terminus (and candidate PEST domain) (del1), the amino terminus and PHD regions (del2), or both PHDs alone as a double internal deletion (dd), as diagrammed in Fig. 1B. In whole cell lysates, transient expression of full-length Jade-1 and the dd and del2 proteins was robust, whereas del1 expression was lower (Fig. 3E, upper panels). VHL expression was comparable in these cotransfections (Fig. 3E, lower panels). Jade-1 immunoprecipitation of these same cell lysates permitted coimmunoprecipitation of VHL with full-length Jade-1, dd, and del1, but not with del2 (Fig. 3F). Likewise, VHL immunoprecipitation allowed coimmunoprecipitation of full-length Jade-1, dd, and del1, but not del2 (Fig. 3G). The VHL interaction with del1 did appear reduced, even taking into account lower del1 expression. As expected, immunoprecipitation in the absence of either partner did not show the interaction (Fig. 3, F and G, end lanes). Thus, these experiments demonstrate that the Jade-1 carboxyl terminus and PHD regions themselves are not absolutely required and that the amino terminus and the inter-PHD region in particular may be most important for interaction with VHL. These findings also support the specificity of the VHL-Jade-1 interaction.

Localization of Jade-1 and VHL—To determine subcellular compartments where Jade-1 might reside, cells were transiently transfected with HA-tagged Jade-1. In 293T17 cells, transiently transfected HA-Jade-1 gives a strong diffuse and speckled cytoplasmic signal (Fig. 4A). Occasionally, intense perinuclear Jade-1 fluorescence was seen, as shown. Cotransfected FLAG-VHL exhibited a nearly identical immunofluorescence pattern (Fig. 4B). Moreover, the Jade-1 and VHL proteins were almost completely colocalized (Fig. 4C). The HA antiserum and FITC-linked anti-rabbit secondary antibodies gave no detectable fluorescence with untransfected or vector transfected cells (data not shown), which indicates signal is highly specific for HA-tagged Jade-1 protein. VHL was detected with a monoclonal antibody and cyst-conjugated anti-mouse secondary antibody, a combination that also showed negligible background or bleedthrough fluorescence to the other fluorophore wavelength (data not shown). In MPT cells, transiently transfected HA-Jade-1 was seen in prominent nuclear speckles, in addition to the diffuse and speckled cytoplasmic pattern (Fig. 4D). Nuclear speckle localization has also been described for other PHD proteins (56, 57).

Endogenous Jade-1 was localized using Jade-1 antiserum 1 that had been affinity-purified against the immunizing peptide. Several lines of evidence suggest this antibody is specific for human Jade-1 in immunofluorescence studies despite its recognition of several bands by Western blotting. First, the antiserum specificity for Jade-1 in immunoprecipitations was high, because no cross-reacting bands detectable by Western blot were immunoprecipitable (see Fig. 2, A and B). Second, although antiserum 1 readily detected 61-kDa mouse Jade-1 by Western blot as well as nonspecific bands, it could not immunoprecipitate any of these proteins (data not shown). Moreover, the mouse Jade-1 immunofluorescence signal was negligible at the antibody dilution used for human cells. These observations suggest strongly that the antiserum 1 Jade-1 immunofluorescence signal corresponds only to the highest affinity target, human Jade-1, and not any lower affinity, non-immunoprecipitable protein, such as mouse Jade-1.

Endogenous Jade-1 appeared in prominent nuclear speckles in 786-O (Fig. 4E) and A498 renal cancer cells (Fig. 4F). In cytoplasm, diffuse, filamentous, and speckled endogenous Jade-1 fluorescence was seen as well. Stably transfected VHL colocalized with endogenous Jade-1 in a subset of Jade-1-positive nuclear, perinuclear, and cytoplasmic speckles (Fig. 4, F–I). Endogenous Jade-1 and stably transfected VHL also colocalized diffusely in the nucleus of this cell. Jade-1- and VHL-positive cytoplasmic speckles were present in roughly equal abundance, however, only about 10% of each protein colocalized with the other (Fig. 4I). Some Jade-1- and VHL-positive cytoplasmic speckles appeared in ring-like groups, as indicated by the circles. Thus, VHL and Jade-1 colocalized in several compartments, although the colocalizing protein fractions were small, which suggests that the protein interactions may be dynamic.

VHL Stabilizes Jade-1 Protein Expression—To establish a biological relationship between VHL and Jade-1 protein, Jade-1 protein expression was analyzed in VHL-deficient renal cancer cell lines and compared with VHL-expressing stable derivatives by Western analysis and immunoprecipitation. In 786-O, UMRC6, and A498 renal cancer cells, endogenous Jade-1 protein expression is 3- to 10-fold higher in VHL stably transfected derivatives than in parental or empty vector lines (Fig. 5A, upper panels). Protein loading for each sample pair is comparable based on Ponceau S membrane staining (Fig. 5A, lower panel). The UMRC6 cells have lower Jade-1 expression than 786-O and A498 cells; consequently, UMRC6 Jade-1 signal detection here required longer exposure. These results have been confirmed by Jade-1 immunoprecipitation (data not shown). Despite expressing even less wild-type VHL protein than many non-cancer renal lines, including 293T17 cells (data not shown), UMRC6 VHL cells still exhibited 3-fold increased Jade-1 expression, This observation supports the notion that elevated Jade-1 protein levels are not merely the result of VHL overexpression. Furthermore, increased Jade-1 expression was readily seen in other VHL-transfected 786-O stable lines in comparison with additional 786-O empty vector lines and was evident regardless of the degree of cell confluence (data not shown). These results establish a consistent biological relationship whereby the presence of VHL increases Jade-1 expression.

To initially assess how VHL might increase Jade-1 expression, Jade-1 was transiently transfected into 293T17 cells with and without VHL. One day following transfection the amount of transfected Jade-1 protein was largely unaffected by VHL cotransfection (Fig. 5B, far left panel). In contrast, VHL co-transfection substantially increased Jade-1 abundance 3 days post-transfection (Fig. 5B, right panels). In HeLa and HT1080 cancer cell lines in particular VHL dramatically increased Jade-1 protein. As controls, cotransfected empty vector or beta galactosidase did not increase Jade-1. In addition, VHL co-transfection did not increase the expression of p53 or VHL-binding proteins PKC ξ and the C2H2 zinc-finger transcription factor Sp1 (Fig. 5C), supporting the notion that VHL specifically increases Jade-1 abundance. VHL-dependent increases in transfected Jade-1 suggest that the VHL effect on Jade-1 is not at the transcriptional or mRNA level, because Jade-1 gene control elements were not present and the regulatory elements in the different expression vectors were similar. Because increased Jade-1 expression was most notable by late post-transfection, we examined whether VHL affects Jade-1 protein stability.

To explore the mechanism whereby VHL increases Jade-1 abundance, pulse-chase metabolic labeling experiments were performed in renal cancer cell lines. 786-O cells stably transfected with wild-type VHL or an empty expression vector were pulsed with radiolabeled [35S]Met and [35S]Cys. Labeled Jade-1 was immunoprecipitated from 786-O cell lysates and subjected to SDS-PAGE, followed by autoradiography and densitometry.
FIG. 4. Jade-1 resides in cytoplasmic and nuclear speckles and partly colocalizes with VHL. A–C, Jade-1 and VHL colocalize in transiently transfected 293T17 cells. 293T17 cells were transiently transfected with HA-Jade-1 and FLAG-VHL. A, anti-HA polyclonal antibody and FITC-labeled anti-rabbit secondary antibody were used to localize transfected Jade-1. B, anti-VHL monoclonal antibody and cy3-labeled anti-mouse secondary antibodies were used to detect VHL. C, merged view of A and B. D, transfected Jade-1 is found in nuclear speckles in mouse proximal tubule cells. MPT cells were transiently transfected with HA-Jade-1. Anti-HA polyclonal antibody and FITC-conjugated anti-rabbit
Jade-1 is a novel protein-coding gene. Initially, the Jade-1 nt
and aa sequences were not found in GenBank™. Subsequently, cDNA clones were obtained with predicted protein coding sequences identical to Jade-1, including "hypothetical protein" FLJ22479, which is represented by protein accession numbers NP_079176 (New Energy Development Organization (Japan) human cDNA sequencing project, Japan) and XP_033946 (NCBI annotation project). Protein FLJ14714 is identical to FLJ22479 and has been assigned accession number AK027620 (New Energy Development Organization (Japan) project, Japan). Another cDNA encodes a putative "unnamed protein product" with accession number BAB55239 (New Energy Development Organization (Japan) project, Japan). No protein expression or functional information accompanies these GenBank™ reports. Characterization of the human Jade-1 cDNA clone identified revealed the presence of a complete 3′-end. Expressed sequence tag sequences were reviewed to determine the Jade-1 initiation codon, and the MKF site was chosen because no clone with substantially more 5′ sequence was found. Correct identification of the initiation codon is supported by the identical sizes of untagged, transfected Jade-1 protein and the endogenous, immunoprecipitable Jade-1 protein. Other Jade-1 transcripts exist as well, as suggested by the prominent 6-kb message on Northern analysis and a 95-kDa Jade-1 immunoreactive band on Western blotting (data not shown). Although such bands may reflect homologs or alternative transcripts, longer Jade-1 coding sequence clones have been deposited in public databases. For example, an 846-aa Jade-1 protein would be predicted based on combined overlapping Celera Genomics cDNA clone CT8385 and clone KIAA1807 (accession number AB058710) (Kazusa DNA Re-

![VHL stabilizes Jade-1 protein](image_url)
search Institute) (58), although their non-coding sequences are incomplete. The predicted 846-aa Jade-1 protein would have a mass near 95 kDa and would contain the two PHD regions but would lack the candidate PEST sequence or any additional recognizable domains. This larger protein also contains internally 13 of the 20 aa in the immunizing peptide, which still might be sufficient for immunoreactivity. Although alternative splicing may change interaction with and regulation by VHL or other functions, 509-aa Jade-1 may be the first member of a group of proteins stabilized by the VHL tumor suppressor.

Several observations suggest Jade-1 is growth suppressive or might participate in apoptosis. Stable Jade-1 overexpression in 786-O renal cancer cells so far has not been possible. We typically achieve 30–40% success rates for stable expression of any transgene and 20% success rates with VHL, which is growth suppressive in these cells. However, 0 of 25 Jade-1-transfected, drug-resistant stable 786-O colonies exhibited increased Jade-1 expression. In addition, transient transfection of Jade-1 into 293T17 cells increases apoptosis by 50%, as measured both by Hoechst fluorescence and direct visualization and by tagged 293T17 cells increases apoptosis by 50%, as measured both by expression. In addition, transient transfection of Jade-1 into drug-resistant stable 786-O colonies exhibited increased Jade-1 expression. However, 0 of 25 Jade-1-transfected, Jade-1 stable cell line. In addition, the closely homologous gene might participate in apoptosis. Stable Jade-1 overexpression in other functions, 509-aa Jade-1 may be the first member of a family that VHL may play a global role in determining protein fate. VHL-mediated control of the deubiquitinating enzyme VDU1 (10) supports this assertion, as does the widening role of VHL in defense against cell stress (28–30, 67).

We pursued a yeast two-hybrid approach to identify low occupancy VHL protein partners that might have importance in renal cancer. This work identifies Jade-1, a novel PEST- and PHD-containing protein, as a strong VHL interactor that may help control gene expression and differentiation in renal cancer precursor cells. Moreover, these studies identify direct protein stabilization as a new VHL function. Further elucidation of the role of Jade-1 in renal tumor suppression and renal epithelial cell growth and development may therefore be of considerable interest.

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