Role of Exon 2-encoded β-Domain of the von Hippel-Lindau Tumor Suppressor Protein*

Received for publication, September 11, 2000
Published, JBC Papers in Press, October 9, 2000, DOI 10.1074/jbc.M008295200

Marie-Eve Bonicalzi, Isabelle Groulx, Natalie de Paulsen, and Stephen Lee‡

From the Department of Cellular and Molecular Medicine and Kidney Research Center, Faculty of Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

Sporadic clear cell renal carcinomas frequently harbor inactivating mutations in exon 2 of the von Hippel-Lindau (VHL) tumor suppressor gene. Here, we examine the effect of the loss of exon 2-encoded β-domain function on VHL biochemical properties. Exon 2-encoded residues are required for VHL-mediated NEDD8 conjugation on cullin-2 and assembly with hypoxia-inducible factor α (HIFα) and fibronectin. These residues are not essential for VHL ability to assemble with elongin BC/cullin-2, to display E3 ubiquitin ligase activity in vitro and to confer energy-dependent nuclear import properties to a reporter protein. Localization studies in HIF-1α-null embryonic cells suggest that exon 2-encoded β-domain mediates transcription-dependent nuclear/cytoplasmic shuttling of VHL independently of assembly with HIF-1α and oxygen concentration. Exon 3-encoded α-β-helical domain is required for VHL complex formation with BC/cullin-2 and E3 ubiquitin ligase activity, for binding to HIFα/fibronectin, but this domain is not essential for transcription-dependent nuclear/cytoplasmic trafficking. VHL−/− renal carcinoma cells expressing β-domain mutants failed to produce an extracellular fibronectin matrix and to degrade HIFα, which accumulated exclusively in the nucleus of normoxic cells. These results demonstrate that exon 2-encoded residues are involved in two independent functions: substrate protein recognition and transcription-dependent nuclear/cytoplasmic trafficking. They also suggest that β-domain mutations inactivate VHL function differently than α-domain mutations, potentially providing an explanation for the relationship between different mutations of the VHL gene and clinical outcome.

Inactivating mutations of the von Hippel-Lindau (VHL) tumor suppressor gene are associated with inherited VHL cancer syndrome, of which afflicted individuals are at risk to develop a wide variety of tumors including clear cell renal cell carcinoma (RCC) (1–3). Biallelic inactivating mutations of the VHL gene are also associated with sporadic RCC, the most common form of kidney cancer in humans (4, 5). Reintroduction of wild-type VHL in RCC−/− RCC cells is sufficient to suppress their ability to form tumors in nude mice (6). The VHL gene contains three exons that code for a 213-residue protein. VHL protein assembles with at least four other associated proteins: elongin B, elongin C, cullin-2, and Rbx (the complex will be hereafter referred to as VBC/Cul-2) (7–11). VBC/Cul-2 is an E3 ubiquitin ligase that targets the α-subunits of hypoxia-inducible factor (HIFα) for oxygen-dependent degradation (12–18). HIFα are stabilized by hypoxia and play an important role in the activation of hypoxia-inducible genes such as the vascular endothelial growth factor and glucose transport-1 (Glut-1) (8, 13, 19–22). HIFα are stable in VHL−/− RCC cells bringing about a constitutive “hypoxia-like” response, regardless of oxygen concentration. The exact mechanism by which VHL can mediate the degradation of HIFα is still unknown but might be related to its ability to shuttle between the nucleus and the cytoplasm (6, 23–27). Another key functional characteristic of VHL is that it binds to fibronectin and is involved in the assembly of an extracellular fibronectin matrix (28).

The crystal structure of VHL has been reported. VHL mainly consists of two independent domains: a large β-domain that spans residues 64–154 and an α-helical domain (α-domain) that encompasses most of the C-terminal part of the protein (residues 157–189) (29). Tumor-derived inactivating mutations (279 entries; Ref. 30) are found across the VHL protein, indicating that both domains play a critical role in VHL tumor suppressor function (29). There is, however, an interesting correlation between the nature and localization of inactivating mutations and the clinical consequences in patients afflicted with inherited VHL syndrome. Individuals with type II VHL syndrome develop pheochromocytoma and have generally inherited a mutation in the exon 3-encoded α-domain. Type I VHL syndrome differs from type II in that patients do not develop pheochromocytoma and are likely to have inherited a mutation in the hydrophobic core of the β-domain (31). There is also an intriguing disparity in the distribution of tumor-derived missense mutations between the inherited and sporadic form of RCC. Mutations in the α-domain of VHL are much more frequent in the inherited form of RCC (5). The role of a few of these residues is well understood, since they correspond to the ones required for VHL binding to elongin C and formation of the VBC/Cul-2 complex (7, 8, 10, 32). In contrast to inherited RCC, sporadic RCC frequently harbor inactivating point mutations in exon 2. This includes point mutations at the exon 2 boundary that cause a splice defect producing a mRNA that lacks exon 2 sequences altogether (5). Exon 2 mutations are rare in VHL patients, and it has been suggested that such mutations might not be easily tolerated and thus not transmit-

* This work was supported by an Operating Grant from the Medical Research Council of Canada (MRC) (to S. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Scholar of the MRC. To whom correspondence should be addressed: Dept. of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, 451 Smyth Rd., Ottawa, Ontario K1H 8M5, Canada. Tel.: 613-562-5800 (ext. 8385); Fax: 613-562-5636; E-mail: slee@uottawa.ca.

‡ The abbreviations used are: VHL, von Hippel-Lindau; RCC, renal cell carcinoma(s); E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; HIF, hypoxia-inducible factor; HIFα, α-subunit(s) of hypoxia-inducible factor; MEF, mouse embryonic fibroblast; GFP, green fluorescent protein; NES, nuclear export signal; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PVDF, polyvinylidene difluoride; CI, calpain inhibitor I; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DRB, 5,6-dichlorobenzimidazole riboside.

This paper is available on line at http://www.jbc.org
This is an Open Access article under the CC BY license.
ted in the germ line (1). The discrepancy in the distribution of mutations between sporadic and inherited RCC suggests that exon 2-associated mutations might inactivate VHL function in a different way than exon 3-associated mutations. Exon 2-encoded residues 114–154 are mostly hydrophobic and form three of the seven β-strands of the β-domain (29). These residues are hypothesized to play a role in substrate protein recognition, although recent in vitro experiments have revealed that they might not be required for VHL binding to HIFα (33). Therefore, the role that exon 2-encoded sequences play in VHL-mediated tumor suppression is still poorly understood. Here, we further examine the role of these sequences in cells by comparing a tumor-derived VHL mutant that lacks residues 114–154 with the known biochemical properties of wild-type VHL and mutant VHL lacking the exon 3-encoded α-domain. We show that the exon 2-encoded β-domain plays two independent roles in binding to HIFα and fibronectin and mediating transcription-dependent nuclear/cytoplasmic trafficking of the VBC/Cul-2 complex. The use of a novel method to examine the energy requirement for nuclear import of proteins will also be discussed in this report. The results presented here support the model that the β-domain of VHL is involved in substrate recognition and nuclear/cytoplasmic trafficking.

MATERIALS AND METHODS

Cell Culture, Transfections, and Adenoviral Infections

The VHL−/− 786-0 RCC cells and HeLa cells were obtained from the American Type Culture Collection (Manassas, VA). The VHL-GFP cell line corresponds to 786-0 cells stably transfected with the VHL-GFP fusion protein as described elsewhere (25). The 117/4 VHL−/− (ref. 25; referred to as 117) cells were a kind gift from Dr. James R. Gnarra (LSU Health Sciences Center, New Orleans, LA). The mouse embryonic fibroblasts (MEFs)’s nullizygous for HIF-1α were a kind gift from Dr. Randy Johnson (Department of Biology, University of California, San Diego) (34). Cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum in a 37 °C, humidified, 5% CO2-containing atmosphere incubator. Transient transfections were performed overnight using a standard calcium phosphate method. Viruses were used as freeze/thaw lysates, and all infections were also performed overnight.

Expression Vectors and Constructs

The human VHL cDNA, which codes for a 213-amino acid VHL protein, was subcloned into pcDNA3.1(−) (Invitrogen) vector. A FLAG epitope tag (DYKDDDDK) was added to the N terminus of the VHL cDNA open reading frame. A cDNA coding for an enhanced fluorescence version of GFP (Fred 25; Ref. 35) was subcloned at the C terminus VHL (34). Two GFPs in tandem were cloned into pcDNA 3.1(−) to produce the ΔE3-GFP fusion protein. An additional deletion mutant of VHL lacking amino acids 114–154 was fused to GFP to produce the ΔE2-GFP fusion protein. Two GFPs in tandem were cloned into pcDNA 3.1(−) to produce the GFP-GFP fusion protein. VHL-GFP-ΔE2-GFP-ΔE2-GFP and GFP-GFP at their C terminus to the strong nuclear export signal (NES) of human immunodeficiency virus REV, LPPLERLT (NES) (36). All constructs were verified by standard DNA sequencing.

Construction of Adenovirus Vectors through Cre-lox Recombination

CRE8 and 293 cells were obtained from Dr. David Park (University of Ottawa, Ottawa, Ontario, Canada) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS). The construction and properties of CRE8 cells are described elsewhere (37). The pAdlov vector and the Δ5 viral DNA were also obtained from Dr. Park. The three different VHL constructions (VHL-GFP, ΔE2-GFP, and ΔE3-GFP) were previously subcloned in the pAdlov vector. Transfections were done according to Graham and van der Eb (38). Typically, a confluent 10-cm diameter dish of CRE8 cells (1.6 × 107) was split into 5–6-cm diameter dishes for transfection 2–4 h later. Each dish received 3 μg of pAdlov vector (containing the foreign VHL construction) and 3 μg of Δ5 viral DNA in a final volume of 0.5 ml of CaPO4, which was applied to the cells for 16 h. The 10% fetal calf serum DMEM was changed for 2% fetal calf serum DMEM 16 h following the transfection. Cells were fed with fresh 2% DMEM after 64 h. Between day 8 and 10, we had a sizable infection in each dish; almost all of the cells were rounded up or floating. Cells were harvested and subjected to freeze/thaw three times with an alternating liquid N2/37 °C water bath. The virus was then passed sequentially through CRE8 cells twice. Finally, a plaque purification assay was performed in order to isolate the recombinant virus expressing adVHL-GFP, adΔE2-GFP, or adΔE3-GFP. The recombinant viruses were then amplified in CRE8 cells to high titer.

Nuclear Import Assay in Living Cells

HeLa cells were plated on a 35-mm dish with a hole at the bottom replaced by a glass coverslip and transfected overnight with VHL-GFP-ΔE3-GFP, ΔE2-GFP-ΔE2-GFP, and GFP-GFP-ΔE2-GFP. Cells were washed with PBS and incubated for 2 h in DMEM at 37 °C with or without metabolic poisons (6 mM 6-deoxyglucose and 0.02% sodium azide), at 4 °C, or at 37 °C with 10 μM leptomycin B (39–40).

In Vitro Ubiquitination Assay

VHL−/− 786-0 RCC cells infected with the three different adenoviruses and 786-0 cells stably expressing VHL-GFP were lysed in the presence of 1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl with protease mixture for 30 min. at 4 °C. Whole cell lysates were first immunoprecipitated with anti-FLAG M2 monoclonal antibody. Precipitates were washed five times with a buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2 mM dithiothreitol. The total volume of the reaction mixture was adjusted to 20 μl. E1 ubiquitin-activating enzyme (100 ng), E2 ubiquitin-conjugating enzyme (200 ng), 0.5 μg of ubiquitin aldehyde, 0.5 μg of ubiquitin, and an ATP-regenerating system (0.5 mM ATP, 10 mM creatine phosphate, and 10 μg of creatine phosphokinase) were added to the reaction mixture. The reaction was stopped after 2 h of incubation at 37 °C by adding 4% SDS loading buffer. Samples were boiled 10 min and separated on an 8% SDS-PAGE and transferred to a PVDF membrane. Blots were blocked and probed in the presence of a mouse anti-ubiquitin antibody (Berkeley Antibody Company). The E1 ubiquitin-activating enzyme and the E2 ubiquitin-conjugating enzyme were kind gifts from Dr. Kazuhiro Iwai (Kyoto University, Kyoto, Japan).

Immunoprecipitations and Immunoblotting

Immunoprecipitation of HIF-1α and HIF-2α—VHL−/− 786-0 cells expressing endogenous HIF-2α or 117-4 cells expressing endogenous HIF-1α were exposed for 4 h to hypoxia (0.1% O2) 16 h after infection. Proteasomal inhibition was performed with 100 μM calpain inhibitor I (CI) for 2 h. When still in the hypoxic chamber, cells were washed several times with PBS and scapped from the Petri dishes in lysis buffer containing 100 mM NaCl, 0.5% Igepal CA630, 20 mM Tris-HCl (pH 7.6), 5 mM MgCl2, and 1 mM sodium orthovanadate with 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 mM 4-(2-aminoethyl)benzene sulfonyl fluoride. Tubes were put back in normoxia and kept at 4 °C for 30 min. with rocking. After clearance by centrifugation, 1-mg aliquots of lysate were incubated for 2 h at 4 °C with anti-FLAG M2 beads (Scientific Imaging Systems, Eastman Kodak Co.). Beads were washed, boiled, and loaded on an 8% SDS-PAGE and blotted onto PVDF membranes using standard methods. Blots were blocked with 3% milk powder in PBS containing 0.2% Tween 20 and were then incubated in the presence of anti-HIF-1α (Transduction Laboratories), anti-HIF-2α antibody (Novus Biologicals), or an anti-FLAG M2 monoclonal antibody (Sigma).

Immunoprecipitation of Culcin-2, NEDD8, and Fibronectin—VHL−/− 786-0 cells and infected 786-0 cells were lysed in 100 mM NaCl, 0.5% Igepal CA630, 20 mM Tris-HCl (pH 7.6), 5 mM MgCl2, and 1 mM sodium orthovanadate with 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μg/ml pepstatin A. After clearance by centrifugation, 1-mg aliquots of lysate were incubated for 2 h at 4 °C with anti-FLAG M2 beads. Beads were washed, boiled, and loaded on an 8% SDS-PAGE and blotted onto PVDF membranes using standard methods. Blots were blocked with 3% milk powder in PBS containing 0.2% Tween 20 and were then incubated in the presence of anti-HIF-1α (Transduction Laboratories), anti-HIF-2α antibody (Novus Biologicals), or an anti-FLAG M2 monoclonal antibody (Sigma).

Immunoprecipitation of Culcin-2, NEDD8, and Fibronectin—VHL−/− 786-0 cells and infected 786-0 cells were lysed in 100 mM NaCl, 0.5% Igepal CA630, 20 mM Tris-HCl (pH 7.6), 5 mM MgCl2, and 1 mM sodium orthovanadate with 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μg/ml pepstatin A. After clearance by centrifugation, 1-mg aliquots of lysate were incubated for 2 h at 4 °C with anti-FLAG M2 beads. Beads were washed, boiled, and loaded on an 8% SDS-PAGE and blotted onto PVDF membranes using standard methods. Blots were blocked with 3% milk powder in PBS containing 0.2% Tween 20 and were then incubated in the presence of anti-HIF-1α (Transduction Laboratories), anti-HIF-2α antibody (Novus Biologicals), or an anti-FLAG M2 monoclonal antibody (Sigma).
The VHL protein, encoded by the VHL gene that causes VHL syndrome (5). To study the role of exon 2-encoded \(\beta\)-domain function while retaining an intact, if not total, loss of \(\alpha\)-helical domain. A tumor-derived truncation of residues 114–154 was fused to GFP (Fig. 1A). Sporadic RCC frequently harbor inactivating mutations in the \(\beta\)-domain, whereas these mutations are relatively rare in individuals afflicted with inherited VHL syndrome (5). To study the role of exon 2-encoded \(\beta\)-domain in VHL tumor suppressor function, a cDNA encoding a tumor-derived truncation of residues 114–154 was fused to GFP to produce \(\Delta\)E2-GFP fusion protein. A cDNA encoding a tumor-derived truncation of residues 114–154 (strike box) was fused to GFP to produce \(\Delta\)E2-GFP. A tumor-derived truncation of the exon 3-encoded \(\alpha\)-domain (strike box) was also fused to GFP to produce \(\Delta\)E3-GFP. A FLAG tag (shadow box) was fused to the N-termini of all three constructs. \(\Delta\)E2-GFP, \(\Delta\)E3-GFP, and VHL-GFP were cloned in pAdlox vector to prepare adenoviruses. ad\(\Delta\)E2-GFP, ad\(\Delta\)E3-GFP, and adVHL-GFP refer to proteins obtained following the infection with the corresponding adenovirus. C, 100% of VHL

RCC cells (786-0) displayed GFP fluorescence following infection with the adenovirus adVHL-GFP. 786-0 cells were infected overnight (16 h), and adVHL-GFP pictures were obtained by a charge-coupled device camera (left panel). Counterstaining with Hoechst 33342 dye (2 mg/ml) (Dako Diagnostic) for 2 min at room temperature. The coverslips were then washed with PBS three times and incubated with Cy\(^{TM}\)3-conjugated anti-rabbit antibody (Jackson ImmunoResearch, PA) diluted 1:1000 for 1 h at room temperature. Coverslips were washed three times with PBS, incubated for 2 min with Hoechst 33342, and mounted with fluoromount-G (Southern Biotechnology Associates) on slides.

For \(\text{HIF-1}\alpha\)–117 cells or transiently transfected 786-0 with \(\text{HIF-1}\alpha\) were grown on coverslip and infected with the three different adenoviruses overnight. Cells were washed three times with PBS, fixed/permeabilized in prechilled 95% ethanol at \(-20^\circ\text{C}\) for 30 min. Ethanol was then aspirated, and the residual ethanol was allowed to air dry at 4 °C. Cells were stained with polyclonal anti-fibronectin antibody (5 μg/ml) (Dako Diagnostic) for 1 h at room temperature. The coverslips were then washed with PBS three times and incubated with Cy\(^{TM}\)3-conjugated anti-rabbit antibody (Jackson ImmunoResearch, PA) diluted 1:1000 for 1 h at room temperature. Coverslips were washed three times with PBS, incubated for 2 min with Hoechst 33342, and mounted with fluoromount-G (Southern Biotechnology Associates) on slides.

For \(\text{HIF-1}\alpha\)–117 cells or transiently transfected 786-0 with \(\text{HIF-1}\alpha\) were grown on coverslip and infected with the three different adenoviruses overnight. Cells were washed three times with PBS, fixed/permeabilized in PBS containing 4% formaldehyde for 30 min at room temperature, washed again three times with PBS, and incubated for 1 h at room temperature with anti-\(\text{HIF-1}\alpha\) antibody (Transduction Laboratories, Lexington, KY) diluted 1:1000 in PBS, 1% Triton X-100, 10% FCS. The cells were washed in PBS and incubated for 60 min in the presence of a Cy\(^{TM}\)3-conjugated anti-mouse antibody (Jackson ImmunoResearch) diluted 1:1000. The cells were washed in PBS, incubated for 2 min in Hoechst 33342, and mounted with fluoromount-G on slides.

**Fluorescence Analysis and Image Processing**

GFP fluorescence images were captured using a Zeiss Axiovert S100TV microscope with a C-Apochromat 40 × water immersion objective, equipped with an Empix digital charge-coupled device camera using Northern Eclipse software. Images were manipulated with Northern Eclipse and Adobe Photoshop software as described elsewhere (25). GFP images were always taken before Hoechst images to minimize any possible bleaching effect.

**RESULTS**

**Biochemical Characterization of the Exon 2-Encoded \(\beta\)-Domain of VHL**—The VHL protein, encoded by the VHL gene that contains three exons, can be divided into three independent domains: an acidic domain, a \(\beta\)-domain, and an \(\alpha\)-domain (Fig. 1A). Sporadic RCC frequently harbor inactivating mutations in the exon 2-encoded part of the \(\beta\)-domain, whereas these mutations are relatively rare in individuals afflicted with inherited VHL syndrome (5). To study the role of exon 2-encoded \(\beta\)-domain in VHL tumor suppressor function, a cDNA encoding a tumor-derived truncation of residues 114–154 was fused to GFP to produce \(\Delta\)E2-GFP fusion protein (Fig. 1B). This truncation mutant is the consequence of point mutations that cause a splice defect producing a mRNA that lacks exon 2 sequences altogether (5). \(\Delta\)E2-GFP is predicted to have a partial, if not total, loss of \(\beta\)-domain function while retaining an intact, exon 3-encoded \(\alpha\)-helical domain. A tumor-derived truncation of the exon 3-encoded \(\alpha\)-helical domain (last 56 C-terminal residues), which retained intact the sequences of the \(\beta\)-domain, was also fused to GFP (\(\Delta\)E3-GFP) (Fig. 1B). \(\Delta\)E2-GFP, \(\Delta\)E3-GFP, and wild-type VHL-GFP were cloned in pAdlox vector, and adenoviruses (ad\(\Delta\)E2-GFP, ad\(\Delta\)E3-GFP, and adVHL-
GFP) were produced to high titers (Fig. 1B) (42). Adenovirus was chosen as a method to reintroduce VHL, since it eliminates the necessity to produce stable clones of different VHL−/− RCC cell lines. VHL−/− RCC cells were infected with very high efficiency, with essentially 100% of cells displaying GFP fluorescence (Fig. 1C). In adenovirus-infected cells, adVHL-GFP was mostly localized to the cytoplasm with some nuclear signal, consistent with data obtained with stable transfectants. In contrast to VHL alone (without GFP; Ref. 43), adVHL-GFP did not restrain proliferation of VHL−/− RCC cells or other cell lines such as 293 cells, even when expressed to very high levels (data not shown). Glut-1 protein levels were significantly decreased in VHL−/− 786-0 RCC infected with adVHL-GFP in normoxia compared with uninfected cells or cells infected with an adenovirus that expressed GFP alone (data not shown). Western blot analysis indicated that adΔE2-GFP accumulated to levels similar to those of adVHL-GFP and adΔE3-GFP, suggesting that adΔE2-GFP is a stable protein (Fig. 1D). We conclude that the adVHL-GFP protein produced from an adenovirus is a functional molecule and shares similar characteristics with VHL.

We next examined the biochemical properties of adΔE2-GFP in comparison with adVHL-GFP and adΔE3-GFP. The β-domain mutant adΔE2-GFP still retained the ability to assemble with cullin-2 (Fig. 2A) and to exhibit E3 ubiquitin ligase activity in vitro (Fig. 2B) to levels similar to those observed for adVHL-GFP. The α-helical domain deletion mutant (adΔE3-GFP) failed to assemble with cullin-2 and to display E3 ubiquitin ligase activity in vitro, as expected. While the experiments described above were being performed, it was noticed that a second band, which migrated slower than cullin-2, was found in the adVHL-GFP lane but was lacking from the adΔE2-GFP lane (Fig. 2A). NEDD8 is a ubiquitin-like molecule, which is conjugated to cullin-2 in a VHL-dependent manner (41, 44). Western blotting with an anti-NEDD8 antibody revealed that the slower migrating form of cullin-2 is conjugated to NEDD8 (Fig. 2C). Therefore, an intact exon 2-encoded β-domain is not required for VHL ability to assemble with cullin-2 and to function as an E3 ubiquitin ligase in vitro but is necessary for VHL-mediated NEDD8 conjugation on cullin-2.

Exon 2-encoded β-Domain Is Required for VHL Binding to Fibronectin and Proper Assembly of a Fibronectin Extracellular Matrix—VHL−/− RCC cells are unable to promote assembly of an extracellular fibronectin matrix, and the reintroduction of VHL was shown to be sufficient to correct this defect (28). Adenovirus-mediated reintroduced adVHL-GFP displayed similar activity as VHL and restored the ability of VHL−/− RCC cells to properly produce a fibronectin extracellular matrix (Fig. 3A; VHL-GFP). In contrast, adΔE2-GFP was unable to rescue this defect (Fig. 3A). Fibronectin was observed in an endoplasmic reticulum-like intracellular distribution in uninfected cells as well as in cells expressing adΔE2-GFP. Immunoprecipitation analysis revealed that adVHL-GFP was able to assemble with fibronectin, whereas adΔE2-GFP failed to do so (Fig. 3B). The adΔE3-GFP was also unable to bind to fibronectin and correct the fibronectin deposition defect of VHL−/− RCC. Therefore, VHL requires an exon 2-encoded β-domain to bind to fibronectin and mediate proper extracellular matrix formation.

Role of Exon 2-Encoded β-Domain of VHL in Oxygen-dependent Degradation of HIFα—It was recently shown that one of the major defects of VHL−/− RCC cells is their inability to mediate oxygen-dependent degradation of HIFα, and reintroduction of wild-type VHL was sufficient to correct this defect (13). In vitro studies have also revealed that truncation mutants of exon 2 and exon 3 of VHL are still able to bind to HIFα (33), which probably assemble with sequences encoded by exon 1 (residues 64–113) (12). Adenovirus-mediated reintroduction of adVHL-
GFP was sufficient to restore VHL^−/− RCC cell line 117 (HIF-1α) and 786-0 (HIF-2α) ability to mediate degradation of HIFα in normoxia (Fig. 4A). HIFα levels were not affected by expression of adΔE2-GFP or adΔE3-GFP (Fig. 4A). We notice that adVHL-GFP assembled with a significant amount of HIFα (1α and 2α) in hypoxia and in the presence of the proteasome inhibitor CI. In contrast to data obtained in vitro, immunoprecipitation analysis revealed that adΔE2-GFP and adΔE3-GFP failed to bind to HIFα in adenovirus-infected cells (Fig. 4B, top panels). We did not detect binding of HIFα to adΔE2-GFP and adΔE3-GFP in cells expressing low to very high levels of the fusion proteins (data not shown). These results indicate that an intact exon 2-encoded β-domain, as well as the α-domain, is required for VHL assembly with HIFα in cells.

It has been hypothesized that HIF-1α requires a hypoxic environment to import in the nucleus most likely assembled into complexes that contain VBC/Cul-2 (33, 45). To further examine the role of hypoxia and VHL in nuclear import of HIFα, the subcellular localization of endogenous HIF-1α was examined by immunofluorescence in VHL^−/− 117 RCC cells uninfected or infected with different VHL constructs. Data shown in Fig. 4C revealed that endogenous HIF-1α accumulated exclusively in the nucleus of uninfected VHL^−/− RCC 117 cell line although these cells were incubated in normoxia (Fig. 4C, a, e, and i). This demonstrates that HIF-1α is able to import in the nucleus even in the presence of oxygen and in the absence of VHL. A strong HIF-1α nuclear signal was also observed in cells expressing adΔE2-GFP (Fig. 4C, c, g, and k) as well as adΔE3-GFP (Fig. 4C, d, h, and l), whereas it was essentially undetectable in cells expressing reintroduced adVHL-GFP (Fig. 4C, b, f, and j). We then examined the subcellular localization of overexpressed HIF-1α in RCC VHL^−/− 786-0 cells (which do not express endogenous HIF-1α). A strong HIF-1α signal was detected exclusively in the nucleus of normoxic RCC VHL^−/− 786-0 cells transiently transfected with HIF-1α cDNA that were either uninfected (Fig. 4C, m), infected with GFP alone (data not shown), or infected with adΔE2-GFP (Fig. 4C, o) and adΔE3-GFP (Fig. 4C, p). The addition of proteasome inhibitors or incubation in hypoxia led to nuclear accumulation of endogenous or overexpressed HIFα regardless of the presence of adVHL-GFP or mutants, as expected (data not shown). HIF-1α was also detected in the nucleus of normoxic RCC VHL^−/− 786-0 cells when co-transfected with different smaller deletion mutants of exon 2, with a substitution
at residue 117 in exon 2 or at residue 98 in exon 1, fused to GFP (Fig. 4D). These results demonstrate that HIF-1α is able to import in the nucleus regardless of oxygen concentration or assembly with VHL.

Exon 2-encoded Residues Mediate Transcription-dependent Nuclear/Cytoplasmic Trafficking of VHL Independently of Assembly with HIFα and Oxygen Concentration—We recently demonstrated that VHL mediates transcription-dependent nuclear/cytoplasmic trafficking of the VBC/Cul-2 complex (25, 46). The addition of 5,6-dichlorobenzimidazole riboside (DRB), an inhibitor of RNA polymerase II activity, causes an important increase of nuclear VBC/Cul-2 by blocking VHL-mediated nuclear import in living cells based on fusing proteins to the energy-dependent human immunodeficiency virus REV NES. NES confers strong nuclear export properties to fusion proteins, leading to their cytoplasmic accumulation at steady state (Fig. 5; compare a with d, b with e, and c with f; see Ref. 46). GFP-GFP-NES rapidly accumulated in the nucleus upon inhibition of NES function at 4 °C or with metabolic poisons, as expected, since this fusion protein is able to passively diffuse in and out of the nucleus (Fig. 5, g and j, and Ref. 46). In contrast, VHL-GFP-NES and ΔE2-GFP-NES strictly remained in the cytoplasm at 4 °C or in the presence of metabolic poisons (Fig. 5, h, i, k, and l), indicating that both fusion proteins are unable to passively diffuse in the nucleus. ΔE2-GFP-NES and VHL-GFP-NES (Fig. 5, n and a) accumulated in the nucleus upon incubation with leptomycin B, a drug that specifically inhibits
both mutants was unaffected by the absence or presence of oxygen tension, and this mutant is unable to bind to HIF-1α, as described under ‘Materials and Methods.’ Cells were either incubated at 37 °C (d–f), at 4 °C for 2 h (g–i), at 37 °C in the presence of 6 deoxyglucose (6-DOG) and sodium azide (SA) for 2 h (j–l), or at 37 °C with leptomycin B (LMB; 10 μM; m–o).

**Fig. 5.** Nuclear import of adΔE2-GFP is energy-dependent. HeLa cells were transiently transfected for 24 h with GFP-GFP, VHL-GFP, and ΔE2-GFP (a–c) or with fusion to NES: GFP-GFP-NES, VHL-GFP-NES, and ΔE2-GFP-NES (d–o), as described under ‘Materials and Methods.’ Cells were either incubated at 37 °C (d–f), at 4 °C for 2 h (g–i), at 37 °C in the presence of 6 deoxyglucose (6-DOG) and sodium azide (SA) for 2 h (j–l), or at 37 °C with leptomycin B (LMB; 10 μM; m–o).

**NES Fusion Proteins**

| Protein        | 37°C | 37°C | 4°C | 37°C/6-DOG+SA | 37°C/LMB |
|----------------|------|------|-----|---------------|----------|
| GFP-GFP        | a    | b    | c   | d             | e        |
| VHL-GFP        | f    | g    | h   | i             | j        |
| ΔE2-GFP        | k    | l    | m   | n             | o        |

NES function (39, 40) at 37 °C, but not at 4 °C, indicating that both fusion proteins contain energy-dependent nuclear import signals. These observations demonstrate that VHL ability to confer energy-dependent nuclear import properties to a reporter GFP is independent of assembly with HIFα and exon 2-encoded β-domain residues.

Exon 2-encoded β-domain mediates transcription-dependent trafficking of VHL and VBC/Cul-2, and the next step was to test if this domain was sensitive to conditions known to affect HIFα stabilization. GFP fluorescence analysis of living cells indicated that the steady state distribution of adΔVHL-GFP was unaffected by oxygen tension (Fig. 6, a and j). The addition of the RNA polymerase II inhibitor DRB caused nuclear accumulation of adVHL-GFP, regardless of oxygen concentration (Fig. 6, b and k). It has been recently suggested that proteasome inhibitors, which prevent proteasome-mediated degradation of ubiquitinated proteins, might also act as general inhibitors of nuclear export (47, 48). Interestingly, a strong shift in the steady state distribution toward the nucleus of adVHL-GFP was observed upon incubation with the proteasome inhibitor CI, or lactacystin (data not shown) in normoxia and hypoxia (Fig. 6, c and l). adΔE3-GFP steady state distribution is more nuclear than adVHL-GFP and is unaffected by oxygen concentration (Fig. 6, g and p). The addition of DRB or CI also caused an important nuclear accumulation of adΔE3-GFP with few cells displaying exclusive nuclear signal (Fig. 6, h, i, q, and r).

In contrast, the localization of the β-domain mutant adΔE2-GFP remained unchanged regardless of oxygen tension, proteasome inhibitors, or RNA polymerase II inhibitors (Fig. 6, d–f and m–o). One possible explanation for adΔE2-GFP insensitivity to DRB and CI is that this mutant is unable to bind to HIFα. These observations led us to test if the effect of DRB and CI on shuttling of VHL is intrinsic to exon 2-encoded residues or if this activity is mediated by HIFα. To test this, VHL shuttling was analyzed in mouse embryonic fibroblasts that do not express endogenous HIFα (Fig. 7). We noticed that adVHL-GFP steady state subcellular localization was unaffected by the absence of HIF-1α (Fig. 7, a and c). Likewise, the addition of DRB caused nuclear accumulation of adVHL-GFP in HIF-1α−/− as well as in HIF-1α−/− cells (Fig. 7, b and d). The localization of both mutants was unaffected by the absence or presence of HIF-1α (Fig. 7, e, g, i, and k). The α-domain mutant adΔE3-GFP accumulated in the nucleus upon incubation with DRB, whereas adΔE2-GFP was unaffected by this treatment in HIF-1α−/− and HIF-1α−/− cells. The effect of CI was essentially the same as DRB (data not shown) on the three fusion proteins (data not shown). The same data were obtained in hypoxia (data not shown). Put together, these results demonstrate that oxygen tension and HIFα have no affect on VHL nuclear/cytoplasmic shuttling properties. They also indicate that exon 2-encoded β-domain plays a role in nuclear/cytoplasmic trafficking of VHL, which is independent of its role in binding to HIFα.

**DISCUSSION**

Inactivating mutations of the VHL tumor suppressor gene are distributed equally between the β- and α-domains, suggesting that both domains play a key role in tumor suppression (29). Yet, the nature and localization of the mutations has a profound effect on the clinical manifestations in inherited VHL syndrome (31). Likewise, sporadic RCC tumors are much more likely to harbor mutations in exon 2, mutations that are rarely found in individuals afflicted with inherited VHL syndrome (5). The discrepancy in the distribution of inactivating mutations between sporadic and inherited RCC implies that exon 2-associated mutations might inactivate VHL function in different ways than exon 3-associated mutations. We show here that loss of exon 2 or exon 3 function essentially gives rise to the same cellular defects in RCC, which includes aberrant nuclear accumulation of HIFα in normoxia and inability to produce an extracellular fibronectin matrix. However, loss of exon 2 function appears to have a lesser effect on the overall activity of the VHL protein compared with loss of α-domain activity. The major defects of the β-domain mutant that we were able to identify were its inability to bind to HIFα and fibronectin and to mediate transcription-dependent shuttling of VHL. The binding results are similar to those recently reported by two other groups, which demonstrated that missense mutations in exon 1-encoded portion of the β-domain also abrogated VHL assembly to HIFα but not to BC/Cul-2 (12, 18). A deletion of the α-domain caused a more complete loss of function, since this mutant failed to assemble with BC/Cul-2 as well as with substrate proteins and act as an E3 ubiquitin ligase. This is not the consequence of a truncation of the α-domain, since a missense mutation at residue 162 in the elongin C-binding box has recently been reported to cause similar defects (8, 29). There is a discrepancy between data obtained in vitro and in culture inasmuch as truncations of exon 2- and exon 3-encoded sequences of VHL are still able to assemble with HIFα in vitro (12, 18, 33). Either ΔE2-GFP and ΔE3-GFP fold in a different way in vivo compared with in vitro, or these mutants have a yet uncharacterized defect that prevents their assembly with HIFα in cells. Interestingly, an alternative spliced mRNA of the VHL
gene that lacks exon 2 sequences has been reported to be produced in several independent tissues and cell lines (1). A VHL protein without exon 2 sequences might change substrate specificity from HIFα to another unidentified protein while still acting as an E3 ubiquitin ligase. An endogenous protein product originating from a mRNA lacking exon 2 sequences still remains to be identified. Nevertheless, the data presented in this report are in good agreement with the proposed model predicted by the crystal structure of VHL that the \( \beta \)-domain of VHL is involved in substrate protein, as well as fibronectin, recognition (29). They also demonstrate that tumor-derived mutations inactivate VHL functions in different ways, which may lead to distinct cellular phenotypes.

The study of ad\( \Delta E2 \)-GFP has also revealed other interesting biochemical aspects of the function of exon 2-encoded sequences, one of which is that it is required for VHL-mediated NEDD8 conjugation on cullin-2. The functional relevancy of this post-translational modification is still unknown, but it has been suggested that it might play a role in protecting cullin-2 from self-ubiquitination (49). Data shown here are somewhat in disagreement with this model, since equal amounts of cullin-2 can be found bound to VHL and ad\( \Delta E2 \)-GFP, regardless of conjugation to NEDD8. NEDD8 conjugation is reported to be a nuclear event (44). ad\( \Delta E2 \)-GFP can be detected in the nuclear compartment at steady state, and the lack of NEDD8 conjugation activity cannot be simply explained by a defect in nuclear import of the VBC/Cul-2 complex. This argument is supported by a novel assay presented here, which enables the analysis of energy requirement for nuclear import of proteins in living cells. Energy expenditure for nuclear import is a hallmark of signal-mediated and -regulated nuclear/cytoplasmic trafficking processes (50–52). The observation that ad\( \Delta E2 \)-GFP retains the ability to import in the nucleus in an energy-dependent manner suggest that other protein/protein interactions involved in nuclear import of the VBC/Cul-2 complex are not affected by loss of function of exon 2-encoded sequences. Likewise, we noticed HIFα signal exclusively in the nucleus of normoxic VHL\(-/-\) cells, indicating that HIFα is able to import even in the absence of hypoxic conditions and assembly with VHL. These data are somewhat surprising, since it is generally believed that HIFα contains a nuclear import signal that is activated only in hypoxia (45). One possible interpretation of...
inhibitors is mediated by exon 2-encoded proteins have been hypothesized to also interfere with general Drugs that inhibit proteasome-mediated degradation of pro-inhibitors, similar to the effect obtained with DRB treatment. accumulated in the nucleus upon incubation with proteasome E3-D2-GFP, and adD2 h. The addition of CI (100 m M) for 2 h essentially gave the same results as DRB (data not shown). These data is that the hypoxia-inducible nuclear import of HIFα is regulated by VHL, which might play a role in retaining HIFα in the cytoplasm in normoxia.

Results shown here suggest that transcription-dependent nuclear/cytoplasmic shuttling and steady state distribution of VHL are not affected by oxygen tension and do not require assembly with HIFα. However, we did find that adVHL-GFP accumulated in the nucleus upon incubation with proteasome inhibitors, similar to the effect obtained with DRB treatment. Drugs that inhibit proteasome-mediated degradation of proteins have been hypothesized to also interfere with general nuclear export processes (47, 48). Sensitivity to proteasome inhibitors is mediated by exon 2-encoded b-domain in a manner reminiscent of DRB. We have previously shown that VHL transcription-dependent shuttling domain acts dominantly on the VBC/Cul-2 complex and that DRB is a good inhibitor of VHL-mediated VBC-Cul-2 nuclear export in living cells and in vitro (46). It is conceivable that CI also blocks exon 2-mediated nuclear export of VHL, leading to nuclear accumulation of VBC/Cul-2. It is unlikely that the observed nuclear accumulation of adVHL-GFP is the consequence of HIFα-mediated nuclear retention, since proteasome inhibitors and DRB have similar effects on VHL in HIF-null MEFs. The presence of adΔE2-GFP in the cytoplasm at steady state might be explained by a fraction of VHL that is not importable at a given time. Alternatively, the existence of other nuclear export signals within the complex might gain dominance upon loss of function of exon 2-encoded residues. Taken together, these results support the model that exon 2-encoded residues are involved in two independent functions: mediating nuclear export of the VBC/Cul-2 complex and binding to substrate proteins. We are still in the process of identifying relevant sequences involved in signal-mediated and Ran-dependent nuclear/cytoplasmic trafficking of the VBC/Cul-2 complex. Identification of these sequences will surely provide important clues in the elucidation of VHL-mediated tumor suppressor function.

Acknowledgments—We sincerely thank Dr. David Park, Ruth Slack, and Steve Callaghan for their help with the adenovirus system and Dr. Randy Johnson for the HIF-1α+/− MEF cell line.

REFERENCES
1. Gnarra, J. R., Duan, D. R., Weng, Y., Humphrey, J. S., Chen, D. Y., Lee, S., Pause, A., Dudley, C. F., Latif, F., Kuzmin, I., Schmidt, L., Duh, F. M., Stackhouse, T., Chen, F., Kishida, T., Mei, W. H., Lerman, M. I., Zbar, B., Klausner, R. D., and Linehan, W. M. (1996) Biochem. Biophys. Acta 1242, 201–210
2. Latif, F., Tory, K., Gnarra, J., Yao, M., Duh, F. M., Orecutt, M. L., Stackhouse, T., Kuzmin, I., Modi, W., Geil, T., Schmidt, L., Zhou, F., Li, H., Wei, M. H., Chen, F., Glenn, G., Choyke, P., Walther, M. M., Weng, Y., Duan, D. S. R., Dean, M., Glavac, D., Richards, F. M., Crosse, P. A., Ferguson-Smith, M. A., Le Paslier, D., Chunakov, I., Cohen, D., Chinault, A. C. R., Maher, E. R., Linehan, W. M., Zbar, B., and Lerman, M. I. (1993) Science 260, 1317–1320
3. Linehan, W. M., Lerman, M. I., and Zbar, B. (1995) J. Nat. Med. Assoc. 273, 564–570
4. Foster, K., Prowse, A., van den Berg, A., Fleming, S., Hubbeek, M. M. F., Crosse, P. A., Richards, P. M., Cairns, P., Affara, N. A., Ferguson-Smith, M. A., Buys, C. H., and Maher, E. R. (1994) Hum. Mol. Genet. 3, 2169–2173
5. Gnarra, J. R., Tory, K., Weng, Y., Schmidt, L., Mei, W. H., Li, H., Latif, F., Liu, P. S., Chen, F., Duh, F. M., Lubensky, I., Duan, D. R., Florence, C., Pazzatti, R., Walther, M. M., Bander, N. H., Grossman, H. B., Brauch, H., Pomer, S., Brooks, J. D., Isaac, W. B., Lerman, M. I., Zbar, B., and Linehan, W. M. (1994) Nat. Genet. 7, 85–90
6. Iliopoulos, O., Kihel, A., Gray, S., and Kaelin, W. G. (1995) Nat. Med. 1, 822–826
7. Duan, D. R., Pause, A., Burgess, W. H., Aso, T., Chen, D. Y. T., Garrett, K. P., Conaway, R. C., Conaway, J. W., Linehan, W. M., and Klausner, R. D. (1995) Science 269, 1402–1406
8. Lonergan, K. M., Iliopoulos, O., Ohh, M., Kamura, T., Conaway, R. C., Conaway, J. W., and Kaelin, W. G. (1996) Mol. Cell. Biol. 16, 732–741
9. Kamura, T., Roop, D. M., Conrad, M. N., Skowrya, D., Moreland, R. J., Iliopoulos, O., Lane, W. S., Kaelin, W. G., Elledge, S. J., Conaway, R. C., Harper, J. W., and Conaway, J. W. (1999) Science 284, 657–661
10. Kihel, A., Iliopoulos, O., DeCaprio, J. A., and Kaelin, W. G. (1995) Science 269, 1444–1446
11. Pause, A., Lee, S., Worrell, R. A., Chen, D. Y. T., Burgess, W. H., Linehan, W. M., and Klausner, R. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2156–2161
12. Cockman, M. E., Masson, N., Male, D. R., Jaakkola, P., Chang, G. W., Clifford, S. C., Maher, E. R., Pugh, C. W., Ratchfile, P. J., and Maxwell, P. H. (2000) J. Biol. Chem. 275, 25735–25741
13. Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Racil, P. J. (1999) Nature 399, 271–275
14. Huang, L. E., Gu, J., Schau, M., and Bunn, H. F. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7987–7992
15. Saldana, S., and Caro, J. (1997) J. Biol. Chem. 272, 22642–22647
16. Iwai, K., Yamamoto, K., Kamura, T., Minato, N., Conaway, R. C., Conaway, J. W., Klausner, R. D., and Pause, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12436–12441
17. Lisztwan, J., Imbert, G., Wirbelauer, C., Gataiger, M., and Krek, W. (1999) Genes Dev. 13, 1822–1833
18. Ohh, M., Park, C. W., Ivan, M., Hoffman, M. A., Kim, T. Y., Huang, L. E., Pavletich, N., Chau, V., and Kaelin, W. G. (2000) Nat. Cell Biol. 2, 425–427
19. Gnarra, J. R., Zhou, S., Merril, M. J., Wagner, J. K., Krumm, A., Papavassiliou, G. A., and Linehan, W. M., Zbar, B., and Lerman, M. I. (1993) Science 260, 1317–1320
Function of the β-Domain of VHL