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

by

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A thesis submitted to the School of Graduate Studies and Research
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0-612-67200-X

Abstract

Sporadic clear cell renal carcinomas (RCC) frequently harbor inactivating mutations in exon 2 of the von Hippel-Lindau (*VHL*) tumor suppressor gene. In this work, we examine the effect of the loss of exon 2-encoded β-domain function on VHL biochemical properties. Exon 2-encoded residues are not essential for VHL ability to assemble with elongin BC/Cullin-2 and to display E3-ubiquitin ligase activity *in vitro*. However, exon 2-encoded β-domain is required for VHL-mediated NEDD8 conjugation on Cullin-2, proper formation of an extracellular fibronectin matrix, assembly with fibronectin and elongation factor-1α (EF-1α), a protein that we recently found to be associated with wild-type VHL *in vivo*. Exon 2-encoded residues are also needed for VHL binding to hypoxia-inducible factor alpha (HIF-α) and for its subsequent ubiquitination. 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. Therefore, we suggest that exon 2-encoded sequences of VHL are essential for VHL nuclear/cytoplasmic shuttling and for substrate HIF-α recognition and ubiquitination.

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List of Abbreviations

ActD	Actinomycin D
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
ATP	Adenosine Triphosphate
bHLH-PAS	Basic Helix-Loop-Helix-Per-ARNT-SIM
BSA	Bovine Serum Albumin
CCD	Charge-Coupled Device
CDK	Cyclin-Dependent Kinase
cDNA	Complementary Deoxyribonucleic Acid
CI	Calpain Inhibitor I
CKI	Cyclin-Dependent Kinase Inhibitor
CNS	Central Nervous System
Cul-2	Cullin-2
DMEM	Dulbecco's Modified Eagle's Medium
DRB	5,6-Dichlorobenzimidazole Riboside
DTT	Dithiothreitol
EF-1α	Elongation Factor-1α
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
Epo	Erythropoietin
ER	Endoplasmic Reticulum

FCS	Fetal Calf Serum
GDP	Guanosine Diphosphate
GFP	Green Fluorescent Protein
GTP	Guanosine Triphosphate
Glut-1	Glucose Transporter-1
GMP-PNP	5'-Guanylyl-Imidodiphosphate Trisodium Salt
HIF-α	Hypoxia-Inducible Factor-α
HIV	Human Immunodeficiency Virus
HRE	Hypoxia-Responsive Element
kDa	Kilodalton
LOH	Loss of Heterozygosity
LMB	Leptomycin B
MEF	Mouse Embryonic Fibroblasts
mRNA	Messenger Ribonucleic Acid
NEDD8	Neural precursor cell Expressed Developmentally Down-regulated 8
NES	Nuclear Export Signal
ODD	Oxygen-Dependent Degradation Domain
PBS	Phosphate Buffered Saline
PVDF	Polyvinylidene difluoride
RCC	Renal Clear Cell Carcinoma
RPTEC	Renal Proximal Tubule Epithelial Cell

SCF	SKP1/Cdc53/F-box Protein
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TGF-α	Transforming Growth Factor-α
tRNA	Transfer Ribonucleic Acid
Ub	Ubiquitin
VBC/Cul-2	VHL/Elongin B/Elongin C/Cullin-2 Complex
VEGF	Vascular Endothelial Growth Factor
VHL	von Hippel-Lindau Gene
VHL	von Hippel-Lindau Protein
ZPR1	Zinc Finger Protein 1

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La mesure de l'homme

"Ce n'est pas celui qui critique qui est important, ni celui qui montre du doigt comment l'homme fort trébuche ou comment l'homme d'action aurait pu faire mieux. L'hommage est dû à celui ou à celle qui se bat dans l'arène, dont le visage est couvert de poussière et de sueur, qui va de l'avant vaillamment, qui commet des erreurs et en commettra encore, car il n'y a pas d'efforts humains sans erreurs et imperfections. C'est à lui ou à elle qu'appartient l'hommage, à celui ou à celle dont l'enthousiasme et la dévotion sont grands, à celui ou à celle qui se consume pour une cause importante, à celui ou à celle qui, au mieux, connaîtra le triomphe du succès, et au pis, s'il échoue, saura qu'il a échoué alors qu'il risquait courageusement.

C'est pourquoi la place de cet homme ou de cette femme ne sera jamais avec ces âmes tièdes et timides qui ne connaissent ni la victoire ni la défaite."

Mahatma K. Gandhi

CHAPTER 1

Introduction

1.1 Kidney Cancer

Renal cell carcinoma is the most common malignancy of the adult kidney. It is diagnosed in about 3900 Canadians each year and is responsible for more than 1450 death per year (Canadian Cancer Society, National Cancer Institute of Canada and Health Canada, 2000). Renal tumors have been divided into two different types based on histologic and morphologic features: 80-85% of renal cancers are of clear cell type (renal clear cell carcinoma-RCC) and 5-10% are of papillary type (Linehan *et al.*, 1995). Renal clear cell carcinoma is most commonly diagnosed between age of 50 and 70. If detected at an early stage, prior to metastasis, many patients can be cured surgically. The estimated 5-year survival for patients with disease confined to the kidney is 90-95% (Linehan *et al.*, 1997). Unfortunately, at the time of diagnosis, approximately one third of patients have already metastatic diseases (Linehan *et al.*, 1995). Because of the sequestered location of kidneys within the retroperitoneum, RCC often remains asymptomatic until the tumor begins to be locally advanced or that metastasis have reached other organs. Moreover, when a metastatic disease develops, the prognosis for long-term survival is poor, with an estimated 5-year survival of 0-20% (Linehan *et al.*, 1997). Therefore, effective treatment strategies are clearly needed.

1.2 The von Hippel-Lindau Tumor Suppressor Gene

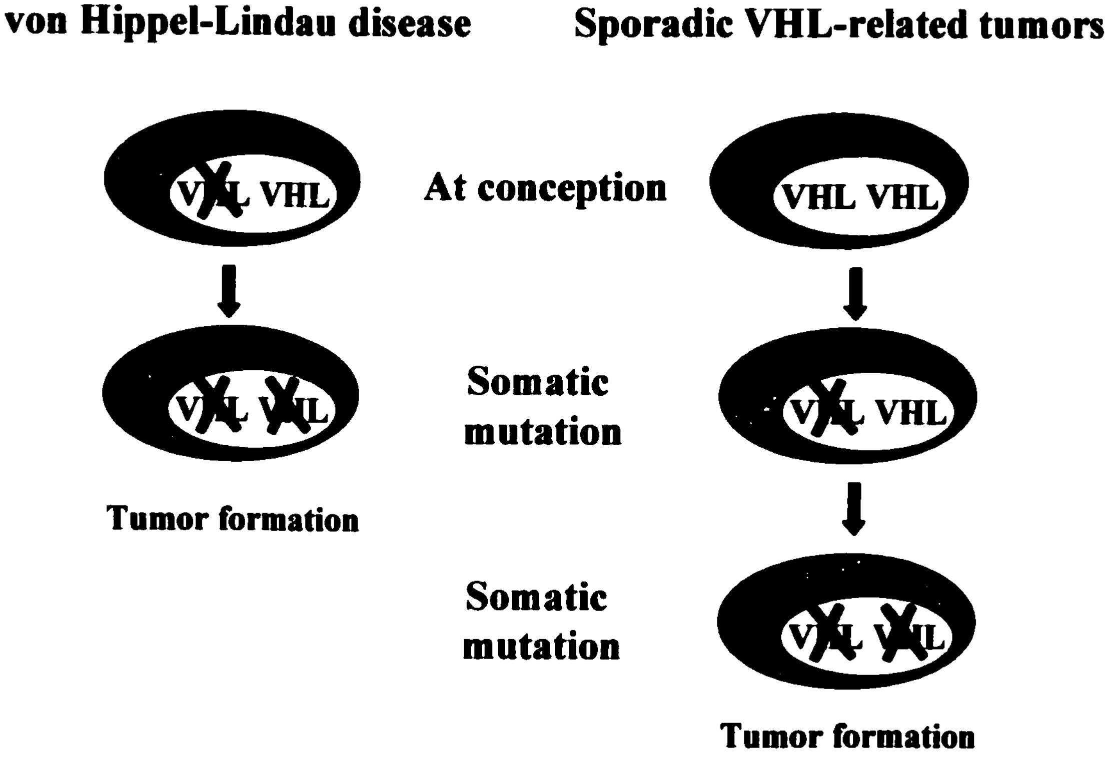
1.2.1 Inherited RCC

The elaboration of new and effective treatment strategies will be possible only if we are in measure to understand the underlying genetic and molecular basis of renal clear cell carcinoma. The fact that inherited forms of renal cancer exist and that some chromosomal abnormalities can be associated with these tumors suggests a heritable genetic determinant for renal cell carcinoma exists. The most studied form of inherited renal cancer is the von Hippel-Lindau syndrome (VHL), an autosomal dominant disorder. Patients afflicted with this disease are predisposed to develop a wide variety of highly vascularized tumors that include retinal angioma, central nervous system (CNS) hemangioblastoma, pheochromocytoma, pancreas cyst and adenoma and renal clear cell carcinoma (RCC) (Gnarra *et al.*, 1996a; Humphrey *et al.*, 1996; Latif *et al.*, 1993; Linehan *et al.*, 1995). The estimated incidence of this disease is one in 36 000 live births and the penetrance is 97% by the age 60 years (Maher *et al.*, 1991; Maher *et al.*, 1990). Between 28-45% of affected VHL patients develop renal clear cell carcinoma and the mean age of diagnosis is 39 years old (Lamiell *et al.*, 1989). The genetic evaluation of tumors from families with hereditary forms of conventional renal cancer has demonstrated the presence of *VHL* gene mutations (Bodmer *et al.*, 1998; Gnarra *et al.*, 1994; Li *et al.*, 1993; Schmidt *et al.*, 1995).

1.2.2 Sporadic RCC

The *VHL* gene is located to chromosome 3p25-p26 (Hosoe *et al.*, 1990; Seizinger *et al.*, 1988). The gene is predicted to be a tumor suppressor gene based on loss of heterozygosity (LOH) studies using tumors from VHL patients. In fact, the chromosome 3p that contains the wild-type *VHL* gene (inherited from the unaffected parent) was deleted in the VHL-associated tumors examined (Tory *et al.*, 1989). Knudson two-hit hypothesis for tumor suppressor gene predicts that both copies of a gene must be inactivated for tumor formation to occur. Therefore, sporadic tumors should arise after somatic inactivation of both copies of the same gene responsible for the corresponding hereditary cancer (Knudson, 1971; Knudson and Strong, 1972). In keeping with Knudson two-hit model, inactivation or loss of both *VHL* alleles has been demonstrated in ~80% of sporadic clear cell renal carcinomas (Foster *et al.*, 1994; Gnarra *et al.*, 1994; Herman *et al.*, 1994; Shuin *et al.*, 1994). This strongly suggests that the loss of VHL protein function (caused by inactivation of both *VHL* alleles) is a crutial step for the development of clear cell carcinoma of the kidney. Figure 1 is a diagram comparing the different steps leading to tumor formation in the case of inherited and sporadic RCC. For VHL patients that are born with a constitutional germline mutation in every cell in their body, only one additional somatic event is enough to initiate tumorigenesis. Individuals afflicted with sporadic RCC have received at conception two normal copies of the *VHL* gene. Therefore, in the case of sporadic form of RCC, tumor formation will arise when a single target cell acquires inactivating mutations on both *VHL* alleles.

Figure 1. VHL is a tumor suppressor gene. According to the two-hit hypothesis of tumor suppressor genes, biallelic inactivations of the VHL gene causes inherited as well as sporadic renal clear cell carcinoma (RCC), the most common malignancy of human kidney.


von Hippel-Lindau disease
Sporadic VHL-related tumors
VHL VHL
At conception
VHL VHL
VHL VHL
Somatic mutation
VHL VHL
Tumor formation
Somatic mutation
VHL VHL
Tumor formation

1.3 VHL: Caretaker or Gatekeeper?

Vogelsein *et al.* proposed a novel concept of tumor suppressor genes. They have suggested that tumor suppressor genes can be divided in two different classes: the caretakers (e.g. *BRCA-1, BRCA-2, p53*) and the gatekeepers (e.g. *VHL, APC, Rb* and *NF-1*) (Kinzler and Vogelstein, 1997). The caretakers are thought to play a role in genome stability and inactivation of those genes does not promote tumor initiation directly. Such genetic alterations will increase the probability of mutations of all genes including the second class of tumor suppressors, the gatekeepers. Gatekeepers are genes that directly regulate the growth of tumors by inhibiting growth or promoting cell death. It is also hypothesized that each cell type may have only one specific gatekeeper gene.

It has been suggested by Vogelstein that *VHL* may exert gatekeeper function in renal proximal tubule epithelial cells, which are thought to give rise to RCC (Kaelin and Maher, 1998; Kinzler and Vogelstein, 1997; Pause *et al.*, 1998). In fact, several evidences are in agreement with this hypothesis:

1-) Patients with VHL syndrome may develop hundred of independent solid tumors during their life span. They have 1000-5000 fold higher chance to develop tumors than the general population. These numbers are high compared to patients affected by other inherited cancer syndrome caused by caretaker genes such as Li-Fraumeni disease (*p53*). These patients have 5-25 fold higher chance to develop one tumor during their lifespan compared to the general population (Kinzler and Vogelstein, 1996).

2-) The second *VHL* allele is found to be mutated in microscopial localized tumors as in advanced tumors indicating that the mutation of the second *VHL* allele is an early event in renal carcinogenesis. Loss of heterozygosity at the *VHL* locus was even observed in early premalignant lesions of the kidney of VHL patients (Lubensky *et al.*, 1996; Zhuang *et al.*, 1995).

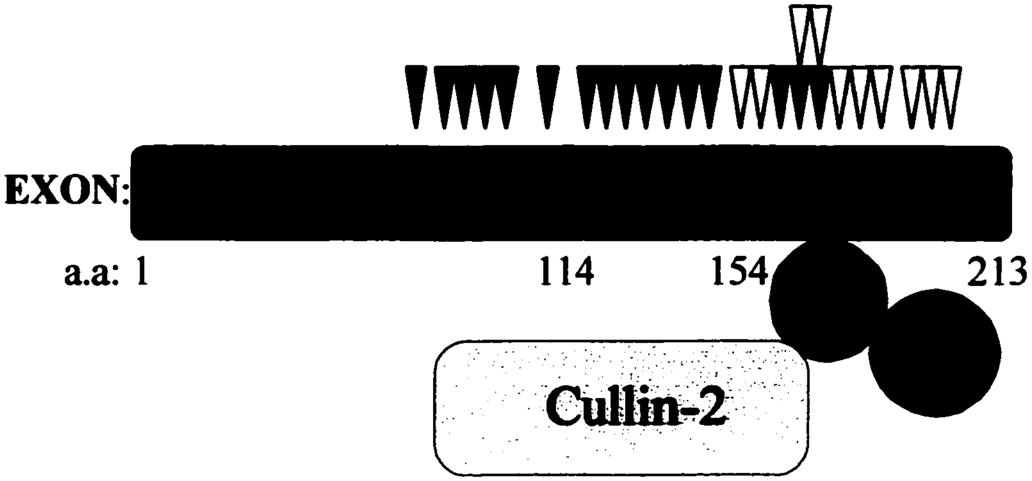
3-) Reintroduction of *VHL* is sufficient to correct several cancer-like phenotypes such as the ability to: grow in serum free media (Pause *et al.*, 1998), form tumors in nude mice (Iliopoulos *et al.*, 1995) and overproduce angiogenic factors such as VEGF, Glut-1 and TGF-α (Gnarra *et al.*, 1996b; Iliopoulos *et al.*, 1996; Lonergan *et al.*, 1998; Siemeister *et al.*, 1996; Knebelmann *et al.*, 1998). Another defect of RCC cancer cells is the inability to properly assemble an extracellular fibronectin matrix (Ohh *et al.*, 1998). This defect can also be corrected by the reintroduction of wild-type, but not mutant, VHL in $VHL^{-/-}$ RCC cells. Fibronectin is an extracellular glycoprotein that binds to heterodimeric cell surface receptors known as integrins (Hynes, 1992; Ruoslahti, 1991). Loss of fibronectin matrix assembly is a recognized feature of cellular transformation (Chen *et al.*, 1976; Hynes and Destree, 1978; Hynes *et al.*, 1978; Lipkin *et al.*, 1978; Ruoslahti, 1991; Vaheri *et al.*, 1978). In normal cells, VHL can normally bind to fibronectin associated with the endoplasmic reticulum (Ohh *et al.*, 1998).

The identification of gatekeeper genes in various tissues and the elucidation of the cellular function of the corresponding proteins will provide crucial information to understand the molecular basis of human cancer.

1.4 Characterization of the *VHL* Gene Product

The *VHL* gene was cloned in 1993 (Latif *et al.*, 1993), contains 3 exons and encodes a 213 amino acid protein (VHL) with an apparent molecular weight of 24-30 kDa following SDS-polyacrylamide gel electrophoresis (Figure 2) (Latif *et al.*, 1993; Iliopoulos *et al.*, 1995). However, a second VHL product is generated by translation initiation from an internal in-frame methionine residue at codon 54 (Blankenship *et al.*, 1999; Iliopoulos *et al.*, 1998; Schoenfeld *et al.*, 1998). This shorter form of VHL is more abundant and has an apparent molecular weight of 18-20 kDa. All functional and biochemical studies performed to date have shown that both products behave similarly. Therefore, both VHL products will be referred to as the VHL protein.

Figure 2. Schematic diagram of VHL protein. VHL has three exons that code for a 213-amino acid protein. VHL assembles with elongin B, elongin C and Cullin-2 to form the VBC/Cul-2 complex. Exon 2 is the site of frequent mutations (blue triangles) in sporadic RCC, but not in the inherited RCC of VHL syndrome. Exon 3 mutations (yellow triangles) are mostly found in familial cases of RCC. Green triangles represent sites of mutations common for both sporadic and inherited forms of RCC. This diagram is not to scale.


EXON:
a.a: 1
114
154
213
Cullin-2

1.4.1 Interaction of VHL With Elongin B, Elongin C and Cullin-2

VHL contains no functional and/or structural motifs that might provide insight as to its function (Foster *et al.*, 1994; Duan *et al.*, 1995a; Gao *et al.*, 1995). Immunoprecipitation experiments have shown that VHL assembles with at least four other proteins: Rbx1, elongin B, elongin C and Cullin-2 (the complex will be hereafter referred to as VBC/Cul-2) (Figure 2) (Duan *et al.*, 1995b; Kibel *et al.*, 1995; Lonergan *et al.*, 1998; Pause *et al.*, 1997). Interaction studies have demonstrated that VHL binds directly to elongin C, which in turn binds with a separate domain to elongin B (Takagi *et al.*, 1997), a small ubiquitin-like protein (Garrett *et al.*, 1995). Moreover, it has been shown that elongin C forms a bridge between VHL and Cullin-2 and that the binding of elongin B to elongin C stabilizes the interaction VHL/elongin C/Cullin-2 (Duan *et al.*, 1995b). VHL assembles with elongin C through residues 157-177 (exon 3) and Cullin-2 interacts with elongin C through its amino terminus (Figure 2) (Pause *et al.*, 1999). I will talk more about Rbx1 later.

1.4.2 Similarities Between VBC/Cul-2 and Yeast SCF Complexes

Elongin C and Cullin-2 share structural homology with yeast (*Saccharomyces cerevisiae*) SKP1 and Cdc53, respectively (Bai *et al.*, 1996; Lonergan *et al.*, 1998; Mathias *et al.*, 1996; Pause *et al.*, 1997). SKP1 and Cdc53 bind to one another and assemble into the SCF complex (SKP1/Cdc53/F-box protein) that target specific protein for polyubiquitination and subsequent degradation by the 26S proteasome (Hopkin, 1997; Jackson, 1996). The similarities between the structure of the SCF complex and the VBC/Cul-2 complex lead to the hypothesis that these proteins complexes might have similar functions (Figure 3) (Pause *et al.*, 1999; Stebbins *et al.*, 1999). In fact, VHL has recently been shown to be a component of an active E3 ubiquitin ligase complex involved in polyubiquitination (Iwai *et al.*, 1999; Lisztwan *et al.*, 1999). Moreover, disease-associated VHL mutants failed to show E3 ubiquitin ligase activity suggesting that this activity is crutial for VHL tumor suppressor function.

1.4.3 E3 Ubiquitin Ligase Activity of the VBC/Cul-2 Complex

Polyubiquitination is a multistep process that depends on the activities of several enzymes and leads to the formation of isopeptide bonds between C-terminal glycine residues of ubiquitin and ε-amino group of lysine residues of an acceptor protein (Figure 4). First, a ubiquitin must be activated by an ATP molecule to form a high-energy thiol ester intermediate with the E1 ubiquitin-activating enzyme. The activated ubiquitin will then be transfered to an E2 ubiquitin-conjugating enzyme from E1. The E3 ubiquitin protein-ligase (role played by the VBC/Cul-2 complex) will recruit the E2 ubiquitin-conjugating enzyme and the ubiquitin will then be transferred to a specific substrate. Finally, the polyubiquitinated protein will be degraded by the 26S proteasome (Figure 4) (Ciechanover, 1994; Hochstrasser, 1996). The SCF complex, the yeast E3 ubiquitin ligase, plays an important role in substrate recognition. The yeast SCF complex targets several substrates including G_1-cyclins, cyclin-dependent kinase (CDK) inhibitors and the CDK-inhibitory kinase SWE1, for ubiquitination (Bai *et* *al.*, 1996; Feldman *et al.*, 1997; Kaiser *et al.*, 1998; Patton *et al.*, 1998a; Skowyra *et al.*, 1997; Willems *et al.*, 1996).

1.4.4 Possible Role of NEDD8 and Rbx1 in the Ubiquitination Process

Recently, the Cdc53 subunit of yeast SCF was shown to be covalently modified by the conjugation of the ubiquitin-like protein RUB1 (Lammer *et al.*, 1998; Liakopoulos *et al.*, 1998). It was also shown that this modification of Cdc53 by RUB1 proceeds analogously to ubiquitination and with similar enzymes but it does not result in the targeting of the Cdc53 protein for degradation (Lammer *et al.*, 1998; Liakopoulos *et al.*, 1998).

NEDD8 (Neural precursor cell Expressed Developmentally Down-regulated 8), a ubiquitin-like protein (Kamitani *et al.*, 1997), is presumed to be the human homolog of RUB1, displaying 59 % homology with the yeast protein. (Kumar *et al.*, 1992). NEDD8 has been shown to modify Cullin-2 of the VBC/Cul-2 complex but only when VHL was present as a part of the complex (Figure 5) (Liakopoulos *et al.*, 1999). In fact, tumorigenic-mutant forms of VHL which are unable to form the VBC/Cul-2 complex, fail to promote NEDD8 conjugation to Cullin-2 (Liakopoulos *et al.*, 1999). However, more recent studies have suggested that VHL promotes, but is not essential for NEDD8 conjugation to Cullin-2 (Wada *et al.*, 1999). Wada *et al.* have observed low level of Cullin-2 neddylation (NEDD8 conjugation) in VHL$^{-/-}$ 786-0 cells, suggesting that VHL is not the only molecule implicated in NEDD8 conjugation to Cullin-2 (Wada *et al.*, 1999). Taken together, the ligation of NEDD8 to Cullin-2 of the VBC/Cul-2 complex appears to be linked to VHL activity.

The regulation and biochemical significance of Cullin-RUB1 or NEDD8 conjugation are not clear at present. However, recent studies have shown that disruption of NEDD8 modification in both mammalian and fission yeast cells, significantly reduced the level of *in vitro* SCF ubiquitin ligase activity of Cullin-1 (Furukawa et al., 2000; Morimoto *et al.*, 2000; Podust *et al.*, 2000; Read *et al.*, 2000). In addition, the same studies have demonstrated that NEDD8 modification of Cullin-1 of the SCF complex stimulates the ability of the complex to support efficient ubiquitin polymerization, leading to degradation of the targeted substrate (Furukawa et al., 2000; Morimoto *et al.*, 2000; Podust *et al.*, 2000; Read *et al.*, 2000; Wu *et al.*, 2000).

Kamura *et al.* have shown that the conjugation of RUB1 to Cdc53 is activated by the presence of another protein, Rbx1 (Kamura *et al.*, 1999). Similarly, NEDD8 modification of human Cullin-2 is also dependent on the presence of Rbx1 (Kamura *et al.*, 1999). The RING-H2 finger protein Rbx1 (also called Hrt1 or ROC1) is a subunit of both the SCF and the VBC/Cul-2 complexes (Bai *et al.*, 1996; Feldman *et al.*, 1997; Lisztwan *et al.*, 1999; Lyapina *et al.*, 1998; Patton *et al.*, 1998ab; Skowyra *et al.*, 1997). Studies on the mechanism of action of Rbx1 suggest that it would also activate the ubiquitination process of both complexes (Kamura *et al.*, 1999). The exact mechanism by which Rbx1 can activate these processes (neddylation and ubiquitination) is still unknown. Since NEDD8 is localized primarily in the nucleus (Kamitani *et al.*, 1997), Furukawa et al. have suggested that Rbx1 facilitates *in vivo* NEDD8 modification of Cullin-1 (Cdc53 homolog) by promoting its nuclear accumulation. Neddylation possibly enhances the ubiquitination process, therefore, Rbx1-mediated Cullin-1 nuclear accumulation might explain the simultaneous effect of Rbx1 on both processes.

Figure 3. Similarities between the VBC/Cul-2 complex and the yeast SCF complex. A) SKP1 and Cdc53 bind to one another and assemble with Cdc4 to form the yeast SCF complex. B) Elongin C, elongin B and Cullin-2 assemble with VHL to form the VBC/Cul-2 complex. Elongin C and Cullin-2 share structural homology with yeast SKP1 and Cdc53, respectively. This diagram is not to scale.

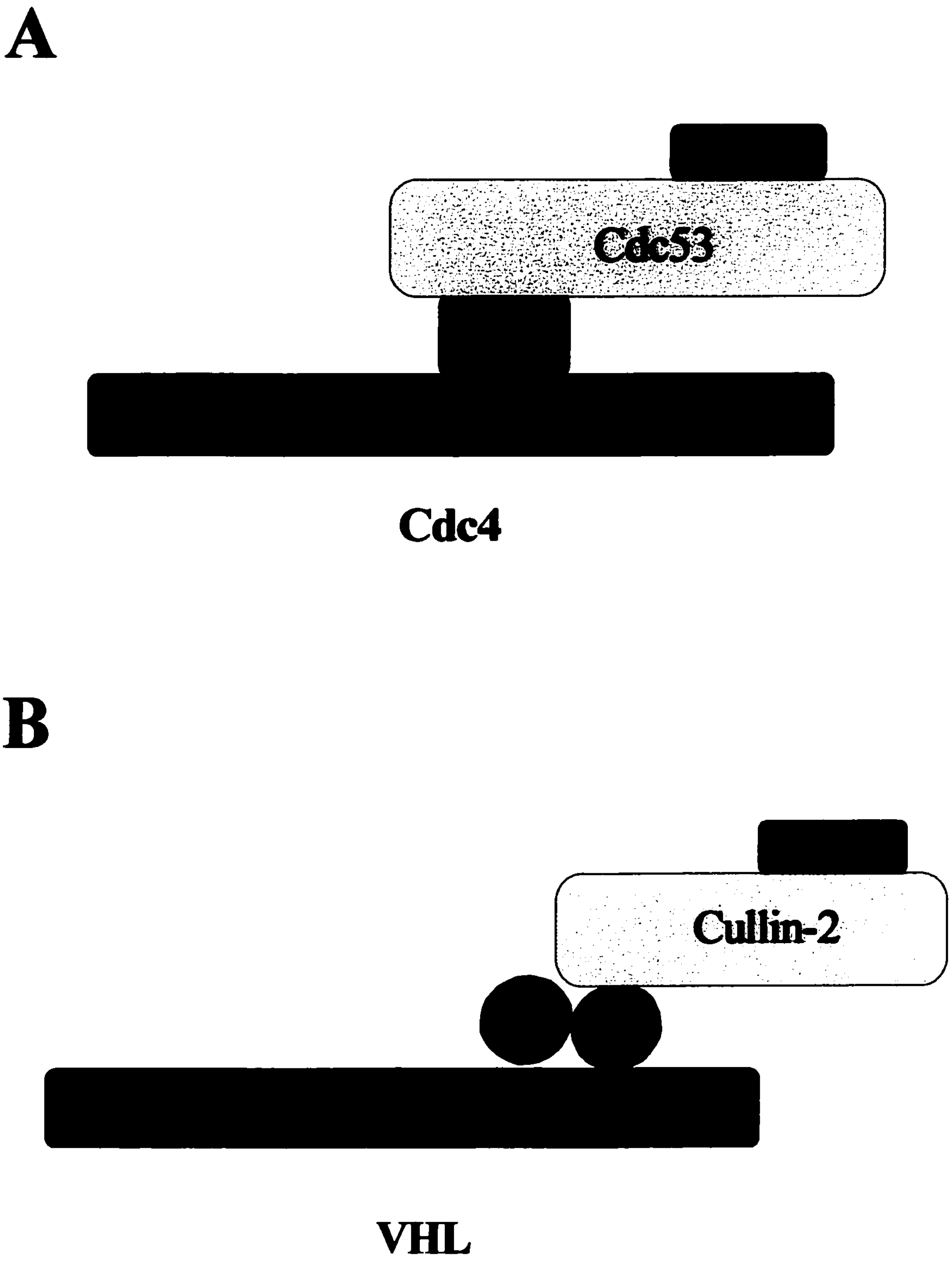

A
Cdc53
Cdc4
B
Cullin-2
VHL

Figure 4. The VHL tumor suppressor protein is a component of an active E3 ubiquitin ligase complex that is involved in the targeting of proteins for ubiquitination. Ubiquitination is a multistep process that depends on the activity of several enzymes. A ubiquitin molecule (Ub) must be first activated by an ATP to form a high-energy thiol ester intermediate with the E1 ubiquitin-activating enzyme (1). The activated ubiquitin molecule will then be transfered to an E2 ubiquitin-conjugating enzyme (2). Finally, in the presence of an E3 ubiquitin protein-ligase (the substrate recognition particule), the E2 ubiquitin-conjugating enzyme will transfer the ubiquitin molecule to a specific substrate (3). At the end, polyubiquitinated proteins are degraded by the 26S proteasome (4-5). This diagram is not to scale.

Ubiquitination: A Multistep Process

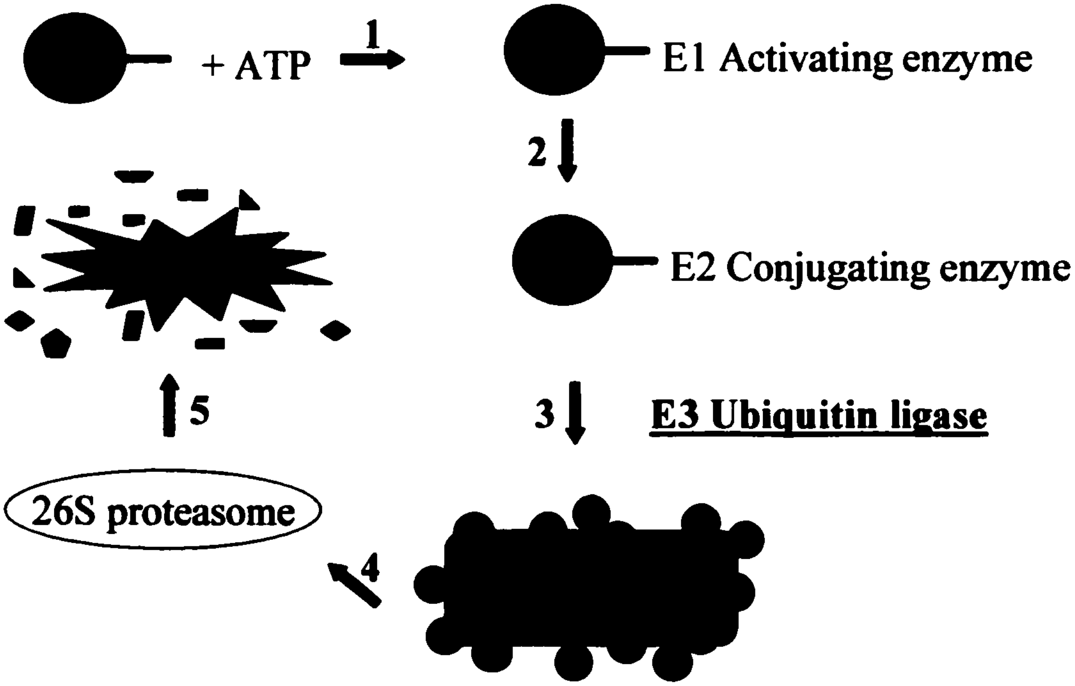
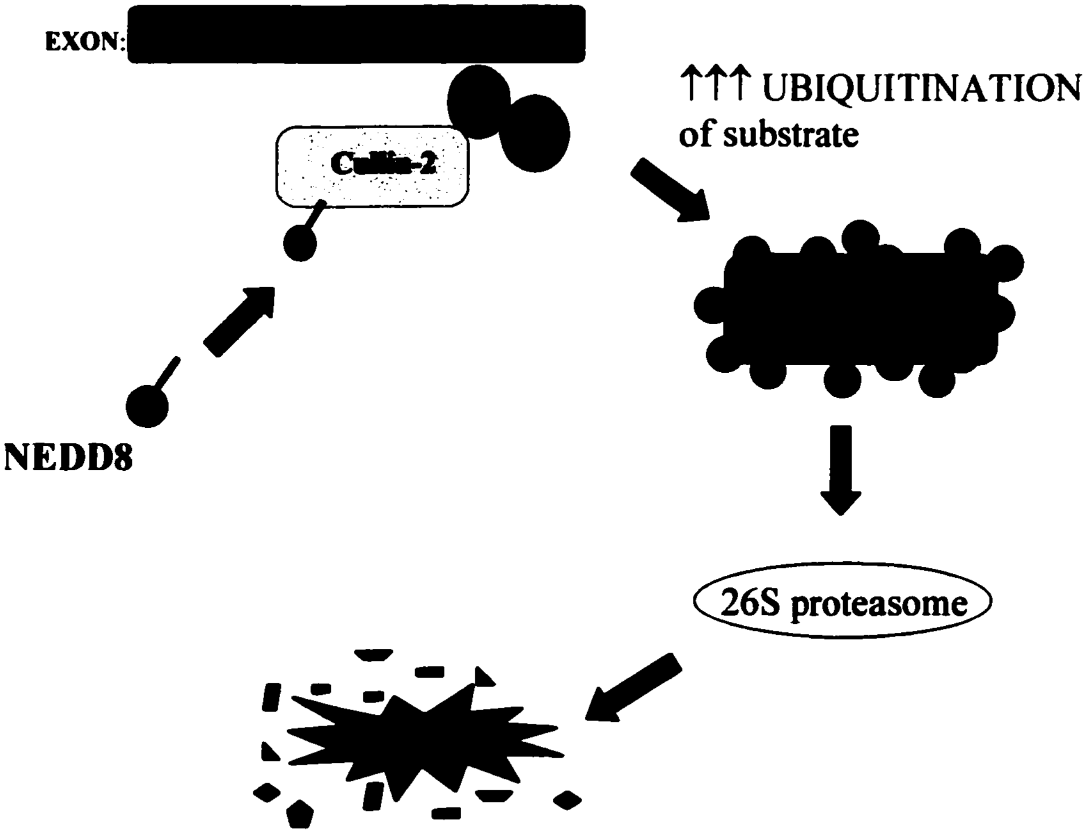


Figure 5. NEDD8 conjugation to Cullin-2: possible role in the ubiquitination process. The ligation of NEDD8 to Cullin-2 of the VBC/Cul-2 complex appears to be linked to VHL activity and might be important for its tumor suppressor function. We suggest a model in which neddylation of Cullin-2 would enhance the ability of the VBC/Cul-2 complex to promote substrate ubiquitination and subsequent degradation. This diagram is not to scale.


EXON:
↑↑↑ UBIQUITINATION
of substrate
Cullin-2
NEDD8
26S proteasome

1.5 Which Protein is Targeted by the VBC/Cul-2 Complex for Ubiquitination?

1.5.1 Post-Transcriptional vs Transcriptional Control of Hypoxia-Inducible mRNAs

A well-established characteristic of VHL-associated tumors is that they are generally highly vascularized (Kaelin and Maher, 1998). In fact, it has been shown that angiogenic factors such as erythropoietin (Epo), glucose transporter-1 (Glut-1), vascular endothelial growth factor (VEGF) and transforming growth factor-alpha (TGF-α) are constitutively expressed in cells lacking VHL (Krieg *et al.*, 1998; Wenger and Gassmann, 1997; Wizigmann-Voos *et al.*, 1995). Those factors are generally produced in larger quantities in normal cells only under hypoxic (low oxygen) conditions. This is not the case for VHL$^{-/-}$ cells, which have high expression levels of those hypoxia-inducible mRNA, even at ambient oxygen concentration (Gnarra *et al.*, 1996b; Iliopoulos *et al.*, 1996; Siemeister *et al.*, 1996; Stratmann *et al.*, 1997). Many hypoxia-inducible genes have been shown to contain hypoxia-responsive elements (HREs) (Guillemin and Krasnow, 1997; Wenger and Gassmann, 1997). Under hypoxic conditions, these HREs are recognized by the transcription activator HIF alpha subunits (hypoxia-inducible factor-α) (Gradin *et al.*, 1996; Wang *et al.*, 1995). HIF-α expression is required for normal physiological response to hypoxia (Carmeliet *et al.*, 1998; Iyer *et al.*, 1998; Ryan *et al.*, 1998; Semenza, 1999). Therefore, it has been suggested that HIF-α would be one potential target for VBC/Cul-2-mediated degradation. In keeping with this hypothesis, upon the loss of VHL function in RCC, HIF-α would accumulate to high levels, regardless of oxygen concentration. This would cause VHL$^{-/-}$ RCC to undergo a constitutive hypoxia-like response associated with the overexpression of angiogenic factors leading to hypervascularization of VHL disease-associated tumors. Nontheless, earlier studies have suggested that hypoxia-inducible mRNAs might be regulated at the level of transcription (Mukhopadhyay *et al.*, 1997) or at the level of mRNA stability (Gnarra *et al.*, 1996b; Iliopoulos *et al.*, 1996; Knebelmann *et al.*, 1998; Lonergan *et al.*, 1998; Siemeister *et al.*, 1996). In fact, Iliopoulos *et al.* did show a 4-fold decrease in VEGF mRNA stability in VHL-expressing 786-0 cells (Iliopoulos *et al.*, 1996). Therefore, other mechanisms might explain why hypoxia-inducible mRNAs are found to be more abundant in VHL$^{-/-}$ RCC cells: 1- VHL fails to degrade HIF-α and an increased transcription of hypoxia-inducible genes occurs, 2- HIF-α protein itself indirectly confers a higher stability to hypoxia-inducible mRNAs, 3- VHL degrades or controls a protein other than HIF-α that would regulate the fate of hypoxia-inducible mRNAs.

1.5.2 α and β Subunits of the Hypoxia-Inducible Factor (HIF)

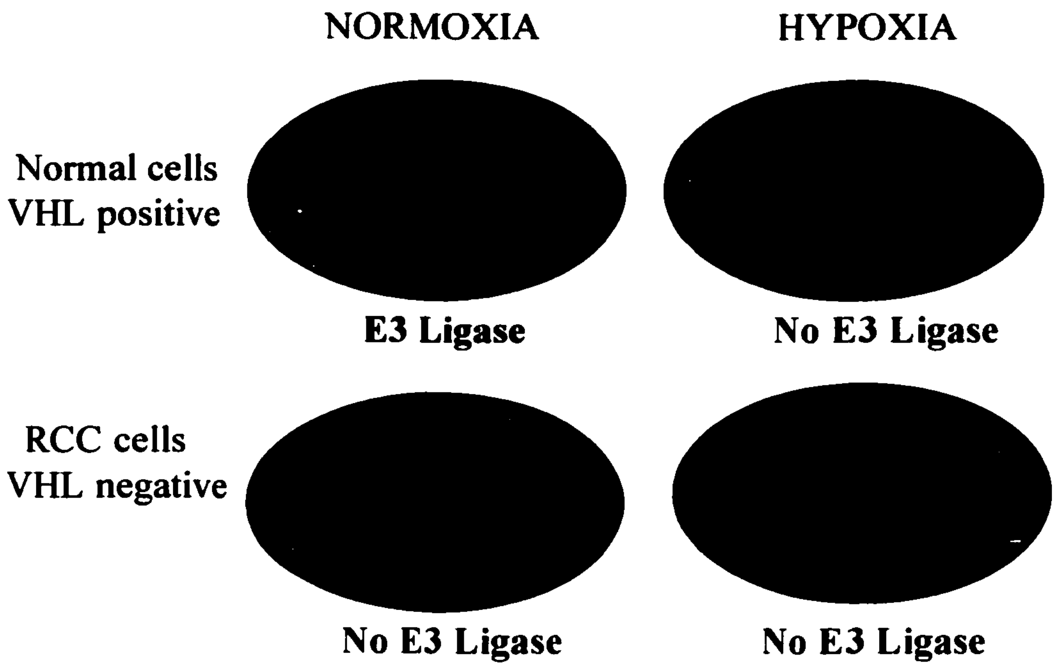
The hypoxia-inducible factor (HIF) is a heterodimeric basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) domain protein that consists of an alpha (HIF-α) and a beta subunit (HIF-β) (Wang *et al.*, 1995). As mentionned above, HIF alpha subunit is present in VHL$^{+/+}$ cells only in hypoxic conditions. In the case of the beta subunit of HIF, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) (Gassmann and Wenger, 1997; Hankinson, 1995; Schmidt and Bradfield, 1996), its expression is oxygen-independent (Huang *et al.*, 1996). Analysis of structure and function of HIF-α have revealed that the N-terminal part of the protein (containing the bHLH-PAS domain) is required for DNA binding (Jiang *et al.*, 1996; Reisz-Porszasz *et al.*, 1994). The C-terminal part of HIF-α contains domains responsible for HIF nuclear localization, protein stabilization and transactivation (Huang *et al.*, 1998; Jiang *et al.*, 1996; Jiang *et al.*, 1997; Kallio *et al.*, 1998). As for HIF-β (ARNT), previous studies have shown that it is indispensable for HIF-α DNA binding and transactivation (Forsythe *et al.*, 1996; Gassmann and Wenger, 1997; Gradin *et al.*, 1996; Salceda *et al.*, 1996; Wood *et al.*, 1996) but is not required for accumulation and nuclear translocation of HIF-α in hypoxia (Chilov *et al.*, 1999).

1.5.3 VHL Targets HIF-α for Oxygen-Dependent Degradation

Studies have shown that hypoxia does not modify HIF-α mRNA levels, suggesting that HIF-α protein content is regulated by a post-transcriptional mechanism involving stabilization of HIF-α protein levels (Huang *et al.*, 1996; Kallio *et al.*, 1997; Salceda and Caro, 1997). Moreover, studies have shown that HIF-α contains an oxygen-dependent degradation domain (ODD) constituted of residues 401 to 603. The removal of this ODD domain of HIF-α was shown to stabilize the protein even in presence of normal concentration of oxygen (20%) (Huang *et al.*, 1998). Maxwell *et al.* have shown that VBC/Cul-2 and HIF-α co-immunoprecipitate, and that VBC/Cul-2 is present in the hypoxic HIF-α DNA-binding complex (Maxwell *et al.*, 1999). Moreover, other studies have clearly shown that residues 530 to 652 of HIF-α are required for the binding to VHL (Ohh *et al.*, 2000). The fact that the domain required for binding to VHL and the ODD domain of HIF-α are overlapping strongly suggests that VHL is implicated in the destabilization of HIF-α protein in normoxia. Many studies have clearly confirmed that the VBC/Cul-2 complex is in fact an E3 ubiquitin ligase that targets the alpha subunit of HIF for oxygen-dependent degradation (Figure 6) (Cockman *et al.*, 2000; Huang *et al.*, 1998; Iwai *et al.*, 1999; Lisztwan *et al.*, 1999; Maxwell *et al.*, 1999; Ohh *et al.*, 2000; Salceda and Caro, 1997).

Figure 6. VBC/Cul-2 mediates the oxygen-dependent proteolysis of the hypoxia-inducible factor-α subunit (HIF-α). The loss of VHL function causes RCC cells to express high levels of the hypoxia-inducible factor α subunit (HIF-α). HIF-α accumulates to high levels regardless of the oxygen concentration since VHL E3 ubiquitin ligase function is abrogated. This results in the overexpression of angiogenic factors such as vascular endothelial growth factor (VEGF), glucose transporter-1 (Glut-1) and transforming growth factor-α (TGF-α).

Oxygen-Dependent Degradation of HIF by VHL



1.6 Why *VHL* Inactivation Would Lead to Carcinogenesis in the Kidney?

1.6.1 Role of VHL in the Regulation of the Cell Cycle Exit

As mentionned above, VHL$^{-/-}$ RCC tumors are highly vascularized because of HIF-α-mediated overproduction of angiogenic factors. However, this characteristic does not explain tumorigenesis. What happens exactly after the inactivation of the second *VHL* allele? A well-known characteristic of the majority of cancer cells is their inability to exit the cell cycle by serum deprivation. In fact, Pause *et al.* have shown that VHL-negative RCC cells as other cancer cells continue to grow in low serum (Pause *et al.*, 1998). They have also shown that the reintroduction of VHL in these cells restores their ability to exit the cell cycle and enter G_0/quiescence in low serum (Pause *et al.*, 1998). Moreover, the cell cycle exit of VHL-expressing RCC cells was accompanied by an increase level of p27, a cyclin-dependent kinase inhibitor (CKI) (Pause *et al.*, 1998). It was previously shown that p27 levels normally increase in quiescent cells and rapidly decrease after serum re-stimulation (Elledge and Harper, 1994; Sherr and Roberts, 1995). VHL was the first tumor suppressor found to be involved in the regulation of cell cycle exit. This finding was consistent with the gatekeeper function of VHL in the kidney.

1.6.2 Overexpression of TGF-α Upon the Loss of VHL may Explain Tumor Initiation in RCC

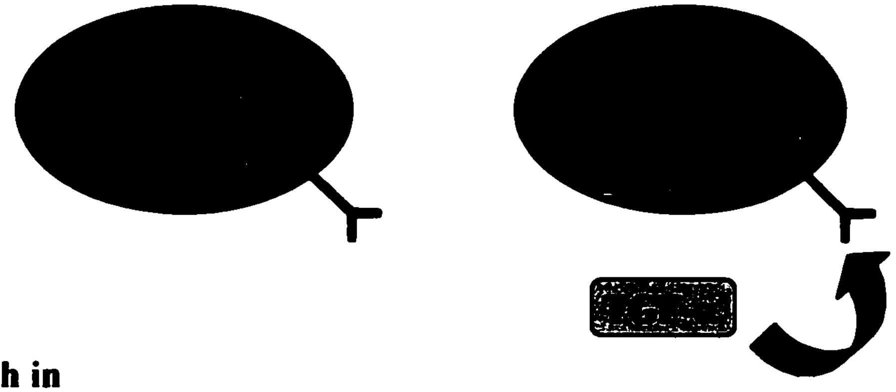
The transforming growth factor-α (TGF-α) is a VHL-regulated growth and angiogenic factor. The reintroduction of VHL in VHL$^{-/-}$ human RCC cell line 786-0 causes a decrease in TGF-α levels in a manner reminescent of VEGF or Glut-1 (Knebelmann *et al.*, 1998). Aside from its established role in angiogenesis (Lee *et al.*, 1995), TGF-α can act as a specific growth factor for renal proximal tubule epithelial cells (RPTECs), which are believed to give rise to RCC (Gomella *et al.*, 1989; Humes *et al.*, 1991). Recent studies have shown in fact that TGF-α stimulates the proliferation of VHL$^{-/-}$ RCC cells through the stimulation of the epidermal growth factor receptor (EGFR) (de Paulsen *et al.*, 2001). Also, it was shown that the high level of TGF-α present in VHL$^{-/-}$ RCC cells is sufficient to promote the growth of those cells without the need for any other exogenous growth factors (Figure 7) (de Paulsen *et al.*, 2001). Moreover, interfering with this TGF-α/EGFR autocrine loop, significantly reduces the ability of VHL$^{-/-}$ RCC cells to grow in low serum (de Paulsen *et al.*, 2001). Another interesting finding in agreement with these results is the fact that the blockade of the EGF receptors prevents RCC tumor formation in nude mice (Ciardiello *et al.*, 1998; Prewett *et al.*, 1998). Therefore, it seems that the loss of VHL in RPTEC, resulting in TGF-α overproduction, may be the event that confers growth advantage to RCC cells and lead to tumor formation.

Figure 7. The transforming growth factor-α (TGF-α) might provide a growth advantage to RCC cells upon the loss of VHL tumor suppressor protein function. Upon the loss of VHL function in RCC, HIF-α accumulates to high levels, regardless of the oxygen concentration. This causes VHL$^{-/-}$ RCC to undergo a constitutive hypoxia-like response associated with the overexpression of vascular endothelial growth factor (VEGF), glucose transporter-1 (Glut-1) and TGF-α. Since TGF-α can act as a specific growth stimulatory factor for kidney cells, it is hypothesized that the loss of VHL function causes RCC by enabling these cells to undergo a TGF-α-mediated autocrine loop.

Transforming Growth Factor-α: TGF- α

Normal cells
VHL positive

RCC cells
VHL negative



Growth in low serum: **NO** **YES**

1.7 Subcellular Localization and Trafficking of VHL

Another characteristic of VHL is the fact that it localizes predominantly to the cytoplasm but with some nuclear signal (Corless *et al.*, 1997; Duan *et al.*, 1995a; Iliopoulos *et al.*, 1995; Lee *et al.*, 1996; Los *et al.*, 1996). Like several proteins that are localized between the nucleus and the cytoplasm, it was shown that VHL shuttles between these two compartments (Corless *et al.*, 1997; Iliopoulos *et al.*, 1995; Lee *et al.*, 1996; Los *et al.*, 1996; Tsuchiya *et al.*, 1996; Ye *et al.*, 1998). Moreover, this nucleocytoplasmic trafficking requires ongoing transcription (Lee *et al.*, 1999). In fact, inhibition of the transcription with a RNA polymerase II-specific inhibitor (5,6-dichlorobenzimidazole riboside-DRB) causes VHL to be redistributed to the nucleus, a consequence of a decreased nuclear export rate (Lee *et al.*, 1999). The same effect can be observed with other transcription inhibitors such as Actinomycin D (ActD) or α-amanitin (Lee *et al.*, 1999). Also, ongoing protein synthesis is not required for the redistribution effect of transcriptional inhibitors (Lee *et al.*, 1999). This suggests that the effect obtained with DRB or ActD treatment on VHL localization is the result of inhibition of new RNA synthesis (Lee et al., 1999). Also, the shuttling of VHL is insentitive to leptomycin B (LMB), a drug that affects CRM1-mediated nuclear export of protein containing a classical, leucine-rich nuclear export signal (NES) (Nishi *et al.*, 1994; Wolff *et al.*, 1997). When VHL is fused to a classical NES and induced to shuttle in a leptomycin-sensitive and transcription-insensitive manner, it is unable to function as a negative regulator of Glut-1 levels (Lee *et al.*, 1999).

These observations suggest that VHL exports from the nucleus through a CRM1-independent pathway and that the proper nucleocytoplasmic shuttling of VHL is required for its tumor suppressor function. More recently, our group has shown that the nuclear export of VHL-fused to the green fluorescent protein (VHL-GFP) is Ran-mediated and ATP hydrolysis-dependent (Groulx *et al.*, 2000). Moreover, the association of VHL with Cullin-2 is not required for the export of VHL-GFP (Groulx *et al.*, 2000). These results suggest that VHL must contain a nuclear export domain, which plays a role in the nuclear export of the VBC/Cul-2 complex.

1.8 Nuclear Export of Many Nuclear Proteins is Required for Their Proteasome-Dependent Degradation

Hdm2 is another E3 ubiquitin ligase that shuttles between the nucleus and the cytoplasm (Honda *et al.*, 1997; Roth *et al.*, 1998). Inhibition of nuclear export of Hdm2 results in the accumulation of p53, suggesting that Hdm2 shuttling is required for p53 degradation in the cytoplasm (Roth *et al.*, 1998; Freedman and Levine, 1998). p53 is a tumor suppressor protein that has an important role in cellular mechanisms required to maintain genomic integrity in response to cellular stress (Ko and Prives, 1996; Levine, 1997). Hdm2 utilizes a classical nuclear export signal (NES) to export from the nucleus. In fact, leptomycin B treatment of various cell lines led to an increase in the steady-state levels of the p53 protein as a result of an increase in its stability (Freedman and Levine, 1998). p53 tumor suppressor protein is present at low levels in the cells. In response to physiological stress, p53 protein levels rise and it becomes active as a transcription factor. p53 induces the transcription of genes such as Bax, p21 and GADD45 that induce growth arrest and apoptosis (el-deiry *et al.*, 1993; Kastan *et al.*, 1992; Miyashita and Reed, 1995; Okamoto and Beach, 1994). p53 protein also induces transcription of Hdm2, its negative regulator, creating an autoregulatory feedback loop (Wu *et al.*, 1993).

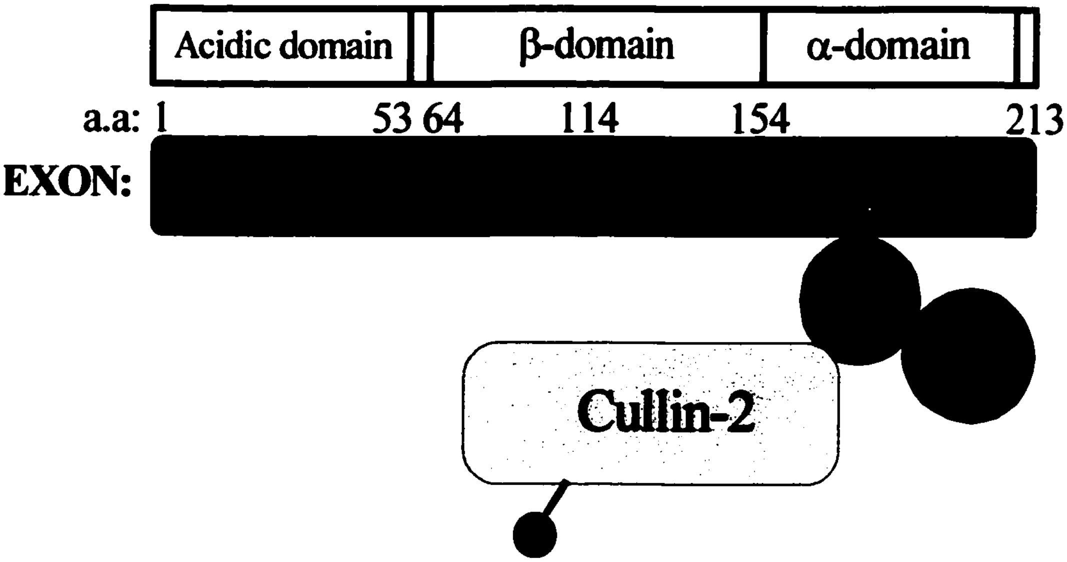
Other proteins also need nuclear export in order to be degraded by the proteasomes. One example is $p27^{kip1}$, a protein that acts at a particular stage of the cell cycle to inhibit another group of cell-cycle proteins. Claret *et al.* have found that $p27^{kip1}$ interacts with an other protein called Jab1 (Claret *et al.*, 1996). They found that increasing the levels of Jab1 causes increased breakdown of $p27^{kip1}$. Tomoda et al. have shown that binding of Jab1 to $p27^{kip1}$ causes relocalization of $p27^{kip1}$ from the nucleus to the cytoplasm (Tomoda *et al.*, 1999). They have also shown that $p27^{kip1}$ is not degraded in the presence of leptomycin B, a drug that affects the nuclear export of proteins from the nucleus. These results clearly indicate that Jab1 controls the activity of $p27^{kip1}$ by promoting its nuclear export and subsequent degradation. Whether Jab1 is a component of the ubiquitin/proteasome system or only a transporter of $p27^{kip1}$ is not yet clear.

These two examples, together with the E3 ubiquitin ligase and shuttling activity of VHL provide indications that nuclear export of many nuclear proteins might be required for their proteasome-dependent degradation.

1.9 Distribution of Tumor-Derived Inactivating Mutations

The crystal structure of VHL has been reported. VHL mainly consists of two independent domains: a large β-domain that spans residues 64 to 154 and an α-helical domain (α-domain) that encompasses most of the C-terminal part of the protein (residues 157-189) (Figure 8) (Stebbins *et al.*, 1999). Tumor-derived inactivating mutations (279 entries; Beroud *et al.*, 1998) are found across the VHL protein indicating that both domains play a critical role in VHL tumor suppressor function (Figure 2) (Stebbins *et al.*, 1999). 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 (Chen *et al.*, 1995). 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 (Gnarra *et al.*, 1994), whereas, sporadic RCC frequently harbor inactivating point mutations in exon 2 (Figure 2). This includes point mutations at the exon 2 boundary that cause a splice defect producing a mRNA that lacks exon 2 sequences altogether (Gnarra *et al.*, 1994). Exon 2 mutations are rare in VHL patients and it has been suggested that such mutations might not be easily tolerated and thus not transmitted in the germline (Gnarra *et al.*, 1996a). The discrepancy in the distribution of mutations between sporadic and inherited RCC suggests that exon 2 and exon 3-associated mutations probably inactivate VHL function in different ways.

Figure 8. The crystal structure of VHL. VHL is a 213 amino acid protein containing an acidic domain, a large β-domain that spans residues 64 to 154 and an α-helical domain (α-domain) that encompasses most of the C-terminal part of the protein (residues 157-189). Mutations in the α-domain are more frequent in the inherited form of RCC whereas sporadic RCC harbor inactivating point mutations in exon 2 (β-domain) of VHL. This diagram is not to scale.


Acidic domain
β-domain
α-domain
a.a: 1
53
64
114
154
213
EXON:
Cullin-2

1.10 Objectives of Study and Hypotheses

As I mentionned before, VHL patients have several naturally occurring mutations within exon 3 of the *VHL* gene. The role of a few residues within this exon is well understood since they correspond to the ones required for VHL binding to elongin C and formation of the VBC/Cul-2 complex (Duan *et al.*, 1995b; Kibel *et al.*, 1995; Kishida *et al.* 1995; Lonergan *et al.*, 1998). However, sporadic cases of RCC frequently harbor inactivating point mutations in exon 2. It has been shown that a splicing defect of exon 2, resulting in a VHL protein lacking amino acids 114-154, is also often found in sporadic RCC (Gnarra *et al.*, 1994).

Exon 2-encoded residues 114-154 are mostly hydrophobic and form three of the seven β-strands of the β-domain (Stebbins *et al.*, 1999). 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 HIF-α (Tanimoto *et al.*, 2000). Also, it was suggested by Lee *et al.* (1999) that transcription-dependent trafficking of VHL is mediated by sequences encoded by exon 2. This statement was based on the observation that a deletion mutant of exon 2, as opposed to wild-type VHL, failed to accumulate in the nucleus upon arrest of transcription with an actinomycin D (Act D) treatment. However, the exact details concerning the role that exon 2-encoded sequences play in VHL-mediated tumor suppression function are not yet elucidated. **Furthermore, it is unclear why inactivating mutations in exon 2 of *VHL* cause a more severe and aggressive form of RCC.**

In this thesis, we further examine the role of these sequences in cells by comparing a tumor-derived VHL mutant that lacks residues 114-154 to the known biochemical properties of wild-type VHL and mutant VHL lacking the exon 3-encoded α-domain. In particular, we wanted to look at the ability of these three VHL constructs to:

1- bind to Cullin-2 and HIF-α

2- act as an E3 ubiquitin ligase

3- properly assemble an extracellular fibronectin matrix

4- shuttle in a transcription-dependent manner

5- mediate oxygen-dependent degradation of HIF-α

Hypotheses:

-Exon 2-encoded β-domain of VHL is involved in substrate recognition and nucleocytoplasmic trafficking.

-Exon 2-encoded β-domain of VHL is required to mediate the oxygen-dependent degradation of HIF-α.

CHAPTER 2

Methods

2.1 Cell Culture, Transfections and Adenoviral Infections

The VHL$^{-/-}$ 786-0 RCC cells and HeLa cells were obtained from the American Type Culture Collection (Rockville, MD). 786-0 renal carcinoma have undergone loss of heterozygosity at the *VHL* locus, and the remaining *VHL* allele contains a frameshift mutation (Gnarra *et al.*, 1994). The VHL-GFP cell line corresponds to 786-0 cells stably transfected with the VHL-GFP fusion protein as described elsewhere (Lee *et al.*, 1999). The 117-4 (VHL$^{-/-}$; referred to as 117) cells were a kind gift from Dr James R. Gnarra (LSU Health Sciences Center, New Orleans, LA, USA). The mouse embryonic fibroblasts (MEF) nullizygous for HIF-1α were a kind gift from Dr. Randy Johnson (Department of Biology, University of California, SD, USA) (Ryan *et al.*, 2000). Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 100 units/ml Penicillin and 100 μg/ml Streptomycin in a 37°C, humidified, 5% CO_2-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.

2.2 Expression Vectors and Constructs

The human VHL cDNA, which codes for a 213-amino acid VHL protein, was subcloned into pcDNA 3.1(-) (Invitrogen) vector in the Nhe I restriction site. 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; Stauber *et al.*, 1995) was subcloned downstream and in-frame of VHL cDNA (between Nhe I and BamHI sites) to produce the VHL-GFP fusion protein. A deletion mutant of the last 56 amino acids was fused to GFP to produce the ΔE3-GFP fusion protein. Another deletion mutant of VHL lacking amino acids 114-154 was fused to GFP to produce the E2-GFP fusion protein. Both deletion mutants were cloned in the NheI sites of pcDNA 3.1(-) using standard PCR, double PCR methods and cloning methods. Two GFP in tandem were also cloned into pcDNA 3.1(-) to produce the GFP-GFP fusion protein. VHL-GFP-NES, ΔE2-GFP-NES and GFP-GFP-NES were produced by fusion of VHL-GFP, ΔE2-GFP and GFP-GFP at their C-terminus to the strong NES of HIV Rev, LPPLERLTL (NES) (Fisher *et al.*, 1995). All constructs were verified by standard DNA sequencing.

2.3 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 (Chen *et al.*, 1996). The pAdlox vector and the Ψ5 viral DNA were also obtained from Dr. Park. VHL-GFP was subcloned into the HindIII and BamH1 sites. Mutant VHL were subcloned in the endogenous Not1 site of VHL at residue 60 and BamH1. Transfections were done according to Graham and van der Eb (1973). Typically, a confluent 10-cm diameter dish of CRE8 cells (1.6×10^7) was split into 5- to 6-cm diameter dishes for transfection 2 to 4 hours later. Each dish received 3 μg of pAdlox vector (containing the foreign VHL construction) and 3 μg of ψ5 viral DNA in a final volume of 0.5 ml of $CaPO_4$, which was applied to the cells on day 1. After sixteen hours, the 10% FCS DMEM was changed for 2% FCS DMEM. Cells were fed again with fresh 2% DMEM after 64 hours. Between day 8 and 10, we had a sizable infection in each dish: almost all the cells were rounded up or floating. Cells were harvest and resuspended in their culture media (no need for trypsin: infected CRE8 cells are detached easily from the bottom of the dish). Lysates were prepared by freezing/thawing three times with an alternating liquid N_2/37°C water bath. Each virus was then passed sequentially through CRE8 cells twice (10-cm diameter dish infected with 200 μl of lysate and cells are harvest after 48 hours, etc.). Finally, a plaque purification assay was performed in order to isolate the recombinant adenovirus expressing adVHL-GFP, adΔE2-GFP or adΔE3-GFP. Plaques containing the recombinant adenovirus were identified by looking at the GFP fluorescence under the inverted microscope. Each recombinant adenovirus were then amplified in CRE8 cells to obtain larger quantities. The amount of lysate required to obtain 90-100% of cells infected was determined experimentally for each adenovirus.

2.4 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-NES, ΔE2-GFP-NES and GFP-GFP-NES. Cells were washed with PBS and incubated for 2 hours 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 (Nishi *et al.*, 1994; Wolff *et al.*, 1997).

2.5 *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 inhibitors (2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A) for 30 min. at 4°C. Whole cell lysates were first immunoprecipitated with anti-FLAG M2 monoclonal antibody. Precipitates were washed 5 times with a buffer containing 20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2 mM DTT. 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 (complete mixture). The reaction was stopped after 2 hours incubation at 37°C by adding 4X SDS loading buffer. Samples were boiled 10 min. and separated on an 8% SDS-PAGE and blotted onto a PVDF membrane. Blots were blocked and incubated in the presence of a mouse anti-ubiquitin antibody (Berkeley Antibody Company). The E1 ubiquitin-activating enzyme and the E2 ubiquitin-conjugating enzyme were a kind gift from Dr Kazuhiro Iwai (Kyoto University, Kyoto, Japan).

2.6 Immunoprecipitation 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 4 hours to hypoxia (0.1% O_2) sixteen hours after infection. Proteasomal inhibition was performed with 100 μM Calpain Inhibitor I (CI) for 2 hours. When still in the hypoxic chamber, cells were washed several times with PBS and scraped from the petri dishes in lysis buffer containing 100 mM NaCl, 0.5% Igepal CA630, 20 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 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 sulphonyl 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 2 h at 4°C with anti-FLAG M2 beads (Scientific Imaging Systems, Eastman Kodak Company, CT). 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 presence of anti-HIF-1α (Transduction Laboratories), anti-HIF-2α antibody (Novus Biologicals) or an anti-FLAG M2 monoclonal antibody (Sigma). Immunoprecipitation of Cullin-2, NEDD8 and Fibronectin: VHL-GFP 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 $MgCl_2$, 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 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 presence of anti-Cullin-2 (Liakopoulos *et al.*, 1999; provided by Dr Arnim Pause, Max-Plank Institute, Germany), anti-NEDD8 (Alexis), anti-fibronectin (Dako Diagnostic) or anti-FLAG M2 monoclonal antibody (Sigma). For total cell lysates, cells were washed several times in PBS, scraped from the petri dishes, centrifuged and resuspended in 4% SDS in PBS (Lee *et al.*, 1996). The samples were boiled for 5 min., and the DNA was sheared by passage of lysates through 19-gauge needles. Protein concentration was determined by bicinchoninic acid method (Pierce) and was use to normalize protein loading in whole-cell immunoblot assay.

Immunoprecipitation of EF-1α: VHL-GFP cells or infected 786-0 cells were lysed in 100 mM NaCl, 0.5% Igepal CA630, 20 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 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 overnight at 4°C with pre-blocked anti-FLAG M2 beads (pre-blocked with bovine serum albumin (BSA) 2 mg/ml for 2 hours). 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 presence of a mouse anti-EF-1α antibody (Upstate Biotechnology) and a mouse anti-FLAG M2 antibody (Sigma).

2.7 Immunofluorescence Staining

For Fibronectin: $VHL^{-/-}$ RCC 786-0 cells, or VHL-GFP cells, were infected and were grown on coverslips for 6 days, washed three times with PBS, and fixed/permeabilized in prechilled 95% ethanol at -20°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) 1 hour 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 hour at room temperature. Coverslips were washed 3 times with PBS, incubated 2 min. with Hoechst 33342 and mounted with fluoromount-G (Southern Biotechnology Associates, AL) on slides. For HIF-1α: 117 cells or transiently transfected 786-0 with HIF-1α 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 30 min. at room temperature, washed again 3 times with PBS and incubated 1 hour at room temperature with anti-HIF-1α antibody (Transduction Laboratories, 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, PA) diluted 1/1000. The cells were washed in PBS, incubated 2 min. in Hoechst 33342 and mounted with fluoromount-G on slides.

2.8 Fluorescence Analysis and Image Processing

GFP fluorescence images were captured using a Zeiss Axiovert S100TV microscope with a C-Apochromat 40X water immersion objective, equipped with an Empix digital charge-coupled device (CCD) camera using Northern Eclipse software. Images were manipulated with Northern Eclipse, and Adobe Photoshop software as described elsewhere (Lee *et al.*, 1999). GFP images were always taken before Hoechst images to minimize any possible bleaching effect.

CHAPTER 3

Results

3.1 Biochemical Characterization of the Exon 2-Encoded β-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 β-domain and an α-domain (Figure 9A). Sporadic RCC frequently harbor inactivating mutations in exon 2-encoded part of the β-domain whereas these mutations are relatively rare in individuals afflicted with inherited VHL syndrome (Gnarra *et al.*, 1994). To study the role of exon 2-encoded β-domain in VHL tumor suppressor function, a cDNA encoding a tumor-derived truncation of residues 114-154 was fused to GFP to produce the ΔE2-GFP fusion protein (Figure 9B). This truncation mutant is the consequence of point mutations that cause a splice defect producing a mRNA that lacks exon 2 sequences altogether (Gnarra *et al.*, 1994). ΔE2-GFP is predicted to have a partial, if not total, loss of β-domain function while retaining an intact, exon 3-encoded α-helical domain. A tumor-derived truncation of the exon 3-encoded α-helical domain (last 56 C-terminal residues), which retained intact the sequences of the β-domain, was also fused to GFP (ΔE3-GFP) (Figure 9B). ΔE2-GFP, ΔE3-GFP as well as wild-type VHL-GFP were cloned in pAdlox vector and adenoviruses (adΔE2-GFP, adΔE3-GFP and adVHL-GFP) were produced to high titers (Figure 9B) (Hardy *et al.*, 1996). 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 90-95% of cells displaying GFP fluorescence (Figure 9C). 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 recombinant adenovirus expressing VHL alone (without GFP; Kim *et al.*, 1998), adVHL-GFP did not restrained 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 to 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 (Figure 9D). We conclude that the adVHL-GFP protein produce from an adenovirus is a functional molecule and shares similar characteristics with VHL.

We next examined the biochemical properties of adΔE2-GFP in comparison to adVHL-GFP and adΔE3-GFP. The β-domain mutant adΔE2-GFP still retained the ability to assemble with Cullin-2 (Figure 10A) and to exhibit E3 ubiquitin ligase activity *in vitro* (Figure 10B) 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 notice 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 (Figure 10A). NEDD8 is an ubiquitin-like molecule, which is conjugated to Cullin-2 in a VHL-dependent manner (Liakopoulos *et al.*, 1999; Kamitani *et al.*, 1997). Western blotting with an anti-NEDD8 antibody revealed that the slower migrating form of Cullin-2 is conjugated to NEDD8 (Figure 10C). 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.

Figure 9. Schematic diagram of VHL fusion proteins and characterization of adenovirus-mediated expression of VHL-GFP. A) Schematic diagram of VHL protein. VHL has three exons that code for a 213-amino acid protein containing an acidic domain, a β-domain and an α-domain. B) Schematic diagram of VHL fusions to GFP. The GFP was fused at the C-terminus (black box [not in scale]), resulting in VHL-GFP fusion protein. A cDNA encoding a tumor-derived truncation of residues 114-154 (strike box) was fused to GFP to produce ΔE2-GFP. A tumor-derived truncation of the exon 3-encoded α-domain (strike box) was also fused to GFP to produce ΔE3-GFP. A FLAG tag (shadow box) was fused to the N-terminal of all three constructs. ΔE2-GFP, ΔE3-GFP and VHL-GFP were cloned in pAdlox vector to prepare adenoviruses. adΔE2-GFP, adΔE3-GFP and adVHL-GFP refer to proteins obtained following the infection with the corresponding adenovirus. C) 90-95% of VHL$^{-/-}$ RCC cells (786-0) displayed GFP fluorescence following infection with the adenovirus adVHL-GFP. 786-0 cells were infected overnight (16 hours) and adVHL-GFP pictures were obtained by CCD camera (left panel). Counterstaining with Hoechst 33342 dye (2 μg/ml for 2 min.) provided staining of all nuclei (right panel). D) adΔE2-GFP, adΔE3-GFP and adVHL-GFP accumulated to similar levels following adenoviral infection of 786-0. Total cell lysates were run on an 8% SDS-PAGE and transferred to a PVDF membrane. The membrane was then blocked and incubated in the presence of a mouse anti-FLAG M2 antibody.

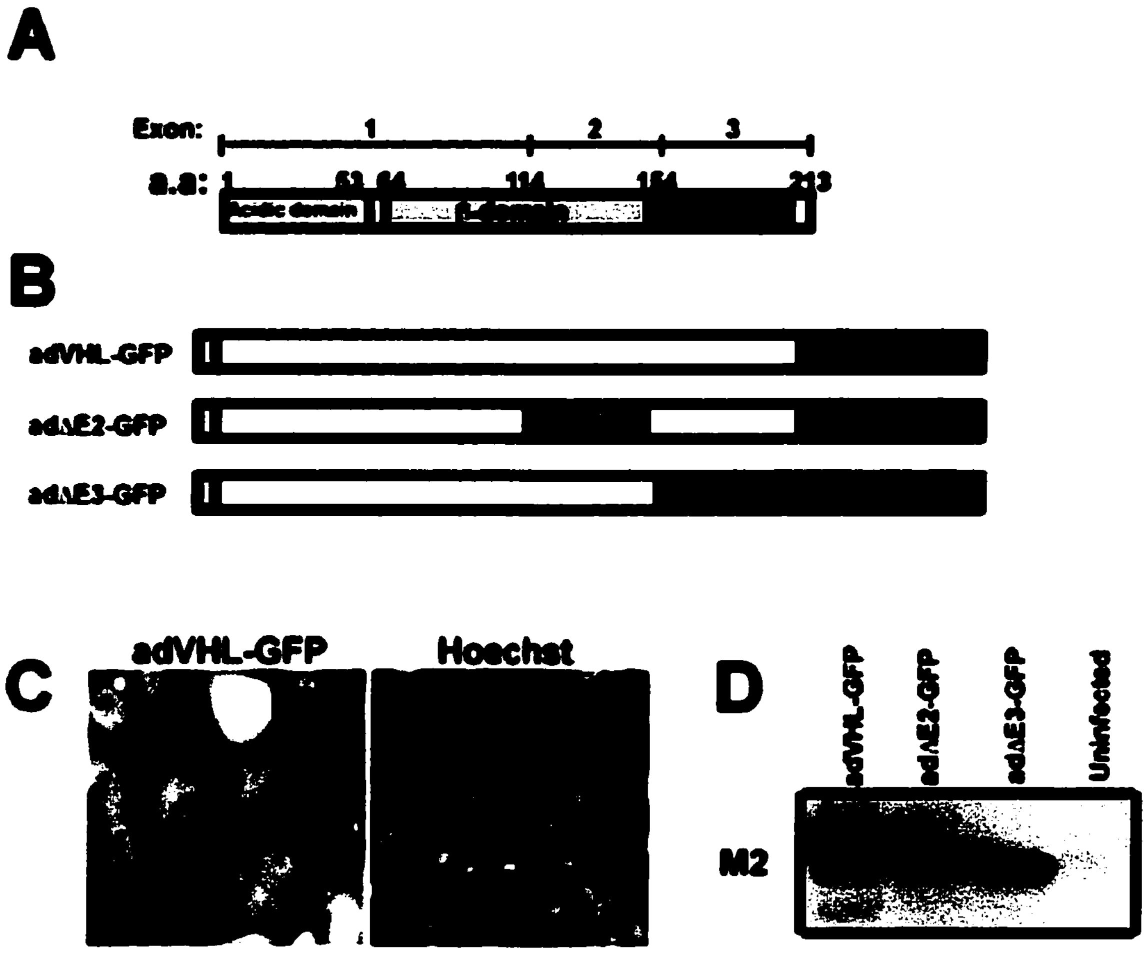
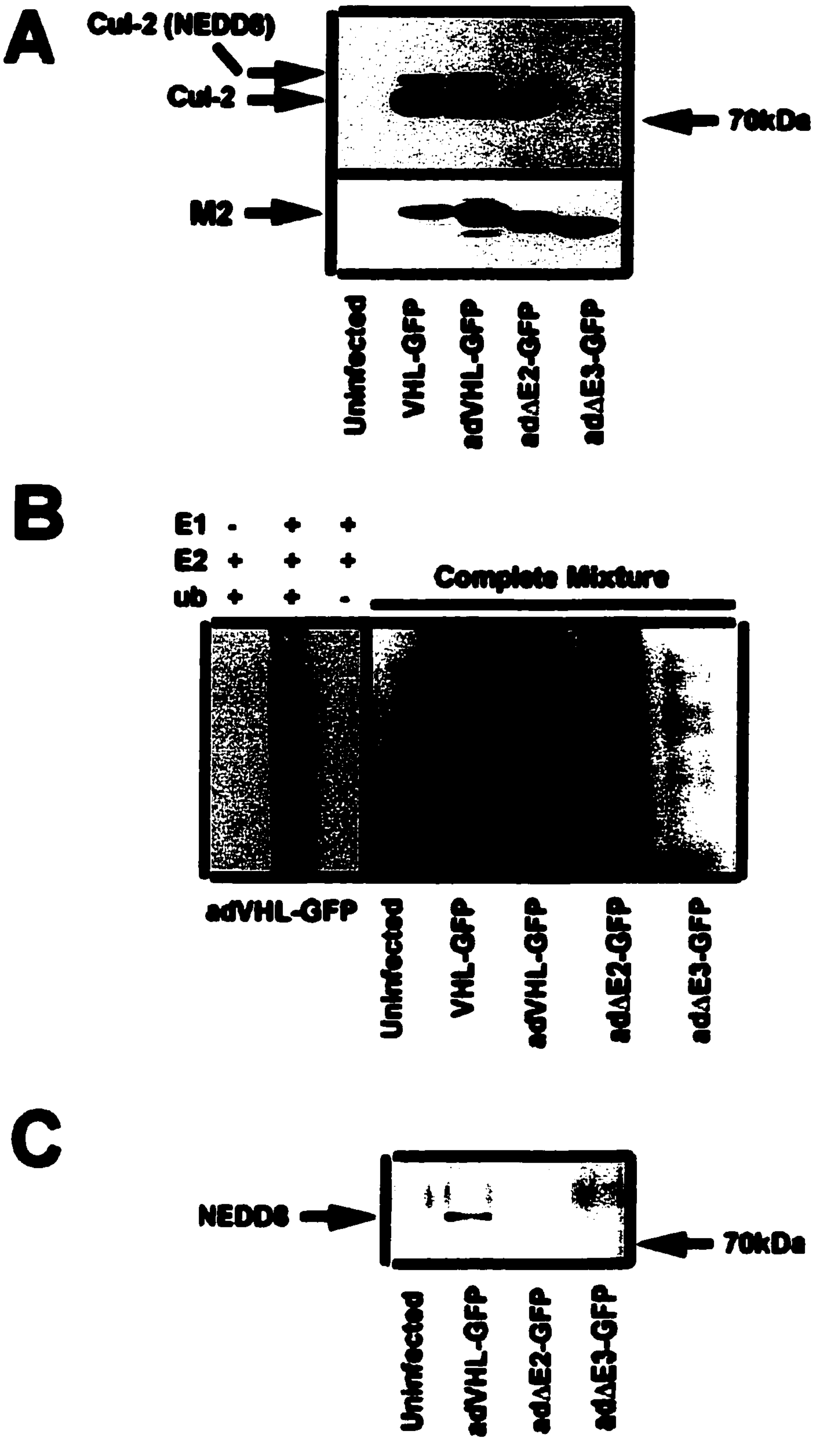

A
Exon:
1
2
3
a.a:
1
63
64
114
154
213
Acidic domain
β-domain
B
adVHL-GFP
adΔE2-GFP
adΔE3-GFP
C
adVHL-GFP
Hoechst
D
adVHL-GFP
adΔE2-GFP
adΔE3-GFP
Uninfected
M2

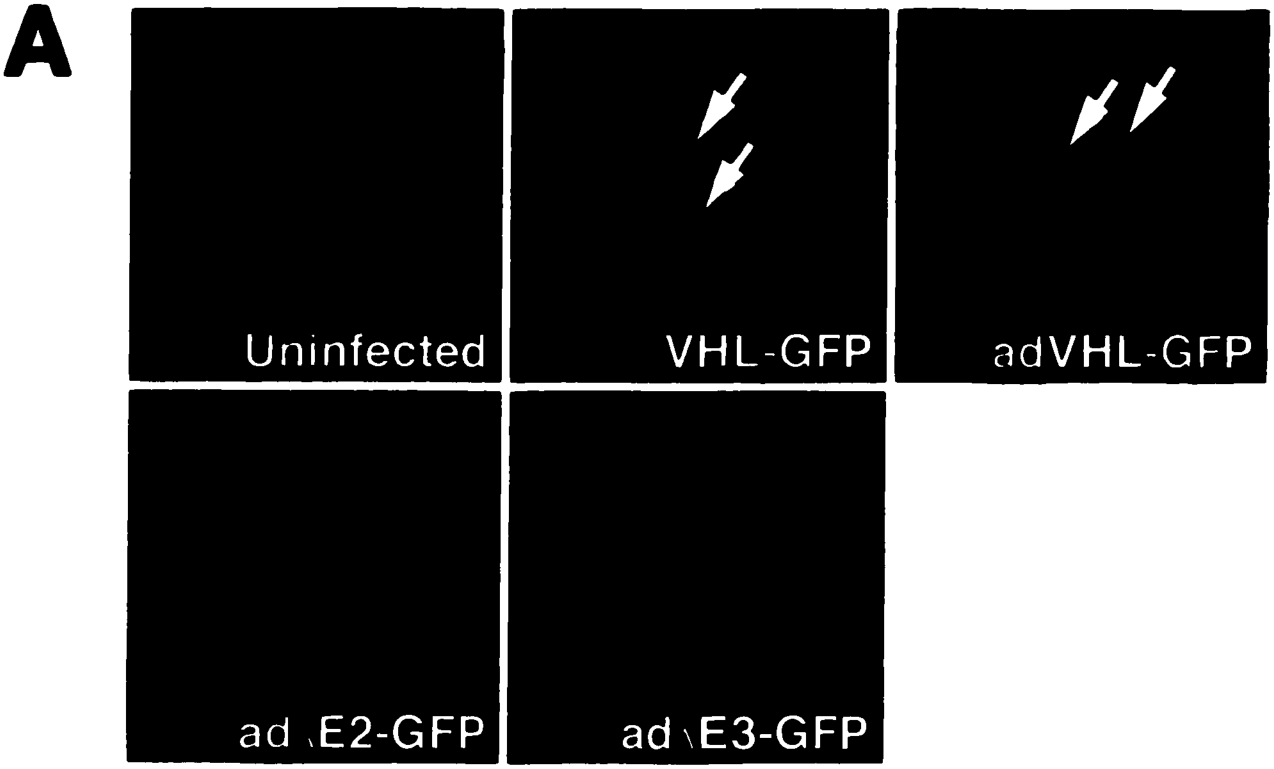
Figure 10. Biochemical characterization of an exon 2-encoded β-domain mutant of VHL. A) An intact β-domain is not required for VHL ability to assemble with Cullin-2. Stable VHL$^{-/-}$ RCC 786-0 cells stably expressing FLAG-tagged VHL-GFP or 786-0 cells infected or not infected with the adenoviruses adΔE2-GFP, adΔE3-GFP and adVHL-GFP were lysed and immunoprecipitated with anti-FLAG M2 beads. Precipitated proteins were run on SDS-PAGE (8% acrylamide) and transferred on PVDF membranes. The membranes were then blocked and incubated in the presence of a rabbit anti-Cullin-2 (top panel) or a mouse anti-FLAG M2 (bottom panel) antibody. Notice that a second band migrates slower than Cullin-2 in the VHL-GFP and adVHL-GFP lanes only. This represents NEDD8 conjugation to Cullin-2 (further confirmed in C)). B) An intact β-domain is not required for VHL ability to function as an E3 ubiquitin ligase *in vitro*. *In vitro* ubiquitination reactions were performed as described in Materials and Methods (complete mixture) except for 2 negative controls: adVHL-GFP was immunoprecipitated with anti-FLAG M2 beads and incubated with the complete mixture except the E1 enzyme (first lane starting from the left) or ubiquitin (third lane starting from the left). Reactions were stopped by adding 4X sample buffer. Samples were electrophoresed in 8% SDS-PAGE and transferred to a PVDF membrane. The membrane was then blocked and incubated in the presence of a mouse anti-ubiquitin antibody. C) Exon 2-encoded β-domain is required for VHL-mediated NEDD8 conjugation to Cullin-2. Immunoprecipitations were performed exactly like for Cullin-2. Immunoprecipitated proteins were run on an 8% SDS/PAGE and transferred to a PVDF membrane. The membrane was then blocked and incubated in the presence of a rabbit anti-NEDD8 antibody.

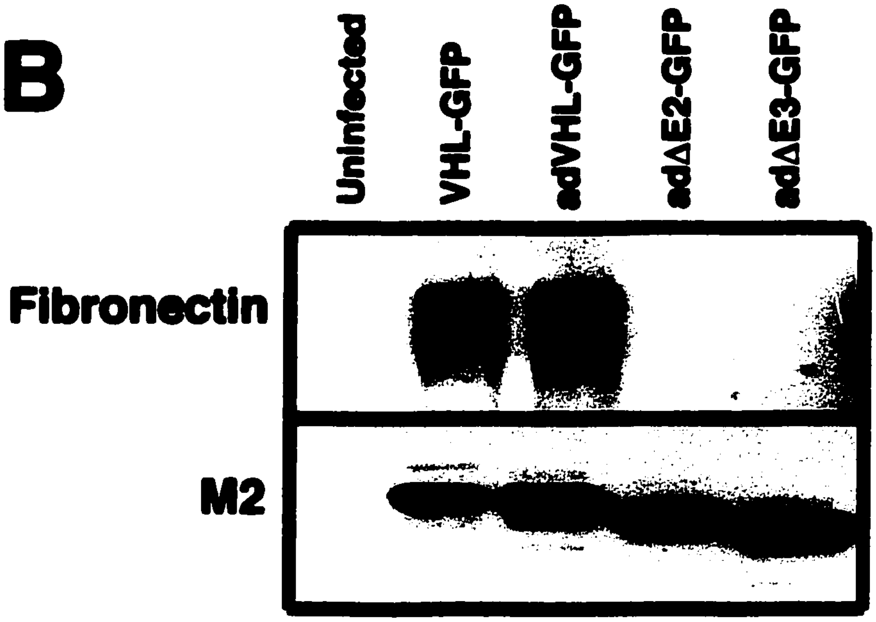

A
Cul-2 (NEDD8)
Cul-2
70kDa
M2
Uninfected
VHL-GFP
adVHL-GFP
adΔE2-GFP
adΔE3-GFP
B
E1 - + +
E2 + + +
ub + + -
Complete Mixture
adVHL-GFP
Uninfected
VHL-GFP
adVHL-GFP
adΔE2-GFP
adΔE3-GFP
C
NEDD8
70kDa
Uninfected
adVHL-GFP
adΔE2-GFP
adΔE3-GFP

3.2 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 (Ohh *et al.*, 1998). We wanted to verify if exon 2-encoded β-domain of VHL is required for binding to fibronectin and for proper formation of a fibronectin extracellular matrix. Adenovirus-mediated reintroduced adVHL-GFP displayed similar activity than VHL and restored the ability of VHL$^{-/-}$ RCC cells to properly produce a fibronectin extracellular matrix (Figure 11A; VHL-GFP). In contrast, adΔE2-GFP was unable to rescue this defect (Figure 11A). Fibronectin was observed in an ER-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 (Figure 11B). 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.

Figure 11. VHL requires an intact β-domain to bind fibronectin and mediate proper extracellular matrix formation. A) adΔE2-GFP is unable to produce a fibronectin extracellular matrix. Uninfected VHL$^{-/-}$ 786-0 cells, VHL$^{-/-}$ RCC 786-0 cells stably expressing FLAG-tagged VHL-GFP or infected with adVHL-GFP, adΔE2-GFP or adΔE3-GFP were grown on coverslips for 6 days. Cells were washed, fixed, incubated with Hoechst for 2 min. (blue) and stained with a rabbit anti-fibronectin antibody (red). Pictures were obtained by CCD camera and superposition of fibronectin and Hoechst-stained nuclei frames was done with Adobe Photoshop. Arrows are pointing at fibronectin deposition. B) adΔE2-GFP is unable to bind fibronectin. Stable VHL$^{-/-}$ RCC 786-0 cells stably expressing FLAG-tagged VHL-GFP or 786-0 cells uninfected or infected with the adenoviruses adΔE2-GFP, adΔE3-GFP and adVHL-GFP were lysed and immunoprecipitated with anti-FLAG M2 beads. Precipitated proteins were run on an 8% SDS-PAGE and transferred on PVDF membranes. The membranes were then blocked and incubated in the presence of a rabbit anti-fibronectin (top panel) or a mouse anti-FLAG M2 (bottom panel) antibody.


A
Uninfected
VHL-GFP
adVHL-GFP
adΔE2-GFP
adΔE3-GFP


B
Uninfected
VHL-GFP
adVHL-GFP
adΔE2-GFP
adΔE3-GFP
Fibronectin
M2

3.3 Role of Exon 2-Encoded β-Domain of VHL in Oxygen-Dependent Degradation of HIF-α

It was recently shown that one of the major defects of VHL$^{-/-}$ RCC cells are their inability to mediate oxygen-dependent degradation of HIF-α and reintroduction of wild-type VHL was sufficient to correct this defect (Maxwell *et al.*, 1999). *In vitro* studies have also revealed that truncation mutants of exon 2 and exon 3 of VHL are still able to bind to HIF-α (Tanimoto *et al.*, 2000), which likely assemble with sequences encoded by exon 1 (residues 64 to 113) (Cockman *et al.*, 2000). Since these experiments were performed *in vitro*, we wanted to look at the ability of wild-type VHL (adVHL-GFP) and the two VHL deletion mutants (adΔE2-GFP and adΔE3-GFP) to bind and degrade endogenous HIF-α. 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 HIF-α in normoxia (Figure 12A). HIF-α levels were not affected by expression of adΔE2-GFP or adΔE3-GFP (Figure 12A). We notice that adVHL-GFP assembled with a significant amount of HIF-α (1α and 2α) in hypoxia and in the presence of the proteasome inhibitor CI (Figure 12B). In contrast to data obtained *in vitro*, immunoprecipitation analysis revealed that adΔE2-GFP and adΔE3-GFP failed to bind to HIF-α in adenovirus infected cells (Figure 12B; top panels). We did not detect binding of HIF-α 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 HIF-α in cells.

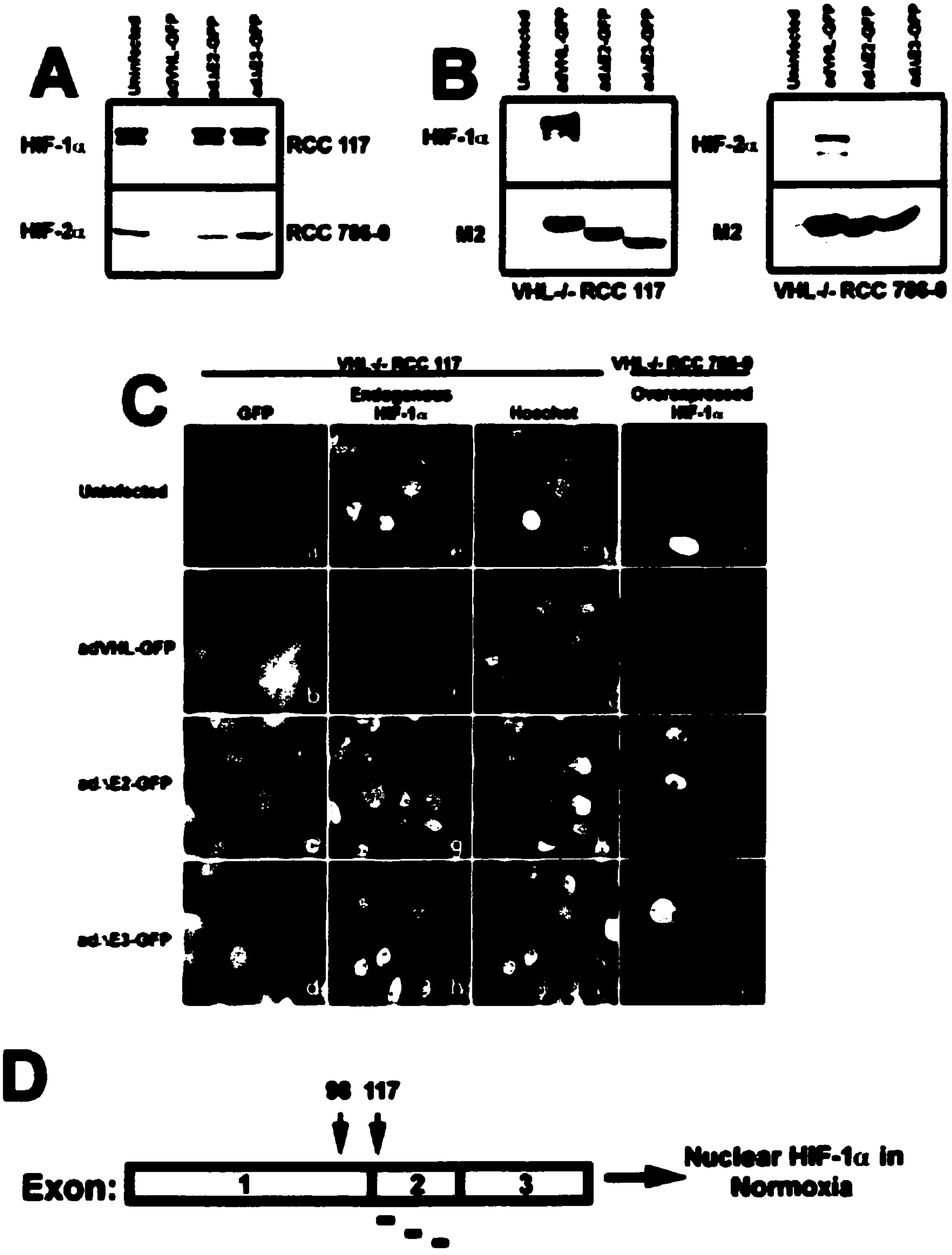
It has been hypothesized that HIF-1α requires a hypoxic environment to import in the nucleus most likely assembled into complexes that contain VBC/Cul-2 (Tanimoto *et al.*, 2000; Kallio *et al.*, 1998). To further examine the role of hypoxia and VHL in nuclear import of HIF-α, the subcellular localization of endogenous HIF-1α was examined by immunofluorescence in VHL$^{-/-}$ 117 RCC cells uninfected or infected with different VHL constructs. Data shown in Figure 12C revealed that endogenous HIF-1α accumulated exclusively in the nucleus of uninfected VHL$^{-/-}$ RCC 117 cell line even though these cells were incubated in normoxia (Figure 12C; a, e, 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. Strong HIF-1α nuclear signal was also observed in cells expressing adΔE2-GFP (Figure 12C; c, g, k) as well as adΔE3-GFP (Figure 12C; d, h, l) whereas it was essentially undetectable in cells expressing reintroduced adVHL-GFP (Figure 12C; b, f, j). We then examined the subcellular localization of overexpressed HIF-1α in RCC VHL$^{-/-}$ 786-0 cells (which express endogenous HIF-2α instead of 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 (Figure 12C; m), infected with GFP alone (data not shown) or infected with adΔE2-GFP (Figure 12C; o) and adΔE3-GFP (Figure 12C; p). Addition of proteasome inhibitors or incubation in hypoxia led to nuclear accumulation of endogenous or overexpressed HIF-α 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 deletions mutants of exon 2 (Δ115-123, Δ128-141 and Δ141-154), with a cancer-causing substitution at residue 117 (W to R) in exon 2, or at residue 98 (Y to N) in exon 1, fused to GFP (Figure 12D). These results demonstrate that HIF-1α is able to import in the nucleus regardless of oxygen concentration or assembly with VHL.

3.4 Exon 2-Encoded Residues Mediate Transcription-Dependent Nuclear/Cytoplasmic Trafficking of VHL Independently of Assembly with HIF-α and Oxygen Concentration

We recently demonstrated that VHL mediates transcription-dependent nuclear/cytoplasmic trafficking of the VBC/Cul-2 complex (Lee *et al.*, 1999; Groulx *et al.*, 2000). 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 export of the complex. The dependence of transcription for trafficking is abolished by a deletion of exon 2-encoded sequences (Lee *et al.*, 1999). We next wanted to determine if exon 2-encoded residues also regulate subcellular trafficking of VHL in conditions known to affect HIF-α stability, such as oxygen concentration and if it is able to do so independently of assembly with HIF-α. adΔE2-GFP is a small molecule (40kDa).

Its presence in the nucleus might be simply the outcome of unregulated passive diffusion through the nuclear pore complex rather or utilization of signal-mediated and regulated energy-dependent processes. Therefore, the first step consisted of determining if the β-domain mutant required energy expenditure for nuclear import before further investigating its role in VHL-mediated shuttling of BC/Cul-2. To do so, we developed a new assay to test for energy requirement for nuclear import in living cells based on fusing proteins to the energy-dependent HIV (human immunodeficiency virus) REV nuclear export signal (NES). NES confers strong nuclear export properties to fusion proteins leading to their cytoplasmic accumulation at steady state (Fisher *et al.*, 1995). 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 (Groulx *et al.*, 2000). In contrast, VHL-GFP-NES and ΔE2-GFP-NES strictly remained in the cytoplasm at 4°C or in the presence of metabolic poisons (data not shown) indicating that both fusion proteins are unable to passively diffuse in the nucleus. ΔE2-GFP-NES and VHL-GFP-NES accumulated in the nucleus upon incubation with Leptomycin B, a drug that specifically inhibits NES function (Nishi *et al.*, 1994; Wolff *et al.*, 1997) at 37°C, but not at 4°C indicating that both fusion protein contain energy-dependent nuclear import signals (data not shown). These observations demonstrate that VHL ability to confer energy-dependent nuclear import properties to a reporter GFP is independent of assembly with HIF-α and exon 2-encoded β-domain residues.

Figure 12. HIF-α fails to bind to an exon 2-encoded β-domain mutant of VHL and accumulates in the nucleus of normoxic cells. A) adΔE2-GFP failed to mediate oxygen-dependent degradation of HIF-α. VHL$^{-/-}$ 786-0 RCC cells (HIF-2α) and 117-4 cells (HIF-1α) were uninfected or infected with adVHL-GFP, adΔE2-GFP and adΔE3-GFP. Total cell lysates were run on an 8% SDS-PAGE, transferred to PVDF membranes. The membranes were blocked and incubated in presence of a mouse anti-HIF-1α (top panel) or a rabbit anti-HIF-2α (bottom panel) antibody. B) adVHL-GFP assembled with endogenous HIF-1α (117 cells) or with endogenous HIF-2α (786-0 cells) but adΔE2-GFP and adΔE3-GFP failed to do so. Cells were put under hypoxic conditions (0.1% O_2) for 4 hours in presence of the proteasome inhibitor Calpain Inhibitor I (CI). Cells were lysed and immunoprecipitation was performed with anti-FLAG M2 beads for 2 hours. Immunoprecipitated proteins were run on an 8% SDS-PAGE and blotted onto PVDF membranes. Membranes were blocked and incubated in the presence of a mouse anti-HIF-1α (top left panel), a rabbit anti-HIF-2α (top right panel), or a mouse anti-FLAG M2 antibody (bottom panels). C) Nuclear import of HIF-α occurs regardless of oxygen tension. VHL$^{-/-}$ RCC 117 cells (endogenous HIF-1α) (a-l) or 786-0 cells transiently transfected with HIF-1α (m-p) were uninfected or infected with the adenoviruses adVHL-GFP, adΔE2-GFP and adΔE3-GFP. Cells were washed, fixed and stained with a mouse anti-HIF-1α antibody. Counterstaining of cells with Hoechst 33342 dye provided staining of all 117 cells nuclei (i-l). D) Line drawing of mutant VHL-GFP, which were co-transfected with HIF-1α cDNA in VHL$^{-/-}$ RCC 786-0 cells. Arrows indicate single amino acid substitutions at residue 98 and 117 whereas black bars indicate small deletion mutants within exon 2. HIF-1α accumulated in the nucleus in normoxia when co-transfected with these VHL mutants.


A
Uninfected
adVHL-GFP
adΔE2-GFP
adΔE3-GFP
HIF-1α
RCC 117
HIF-2α
RCC 786-0
B
Uninfected
adVHL-GFP
adΔE2-GFP
adΔE3-GFP
HIF-1α
M2
VHL-/- RCC 117
HIF-2α
M2
VHL-/- RCC 786-0
C
VHL-/- RCC 117
VHL-/- RCC 786-0
GFP
Endogenous HIF-1α
Hoechst
Overexpressed HIF-1α
Uninfected
adVHL-GFP
adΔE2-GFP
adΔE3-GFP
a
b
c
g
h
d
h
D
98
117
Exon:
1
2
3
Nuclear HIF-1α in Normoxia

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 HIF-α stabilization. GFP fluorescence analysis of living cells indicated that the steady state distribution of adVHL-GFP was unaffected by oxygen tension (Figure 13; a, j). Addition of the RNA Polymerase II inhibitor DRB caused nuclear accumulation of adVHL-GFP, regardless of oxygen concentration (Figure 13; b, 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 (Scheffner, 1999; Tomoda *et al.*, 1999). 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 (Figure 13; c, l). adΔE3-GFP steady state distribution is more nuclear than adVHL-GFP and is unaffected by oxygen concentration (Figure 13; g, p). Addition of DRB or CI also caused an important nuclear accumulation of adΔE3-GFP with few cells displaying exclusive nuclear signal (Figure 13; h, i, q, 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 (Figure 13; d-f, m-o). One possible explanation for adΔE2-GFP insensitivity to DRB and CI is that this mutant is unable to bind to HIF-α. These observations led us to test if the effect of DRB and CI on shuttling of VHL are intrinsic to exon 2-encoded residues or if this activity is mediated by HIF-α. To test this, VHL shuttling was analyzed in mouse embryonic fibroblasts that do not express endogenous HIF-α (Figure 14). We noticed that adVHL-GFP steady state subcellular localization was unaffected by the absence of HIF-1α (Figure 14; a, c). Likewise, addition of DRB caused nuclear accumulation of adVHL-GFP in HIF-1α$^{-/-}$ as well as in HIF-1α$^{+/+}$ cells (Figure 14; b, d). The localization of both mutants was unaffected by the absence or presence of HIF-1α (Figure 14; e, g, i, 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 on the three fusion proteins (data not shown). The same data was obtained in hypoxia (data not shown). Put together, these results demonstrate that oxygen tension and HIF-α have no effect 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 HIF-α.

Figure 13. Effect of oxygen tension and proteasome inhibitors on the subcellular localization and nuclear/cytoplasmic trafficking properties of adVHL-GFP. Subcellular localization of adVHL-GFP and mutants in cells grown in normoxia (20% O_2) and hypoxia (0.1% O_2) in the presence of absence of DRB or CI. VHL$^{-/-}$ RCC 786-0 cells were infected with adVHL-GFP, adΔE2-GFP and adΔE3-GFP and incubated in normoxia (a-i) or for 4 hours in hypoxia (j-r). Cells were grown without further treatments (a, d, g, j, m, p), were treated with DRB (25μM) for two hours (b, e, h, k, n, q) or with calpain inhibitor I (CI; 100μM) for 2 hours (c, f, i, l, o, r). GFP fluorescence images of living cells were captured using a Zeiss Axiovert S100TV microscope equipped with an Empix digital charged-couple device (CCD) camera.

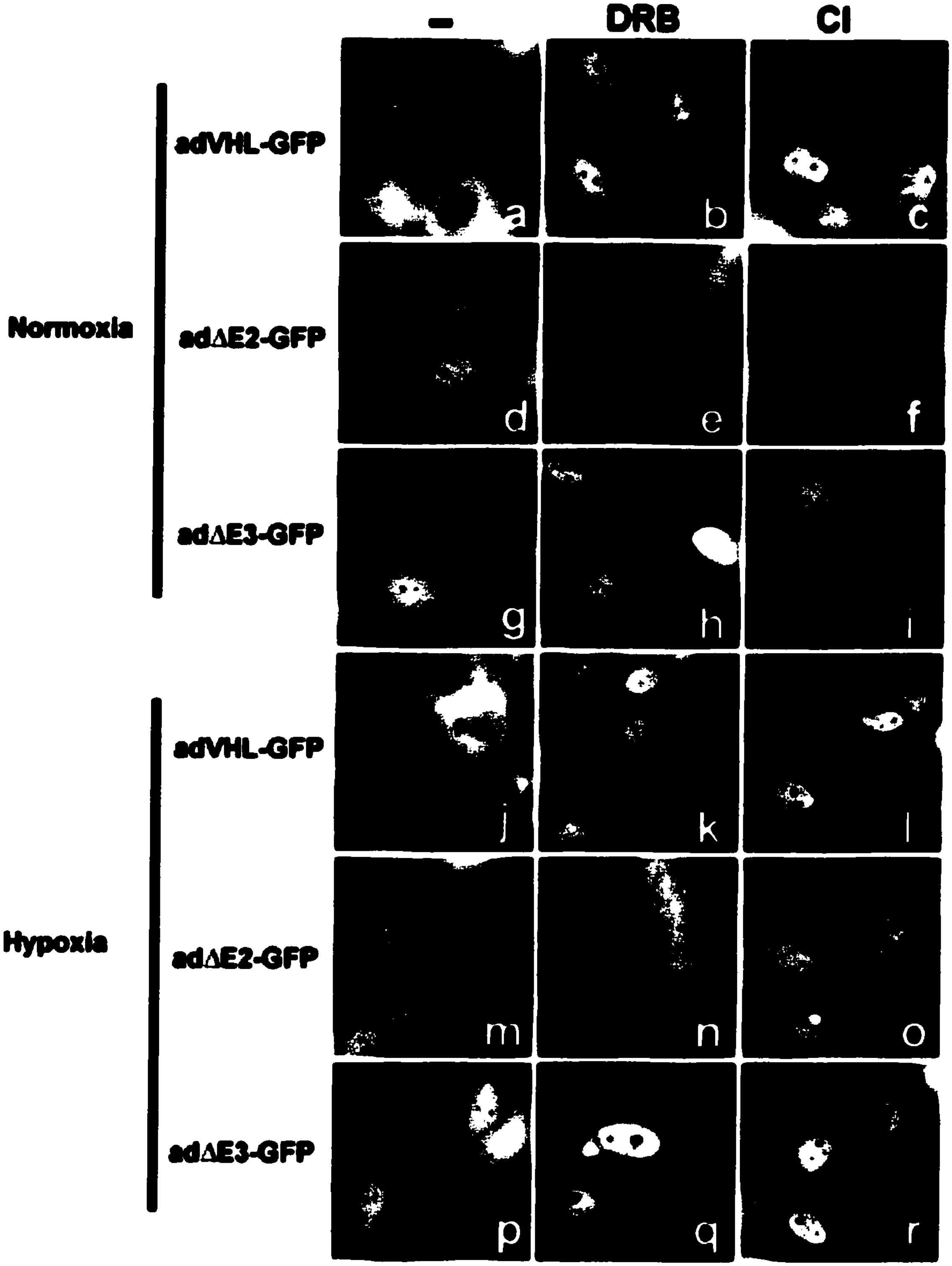

–
DRB
CI
Normoxia
Hypoxia
adVHL-GFP
adΔE2-GFP
adΔE3-GFP
adVHL-GFP
adΔE2-GFP
adΔE3-GFP
a
b
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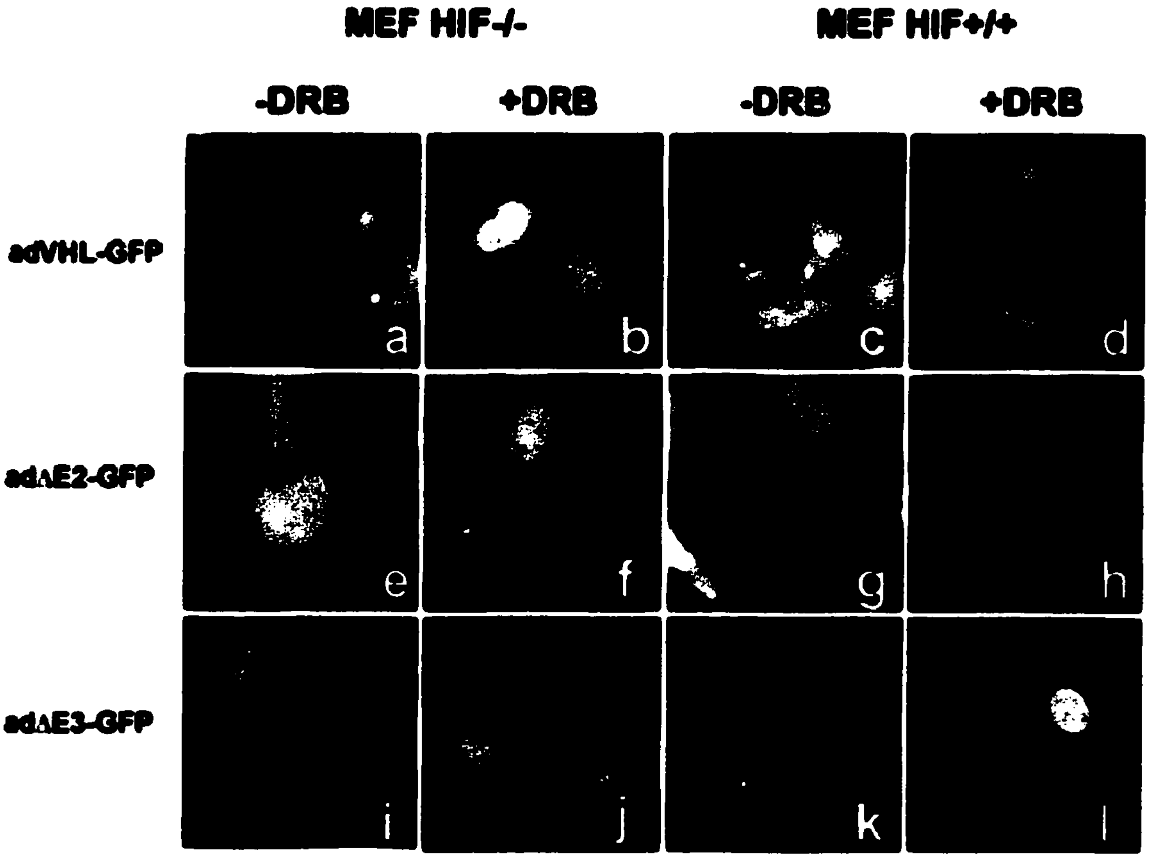
Figure 14. Exon 2-encoded residues mediate transcription-dependent trafficking of VHL independently of assembly with HIF-α. HIF-1α$^{-/-}$ or HIF-1α$^{+/+}$ mouse embryonic fibroblasts (MEF) cells were infected with adVHL-GFP, adΔE2-GFP and adΔE3-GFP and incubated in normoxia in the presence or absence of DRB (25μM) for 2 hours. Addition of CI (100μM) for 2 hours essentially gave the same results as DRB (data not shown). The exact same data were also obtained for cells incubated in hypoxia (data not shown).


MEF HIF-/-
MEF HIF+/+
-DRB
+DRB
-DRB
+DRB
adVHL-GFP
adΔE2-GFP
adΔE3-GFP
a
b
c
d
e
f
g
h
i
j
k
l

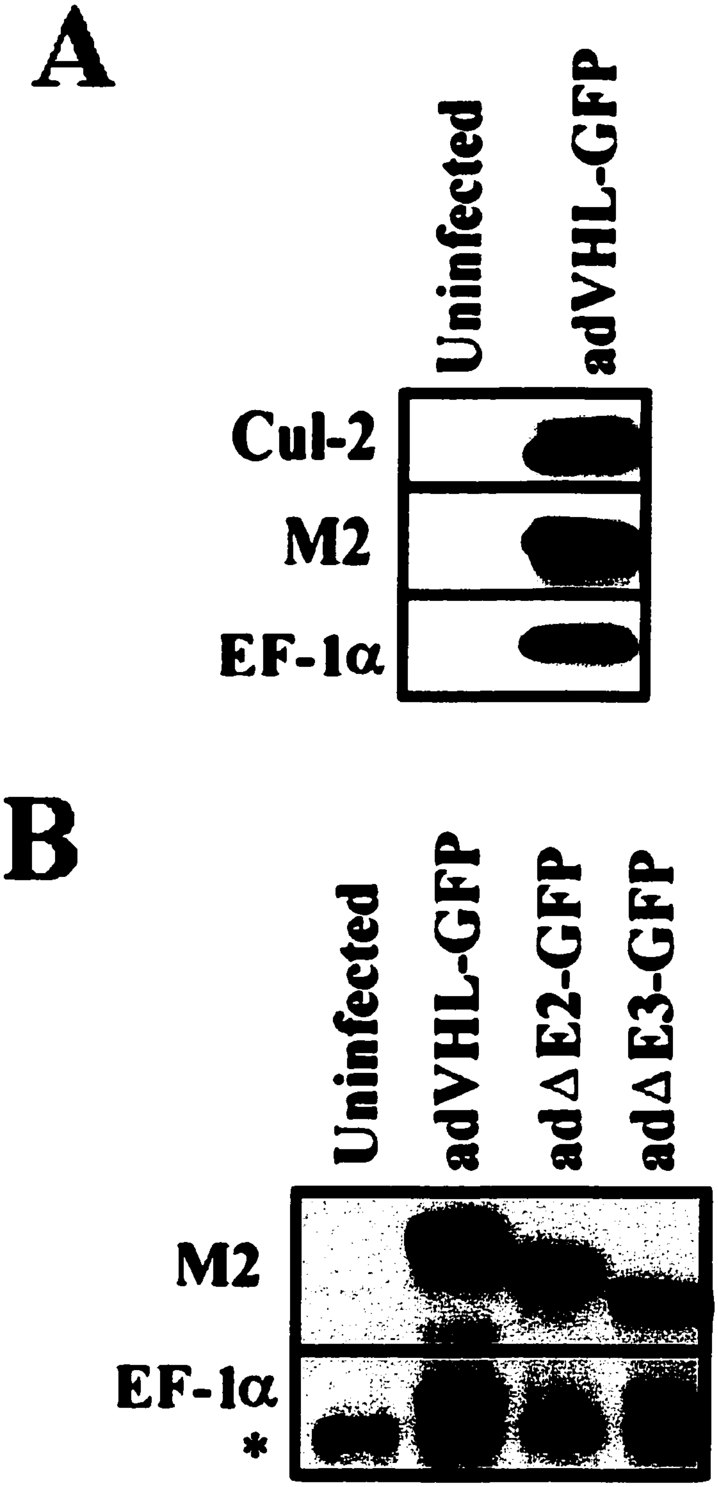
3.5 Exon 2-Encoded β-Domain is Required for VHL Binding to Elongation Factor-1α (EF-1α), a new VHL-Associated Protein

Previous work has shown that exon 2-encoded residues are needed for transcription-dependent shuttling of VHL (Lee *et al.*, 1999). Therefore, it has been hypothesized at the time that other proteins might regulate VBC/Cul-2 cellular trafficking via assembly with VHL "shuttling domain", such as exon 2. Since the exon 3 deletion mutant retains the ability to shuttle in a transcription-dependent manner, immunoprecipitation experiments were performed with this mutant in order to find novel associated proteins. As predicted, Dr Lee found a protein of 50 kDa (p50) that assembled strongly with this exon 3 deletion mutant of VHL but only weakly with the VBC/Cul-2 complex. This later observation is not surprising since proteins involved in trafficking and localization often interact weakly with their respective complexes (Gorlich and Mattaj, 1996; Ohno *et al.*, 1995; Pollard *et al.*, 1996). Since exon 3 deletion mutants lack the ability to bind to BC/Cul-2, we suggest that they might be more able to associate with p50.

p50 was purified and was sent for microsequencing (Harvard Microchemistry Facility, Boston). It was identified as the translation elongation factor-1α (EF-1α). EF-1α is a GTP-binding protein known to be involved in diverse cellular processes including protein biosynthesis (Moldave, 1985), embryogenesis (Krieg *et al.*, 1989), cell proliferation (Sanders *et al.*, 1996), organization of the cytoskeleton (Condeelis, 1995). EF-1α was also shown to be an ubiquitin isopeptidasc essential for ubiquitin-dependent degradation of certain proteolytic substrate (Gonen *et al.*, 1996). More interestingly, EF-1α was recently shown to be involved in the aminoacylation-dependent export pathway of nuclear tRNA in yeast (Grosshans *et al.*, 2000). Furthermore, the interaction of zinc finger protein (ZPR1) with EF-1α upon epidermal growth factor (EGF) treatment causes the redistribution of both proteins to the nucleus (Gangwani *et al.*, 1998). These latter results are appealing since they implicate EF-1α in nucleocytoplasmic trafficking.

Here, we wanted to see if endogenous EF-1α was able to bind to the VBC/Cul-2 complex obtained by infection of VHL$^{-/-}$ RCC 786-0 cells with adVHL-GFP. Indeed, immunoprecipitation experiment revealed that adVHL-GFP was able to assemble with endogenous EF-1α (Figure 15A). In contrast, adΔE2-GFP failed to do so, suggesting that the exon 2-encoded β-domain of VHL is required for its assembly with EF-1α (Figure 15B). In addition, we confirmed that the exon 3-encoded α-domain is not required for this interaction since the exon 3 deletion mutant (adΔE3-GFP) is also able to bind to EF-1α (Figure 15B).

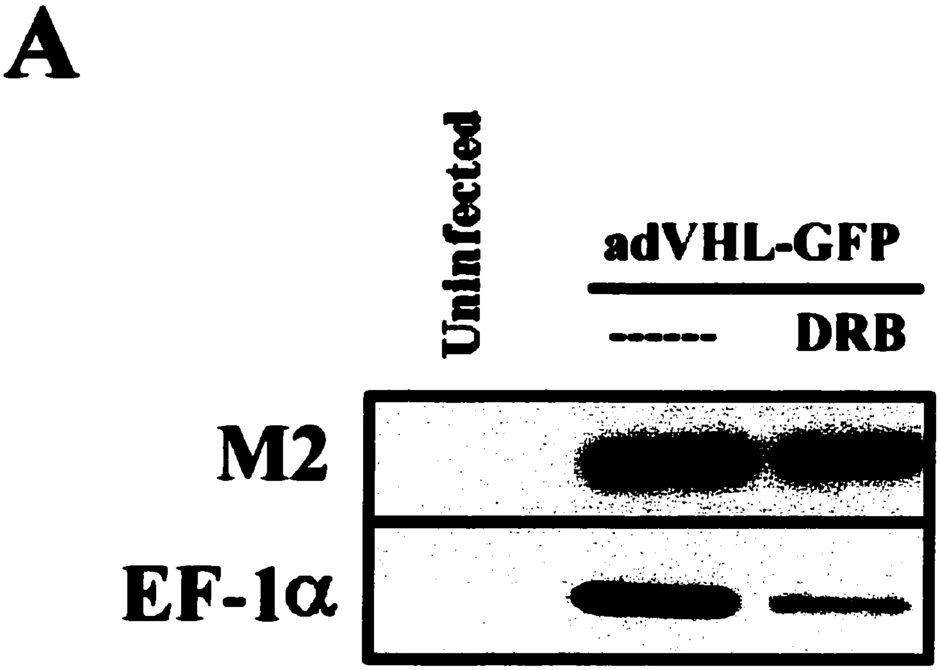
Figure 15. An exon 2-encoded β-domain mutant of VHL fails to bind to endogenous elongation factor-1α (EF-1α). A) VHL$^{-/-}$ RCC 786-0 cells infected or not infected with the adenovirus adVHL-GFP were lysed and immunoprecipitated with pre-blocked anti-FLAG M2 beads overnight at 4°C. Precipitated proteins were run on SDS-PAGE (8% acrylamide) and transferred on PVDF membranes. The membranes were then blocked and incubated in the presence of a rabbit anti-Cullin-2 (top panel), a mouse anti-FLAG M2 (middle panel) or a mouse anti-EF-1α antibody (bottom panel). B) VHL$^{-/-}$ RCC 786-0 cells infected or not infected with the adenoviruses adΔE2-GFP, adΔE3-GFP and adVHL-GFP were lysed and immunoprecipitated with pre-blocked anti-FLAG M2 beads overnight at 4°C. Precipitated proteins were run on SDS-PAGE (8% acrylamide) and transferred on PVDF membranes. The membranes were then blocked and incubated in the presence of a mouse anti-FLAG M2 (top panel) or a mouse anti-EF-1α (bottom panel) antibody. * The band observed in all 4 lanes represents the heavy chain of the M2 antibody used for immunoprecipitation.


A
Uninfected
adVHL-GFP
Cul-2
M2
EF-1α
B
Uninfected
adVHL-GFP
adΔE2-GFP
adΔE3-GFP
M2
EF-1α
*

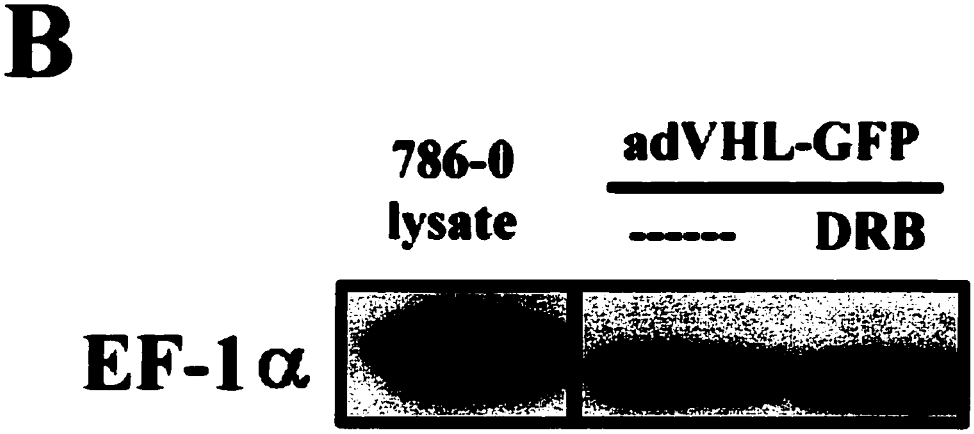
Since exon 2-encoded β-domain of VHL seems to be required for its assembly with EF-1α, this suggests that EF-1α might be implicated in the transcription-dependent shuttling of VHL. Therefore, we next wanted to see if inhibition of the transcription with DRB would affect the interaction between EF-1α and VHL. Indeed, inhibition of the transcription decreased the amount of EF-1α bound to adVHL-GFP (Figure 16A, bottom panel), without affecting the total cellular levels of adVHL-GFP and EF-1α and the amount of adVHL-GFP immunoprecipitated (Figure 16Aand 16B). We can therefore suggest a model by which EF-1α would be responsible for the transcription-dependent nuclear export of the VBC/Cul-2 complex. According to this model, inhibition of transcription would dissociate EF-1α from the complex and result in nuclear accumulation of the VBC/Cul-2 complex.

Most proteins involved in cellular trafficking use the GTP/GDP cycle as source of energy. Since EF-1α is a GTP-binding protein, we first looked at the ability of EF-1α to bind VHL in the presence of GMP-PNP (a non-hydrolyzable analog of GTP) or GDP. We found that EF-1α failed to assemble with adVHL-GFP in the presence of GMP-PNP but GDP had no effect on complex assembly (Figure 17A). These results suggest that a GTP/GDP cycle might regulate the interaction between adVHL-GFP and EF-1α. Also, we wanted to test if other conditions would have an effect on the association of EF-1α with the VBC/Cul-2 complex. We looked at the effect of oxygen concentration on the EF-1α/adVHL-GFP interaction. We have incubated stably transfected VHL$^{-/-}$ RCC 786-0 cells with VHL-GFP in normoxia or hypoxia for 4 hours. Immunoprecipitation analysis revealed that low level of oxygen tends to increase EF-1α binding to VHL (Figure 17B). The amount of EF-1α bound to VHL-GFP in normoxia in the stable cell line is very low compared to the amount bound to adVHL-GFP in normoxia with 786-0 cells infected with the adenovirus. This is the result of the different levels of expression of VHL-GFP that we obtain with the two methods (stably transfected cell lines vs adenoviral infection). Therefore, we conclude that the binding of EF-1α to the VBC/Cul-2 complex seems to be regulated by different factors. EF-1α is the first protein which assembly to VHL is regulated by environmental conditions such as oxygen concentration or forms of energy available. However, more work needs to be done in order to understand the details and the biochemical significance of this regulated interaction.

Figure 16. The binding of EF-1α to VHL is affected by inhibition of transcription with DRB. A) VHL$^{-/-}$ RCC 786-0 cells infected (or not) with adVHL-GFP were treated or not treated with DRB at a final concentration of 25 μM for 2 hours. Cells were lysed and immunoprecipitations were performed with pre-blocked anti-FLAG M2 beads overnight at 4°C. B) VHL$^{-/-}$ RCC 786-0 cells infected (or not) with adVHL-GFP were treated as in A) but were lysed in 4% SDS in PBS. Immunoprecipitates (A) and total cell lysates (150μg) (B) were run on an 8% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked and incubated in presence of a mouse anti-FLAG M2 or a mouse anti-EF-1α antibody. 786-0 lysate correspond to total cell lysate of 786-0 cells that were lysed in 4% SDS in PBS (150 μg).

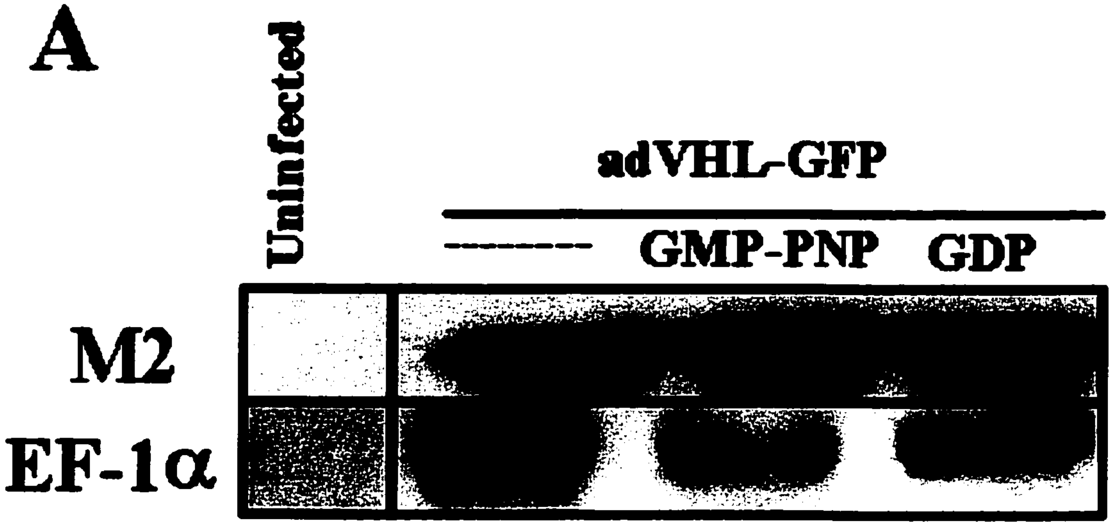

A
Uninfected
adVHL-GFP

DRB
M2
EF-1α

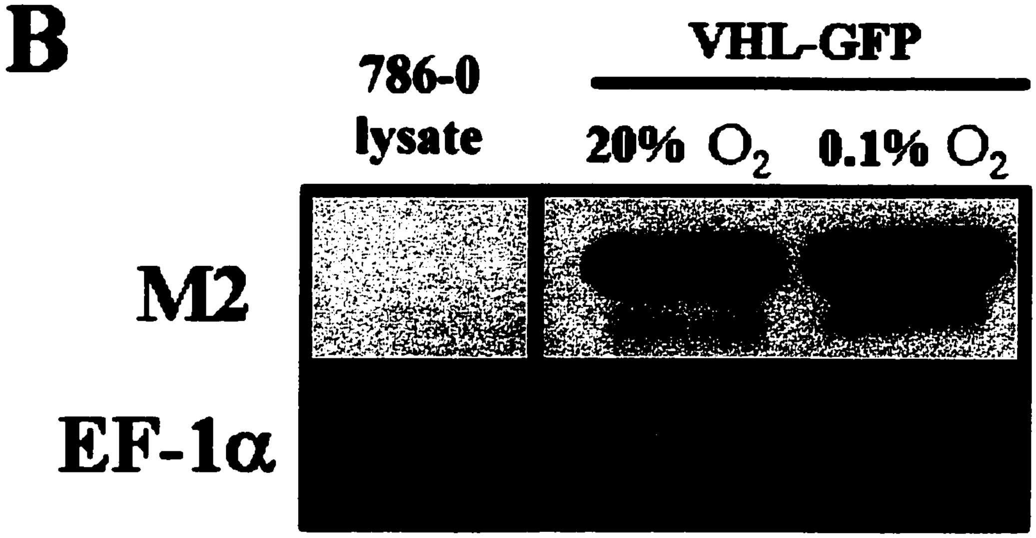

B
786-0
lysate
adVHL-GFP

DRB
EF-1α

Figure 17. The binding of EF-1α to VHL is affected by the presence of GMP-PNP (non-hydrolyzable analog of GTP), GDP and the presence of oxygen. A) VHL$^{-/-}$ RCC 786-0 cells infected (or not) with adVHL-GFP in normoxia were lysed and immunoprecipitations were performed with pre-blocked anti-FLAG M2 beads in the presence of GMP-PNP or GDP (2 mM) overnight at 4°C. Immunoprecipitates were run on an 8% SDS-PAGE and blotted onto PVDF membranes. Membranes were blocked and incubated in the presence of a mouse anti-FLAG M2 (top panel) or a mouse anti-EF-1α (bottom panel) antibody. B) VHL$^{-/-}$ RCC 786-0 cells stably transfected (or not) with VHL-GFP were incubated in normoxia or in hypoxia for 4 hours. Cells were lysed and immunoprecipitations were performed with pre-blocked anti-FLAG M2 beads overnight at 4°C. Immunoprecipitates were run on an 8% SDS-PAGE and blotted onto PVDF membranes. Membranes were blocked and incubated in the presence of a mouse anti-FLAG M2 (top panel) or a mouse anti-EF-1α (bottom panel) antibody. 786-0 lysate correspond to total cell lysate of 786-0 cells that were lysed in 4% SDS in PBS (150 μg).


A
Uninfected
adVHL-GFP

GMP-PNP
GDP
M2
EF-1α


B
786-0
lysate
VHL-GFP
20% O2
0.1% O2
M2
EF-1α

CHAPTER 4

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 (Stebbins *et al.*, 1999). Yet, the nature and localization of the mutations has a profound effect on the clinical manifestations in inherited VHL syndrome (Chen *et al.*, 1995). Likewise, sporadic RCC tumors are much more likely to harbor mutations in exon 2, which mutations are rarely found in individuals afflicted with inherited VHL syndrome (Gnarra *et al.*, 1994). 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 HIF-α 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 to 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 HIF-α and fibronectin and to mediate transcription-dependent shuttling of VHL. The binding results are similar to those recently reported by two other groups, which also demonstrated that missense mutations in exon 1-encoded portion of the β-domain also abrogated VHL assembly to HIF-α but not to BC/Cul-2 (Cockman *et al.*, 2000; Ohh *et al.*, 2000). 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 (Lonergan *et al.*, 1998; Stebbins *et al.*, 1999). 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 HIF-α *in vitro* (Cockman *et al.*, 2000; Ohh *et al.*, 2000; Tanimoto *et al.*, 2000). Either ΔE2-GFP and ΔE3-GFP fold in a different way *in vivo* compared to *in vitro* or these mutants have a yet uncharacterized defect that prevents their assembly with HIF-α 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 (Gnarra *et al.*, 1996a). A VHL protein without exon 2 sequences might change substrate specificity from HIF-α 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 β-domain of VHL is involved in substrate protein, as well as fibronectin, recognition (Stebbins *et al.*, 1999). They also demonstrate that tumor-derived mutations inactivate VHL functions in different ways, which may lead to distinct cellular phenotypes.

The study of adΔE2-GFP has also revealed other interesting biochemical aspects about 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 (Schoenfeld *et al.*, 2000). Data shown here are somewhat in disagreement with this model since equal amounts of Cullin-2 can be found bound to VHL and adΔE2-GFP, regardless of conjugation to NEDD8. NEDD8 conjugation is reported to be a nuclear event (Kamitani *et al.*, 1997). adΔE2-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 (Moore and Blobel, 1992; Gorlich and Mattaj, 1996; Nigg, 1997). The observation that adΔE2-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 HIF-α signal exclusively in the nucleus of normoxic VHL$^{-/-}$ cells indicating that HIF-α 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 HIF-α contains a nuclear import signal that is activated only in hypoxia (Kallio *et al.*, 1998). One possible interpretation of these data is that the hypoxia-inducible nuclear import of HIF-α is regulated by VHL, which might play a role in retaining HIF-α 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 does not require assembly with HIF-α. However, we did find that adVHL-GFP accumulated in the nucleus upon incubation with proteasome inhibitors, similarly 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 (Scheffner, 1999; Tomoda *et al.*, 1999). Sensitivity to proteasome inhibitors is mediated by exon 2-encoded β-domain in a manner reminiscent to DRB. We have previously shown that VHL transcription-dependent shuttling domain act 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* (Groulx *et al.*, 2000). 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 HIF-α-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, less efficient, nuclear export signals within the complex might gain dominance upon loss of function of exon 2-encoded residues.

As mentionned earlier, VHL does not share sequence homology with other known protein, not even a leucine-rich region for nuclear export (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997). In fact, Groulx *et al.* (2000) have shown that VHL shuttling is not affected by a treatment with leptomycin B, a drug that affects CRM1-mediated nuclear export of protein containing a classical, leucine-rich nuclear export signal (NES) (Nishi *et al.*, 1994; Wolff *et al.*, 1997). Results presented in this thesis are in agreement with previous studies suggesting that exon 2-encoded β-domain of VHL is the "transcription-dependent trafficking domain" (Lee *et al.*, 1999). It is possible that new sequences involved in transcription-dependent nuclear/cytoplasmic trafficking will eventually be characterized. However, VHL might also interact (through exon 2-encoded residues) with other proteins that would play a role in VBC/Cul-2 cellular trafficking. Groulx *et al.* have shown that VHL exports from the nucleus through a Ran-mediated and ATP hydrolysis-dependent manner. Nevertheless, the hypothesis that other factors might be required for VHL nuclear export still remains.

In this perspective, the finding that VHL exon 2-encoded β-domain is required for the interaction with endogenous EF-1α is very interesting. One hypothesis is that EF-1α might regulate the transcription-dependent shuttling activity of VHL through its association with the VBC/Cul-2 complex. The results presented here are in agreement with this hypothesis. We have shown that a decreased amount of EF-1α is bound to the complex upon treatment with DRB. According to this model, inhibition of transcription with DRB would cause a disruption of the interaction between VHL and EF-1α and would result in a defective nuclear export of the complex. This would explain the nuclear accumulation of the VBC/Cul-2 complex that we observe upon DRB treatment. Alternatively, EF-1α, a protein mostly localized in the cytoplasm at steady state, might play a role of cytoplasmic retention. In this case, a treatment with DRB would also result in nuclear accumulation of the complex due to the lost of cytoplasmic anchory.

Previous studies have suggested that VHL-GFP does not require assembly with Cullin-2 to engage in nuclear export (Groulx *et al.*, 2000). However, its presence is necessary for the E3 ubiquitin ligase activity of the VBC/Cul-2 complex. Similarly to studies on Cullin-2 interaction with VHL, it will be important in the near future to determine how and when EF-1α binds to VHL. Experiments will be needed to try to find out if EF-1α can also mediate nuclear export of VHL without its other partners (BC/Cul-2). EF-1α is a very abundant protein and is essential for cellular survival because of its major role in protein translation. Therefore, new stategies will have to be established in order to study EF-1α functional role in intact cells, since no EF-1α knock out cell lines are viable.

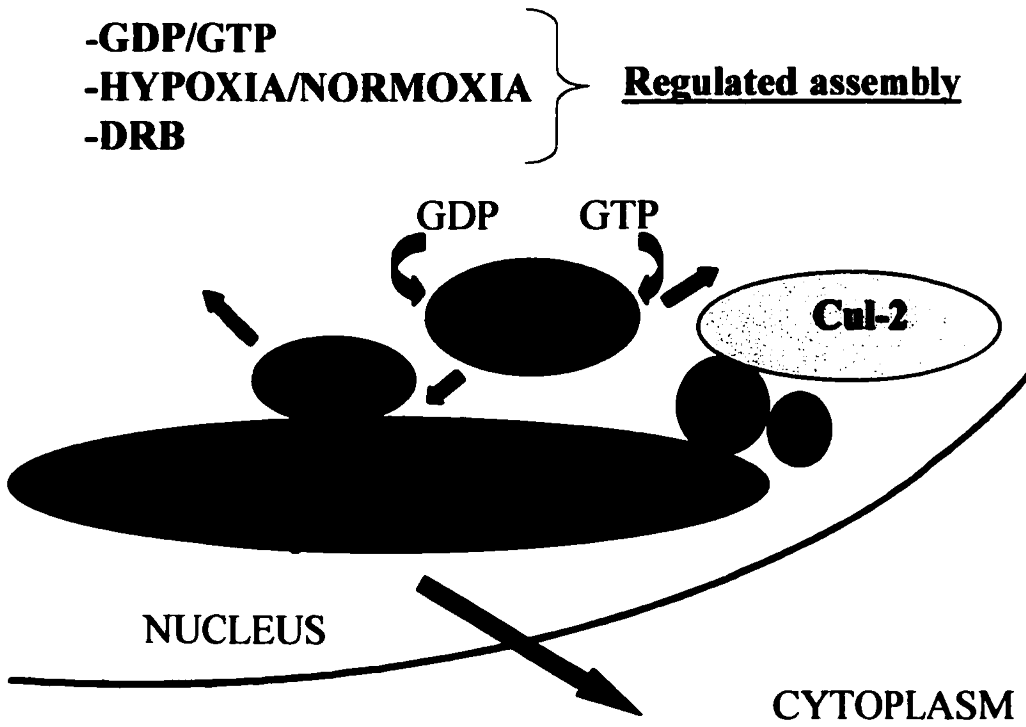
Other experiments will be performed in order to:

1- Precise the sequences involved in the interactions VHL/HIF-α and VHL/EF-1α, respectively. Since the exon 2-encoded β-domain seems to be required for the interaction with both proteins, we can hypothesize that HIF-α and EF-1α bind to very close sequences and that binding of one of these proteins prevents VHL interaction with the other. We can hypothesize that EF-1α would be a "chaperone", and that binding of EF-1α to VHL would prevent binding of HIF-α.

2- Clarify if EF-1α is involved in the oxygen sensing mechanism related to VHL activity and subsequent degradation of HIF-α. In this work, I have shown that EF-1α binding to VHL can be affected by many conditions, in particular hypoxia. Hypoxia seems to increase the amount of EF-1α bound to VHL. It is generally accepted by scientifics working in this field that hypoxia have a direct effect on the E3 ubiquitin ligase activity of the VBC/Cul-2 complex. However, there is not yet clear evidence that it is the case. Some studies have shown that ubiquitination of HIF-α is inhibited by desferroxamine, a compound used to mimic hypoxia (Ohh *et al.*, 2000). Whether hypoxia and desferroxamine stabilize HIF-α through the same molecular mechanism(s) remains to be determined. Does the increased binding of EF-1α to VHL in hypoxia occur to prevent binding to HIF-α? HIF-α ubiquitination? Since the hypoxic condition is highly related to the energy forms available, it will be also important to look at the energy requirement for EF-1α binding to VHL. The results that I have presented here suggest that EF-1α binds more to VHL when put in presence of GDP. Therefore, it will be necessary to determine if the binding of EF-1α to VHL is affected mainly by the oxygen concentration itself or by the forms of energy present under hypoxia. More experiments need to be performed in order to answer all of these questions.

Put together, the results presented in this thesis support the model that exon 2-encoded residues are involved in two independent functions: mediating transcription-dependent nuclear/cytoplasmic trafficking of the VBC/Cul-2 complex and binding to HIF-α substrate. We are still in the process of identifying relevant sequences involved in signal-mediated and Ran-dependent nuclear/cytoplasmic trafficking of VBC/Cul-2 complex. Also, we plan to further examine the role of EF-1α in intact cells. This will surely provide important clues in the elucidation of VHL-mediated tumor suppressor function.

Figure 18. Hypotheses concerning the role of assembly of EF-1α with VHL. EF-1α regulated assembly with VHL suggests that EF-1α might be implicated in the transcription-dependent export of VHL or that EF-1α might act as a chaperone which prevents the interaction between VHL and substrate HIF-α. This diagram is not to scale.


-GDP/GTP
-HYPOXIA/NORMOXIA
-DRB
Regulated assembly
GDP
GTP
Cul-2
NUCLEUS
CYTOPLASM

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