A new role for the von Hippel-Lindau tumor suppressor protein: stimulation of mitochondrial oxidative phosphorylation complex biogenesis

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Although mitochondrial cancer has been described by Warburg, many years ago, the mechanisms underlying this impairment remain essentially unknown. Many types of cancer cells are concerned and, in particular, clear cell renal carcinoma (CCRC). In this cancer, the tumor suppressor gene, VHL (von Hippel-Lindau factor) is invalidated. Previous studies have shown that the transfection of the VHL gene in VHL-deficient cells originating from CCRCs could suppress their ability to form tumors when they were injected into nude mice. However, various additional genetic alterations are observed in such cancer cells. In order to investigate whether VHL invalidation was related to the mitochondrial impairment, we have studied the effects of wild-type VHL transfection into VHL-deficient 786-0 or RCC10 cells on their oxidative phosphorylation (OXPHOS) subunit contents and functions. We show that the presence of wild-type VHL protein (pVHL) increased mitochondrial DNA and respiratory chain protein contents and permitted the cells to rely on their mitochondrial ATP production to grow in the absence of glucose. In parallel to mtDNA increase, the presence of pVHL up regulated the mitochondrial transcription factor A, as shown by western blot analysis. In conclusion, in CCRCs, pVHL deficiency is one of the factors responsible for down-regulation of the biogenesis of OXPHOS complexes.

Introduction

Clear cell renal carcinomas (CCRC) are the most common renal malignancy and represent >70% of kidney cancers. These cancers are characterized by bi-allelic inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene, located at the level of the chromosome 3 short arm, at locus 3p25-26 (1–3). von Hippel-Lindau protein (pVHL) forms a complex with several proteins including elongins C and B, Cul2 and Rbx1 (also called Rox1 or Hrt1) (3). This complex displays E3 ubiquitin ligase activity that targets the alpha subunit of the heterodimeric transcription factor HIF (hypoxia-inducible factor) for ubiquitinylation, leading to its rapid proteasomal destruction (4). Three human HIFα proteins: HIF1α, HIF2α and HIF3α have been described. In the presence of oxygen, the HIFα chains are poly-hydroxylated on conserved prolyl and arginyl residues by oxygen-dependent prolyl (5–7) and arginyl hydroxylases (8). Once hydroxylated, the HIFα proteins bind to the pVHL complex to be ubiquitinylated (see review in ref. 9). Under low oxygen conditions (hypoxia, ischemia), hydroxylation is reduced and the stabilized HIFα chains accumulate in the nucleus where they form a complex with the constitutively expressed HIFβ. The latter complex can activate the transcription of >40 genes whose protein products play crucial roles in cellular adaptation to a low oxygen environment (10). It increases the synthesis of erythropoietin, of vascular endothelial growth factor, of glucose transporters and glycolytic enzymes in order to maintain ATP production despite the low efficiency of anaerobic metabolism compared with oxidative metabolism (11). It also shifts the balance between pro- and anti-apoptotic factors to promote their survival, favors cell proliferation (tumor growth factors and TGFβ), stimulates synthesis of genes involved in iron regulation, in proteolysis (metalloproteinase) (12) or else in acid–base controlling metabolic pathways (carbonic anhydrase IX) (13). In cells lacking wild-type pVHL, these genes are constitutively expressed and contribute to tumor proliferation.

Recent studies in our laboratory (14) have shown that in CCRCs, the expression of most proteins involved in oxidative phosphorylation (OXPHOS) is strongly decreased while, in renal oncocytoma, OXPHOS complex proteins are all increased except those of complex I (15). Similarly, Cuezva et al. have shown that in CCRCs as well as in cancers from other tissues, a decreased bioenergetic function of mitochondria is associated with an increased glycolytic rate in accordance with the Warburg hypothesis (16). For example, the expression of the β subunit of the mitochondrial H⁺-ATP synthase was found to be depressed in liver, kidney and colon carcinomas (17) or in lung, breast, esophagus or stomach cancers (18) while glycolytic markers were increased. This bioenergetic deficiency was the highest in patients with a poor prognosis. The glycolytic phenotype is well recognized in many cancer cells (19,20) but the mechanism by which mitochondrial deficiencies may be related to tumor development is poorly understood (21). It depends on the cancer type, as carcinogenesis in the liver involves a depletion of the whole cellular mitochondrial content whereas in kidney and colon carcinomas, OXPHOS subunits are more specifically reduced (17). As mentioned above, the up-regulation of glycolytic enzymes in renal carcinomas is due to the well-documented transcriptional activation of glycolytic enzymes by HIF in VHL-deficient cells after binding of HIF on hypoxia-responsive element sites.
(HRE) present in the promoter of genes encoding such enzymes (20). However, it is not known whether, and how, HIF could also inhibit mitochondrial biogenesis. Besides, to our knowledge, HRE-like sites that might induce a silencing effect on the expression of OXPHOS genes have not been identified.

In addition to the VHL modifications, other genetic alterations (22–25), often associated with higher grades of tumors, have been observed in CCRCs. A multi-step process in which modifications of several chromosomes come into play to reach full malignancy characterizes these CCRCs. Although mitochondrial deficiency is not much more important in high than in low tumor grade CCRCs (14), it cannot be excluded that some of these additional genetic alterations might be responsible for the mitochondrial deficiency. In the present study, we have used CCRC cells initially devoid of active pVHL but transfected with ectopic wild-type VHL or with a void vector to test the specific influence of pVHL on the expression of proteins involved in OXPHOS. We show that the presence of pVHL, known to prevent the formation of tumors after injection in nude mice (26), stimulated the expression of OXPHOS proteins and restored the cell capacity to grow in the absence of glucose, that is under conditions in which mitochondria are the essential source of energy for the cell.

Materials and methods

Cell lines originating from CCRC patients

pVHL-deficient 786-0 cell clones transfected either with an empty vector (clone PRC3) or with wild-type VHL (clones WT5 and WT10) (26) were kindly provided by William Kaelin (Dana-Farber Institute, Boston, MA). pVHL-negative RCC10 cells and a clone stably transfected with wild-type VHL (clone 53) were generous gifts of Dr Karl H.Plate (Johann Wolfgang Goethe University, Frankfurt, Germany) (27). The CCRC cell lines are derived from human sporadic CCRCs and express inactive pVHL.

Cell culture

Cells were grown in RPMI 1640 medium with 10% fetal calf serum at 37°C in 5% CO2 and routinely used at 70% confluence. For metabolic studies, the cell number was estimated by counting in the presence of 0.04% Trypan Blue and was followed for 2-6 days in culture medium containing either 2 g/l of d-glucose or 2 g/l of d-galactose. Nuclear morphology was assessed in re-suspended cells stained either with Hoechst and fixed with 3% formaldehyde or with acridine orange and ethidium bromide as described by Ghelli et al. (28).

Tissue samples

Tumors from CCRCs and their adjacent normal renal cortex were obtained from patients and their grades were characterized as described (14).

Mitochondrial enzyme activities

Activities of citrate synthase (29) and succinate cytochrome c reductase (30) were determined in cells spun down at 200 x g for 5 min, frozen at ~80°C, thawed and homogenized in NaCl, phosphate medium (1.54 mM KH2PO4, 0.15 M NaCl, 2.7 mM Na2HPO4 - 7H2O, pH 7.4). ATPase activity was determined spectrophotometrically in an ATP-regenerating system as described previously (31). About 5 x 105 frozen cells were permeabilized for incubation for 10 min in 1 ml ice-cold medium D (0.25 M sucrose, 20 mM Tris-HCl, pH 7.2, 40 mM KCl, 2 mM EGTA, 1 mg bovine serum albumin/ml) to which 0.01% digitonin and 10% PercollTM were added; cells were spun down at 2200 g for 5 min, washed in 1 ml medium D and spun again at 6000 g for 5 min. The final pellet was homogenized in 80 µl of medium D. The mitochondrial ATPase activity was calculated as the difference between the rates measured before and after addition of 1 µg oligomycin and 2 µM aurovertin.

Polarographic measurements

Oxygen consumption of cells was measured at 30°C in K medium (80 mM KCl, 10 mM Tris–HCl pH 7.4, 3 mM MgCl2, 1 mM EDTA, 5 mM KH2PO4) as described in (32) using a high resolution OROBOROSTM oxygraph.

Cytofluorometric analysis of mitochondrial membrane potential

About 5 x 103 cells were diluted to 1 mg protein/ml in K medium and then permeabilized with 0.1 mg digitonin/ml of protein for 15 min at room temperature in the presence of 20 nM TMRE. Analyses were performed as described before in (32).

Western blotting

Total proteins of frozen tissues or cells were extracted in lysis buffer: 10 mM Tris, 1 mM EDTA, pH 8, 1 mM PMSF, 2 µg/ml anti-proteases (Sigma-AldrichTM), 1 mM sodium vanadate, 1% deoxycholate. After protein quantification with the Bradford assay (from Bio-RadTM), dithiothreitol (0.1 M) was added and the mix was incubated for 10 min on ice. Lysis was then achieved after adding 0.2% Triton X-100 and 1% SDS (final concentrations). Proteins (10-30 µg) were separated in a 12.5% acrylamide/bisacrylamide gel and transferred to a nitrocellulose BA83 membrane (Schleicher and SchuellTM) that was pre-incubated with 5% skim milk in NaCl-phosphate buffer. The membrane was incubated for 2 h at room temperature with various primary antibodies (except anti-pVHL that was incubated overnight at 4°C), washed four times with buffer A (NaCl-phosphate buffer containing 0.1% Tween 20) and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-RadTM, 1/10 000) or anti-mouse IgG (Bio-RadTM, 1/3000) and washed with buffer A. Anti-13.4 kDa (1/3000), anti-core II (1/10 000), anti-iron sulfur protein, (anti-ISP) (1/25 000), antibodies were prepared as described by Logers et al. (33) against the corresponding proteins that belong to complex III (34). Anti-ATPase subunit 6 (1/500) was prepared as described by Dubot et al. (35). Anti-adenine nucleotide translocator, anti-Cox IV (1/2000) and anti-mitochondrial transcription factor A (TFAM) (1/10000) were generous gifts of Dr Brandolin (36), Dr Taanman (37) and Dr Rojo (38), respectively. Anti F1-ATPase alpha subunit (1/2000) was from Morad-Ameli and Godinot (39), and anti-pVHL (1/250) from Becton DickinsonTM.

Table I. PCR conditions used for mtDNA content analysis

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Forward primers</th>
<th>reverse primers</th>
<th>Temperature (°C)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SURF1</td>
<td>5’CTTGTCTGCCACGTCTGTTCTTA3’</td>
<td>5’AGACTCTCGGTAATGCTCAAGGC3’</td>
<td>60</td>
<td>82</td>
</tr>
<tr>
<td>Cytochrome b</td>
<td>5’GCCCTACTCTCTCTCTCTCTCT3’</td>
<td>5’TCCAGTCTTGTGTAAGGACGG3’</td>
<td>60</td>
<td>117</td>
</tr>
</tbody>
</table>

mtDNA quantification using real time PCR

Total DNA was extracted using the genomic DNA kit from Sigma-AldrichTM. To determine the mitochondrial to nuclear DNA ratio, the SURF1 gene present as a single copy in each chromosome 9 allele of nuclear DNA and the CYTB gene present once in each of the multiple mtDNA copies were amplified using primers indicated in Table I. The PCR reaction was performed in a Roche Light Cycler apparatus, using the Faststart DNA master SYBR Green kit (Roche ManheimTM, Germany). DNA (2 µl) was mixed with a buffer containing 5 mM MgCl2, 0.2 mM dNTPs, 0.5 µM of forward and reverse primers, SYBR green I dye and 0.25 U Hot Start Taq DNA polymerase in a final volume of 20 µl. The reactions were performed as follows: initial denaturation at 95°C for 8 min, and 45 cycles at 95°C for 15 s, 60°C for 5 and 72°C for 10 s. Each measurement was done four times and normalized in each experiment with serial dilutions of a control DNA sample.

Statistical analysis

Means from three or more independent experiments are given. Statistical comparison between different enzyme activities, mitochondrial DNA levels and TFAM content was performed by one way analysis of variance followed by Tukey’s post hoc comparison for multiple data comparisons or by Student’s paired t-test for paired data, as appropriate, using the Instat3TM software. Statistical significance was set at *P < 0.05, **P < 0.01 or ***P < 0.005.

Results

Stimulation of mitochondrial respiratory chain activities and OXPHOS capacity by transfection of wild-type VHL in VHL-deficient cells

The citrate synthase activity, often considered as proportional to the amount of mitochondria, has been measured in VHL-deficient cells or in cells transfected with wild-type VHL. Figure 1A shows that the presence of pVHL did not significantly modify the citrate synthase activity. The activity of complex II + III was determined by the succinate cytochrome c
VHL transfected with wild-type different parental CCRC cell lines (786-0 and RCC10) and in the same cells/C6 VHL/C3/C3 stars:

c succinate-cytochrome c reductase activity that could be suppressed by malonate or by antimycin A (Figure 1B). It was ~2-fold higher in VHL-deficient cells and could be suppressed by malonate or antimycin A

Effect of wild-type Fig. 1.

were observed when RCC10-53 cells transfected with VHL or in PRC3 cells transfected with a void vector. Similar results were compared with parental, PRC3) and VHL-deficient RCC10 parental cells.

To evaluate the overall OXPHOS capacity of 786-0 cells transfected or not with wild-type VHL, their rate of oxygen consumption was monitored after cell permeabilization with 0.1 mg digitonin/mg of protein in the presence of substrates and inhibitors of OXPHOS (Figure 2). Although in each experiment, there was a tendency for up-regulation in the rates of pyruvate + malate and succinate oxidation [average increase of 38\% in the presence of ADP and of 11\% in the presence of carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP)], and of cyanide-sensitive oxidation of ascorbate + N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), reflecting the cytochrome c oxidase (COX) activity, the differences were not statistically significant because relatively large variations in oxygen consumption rates were observed from one experiment to the other.

The ATPase activity, sensitive to oligomycin that inhibits only the whole F0-F1 complex, and to aurovertin known to inhibit as well F0-F1 complex as free F1 or F1 not fully assembled with all other F0 subunits was similar in 786-0 cells devoid of VHL or transfected with VHL. This activity was of 0.166 ± 0.019 μmol of ATP hydrolyzed per minute and per milligram of protein in VHL-deficient 786-0 cells (mean of nine assays±SEM) and of 0.165 ± 0.009 (15 assays) for cells transfected with VHL (WT-8). The percentage of ATPase activity that was sensitive to aurovertin but insensitive to oligomycin did not appear to be modified by the presence of pVHL.

Fig. 2. Increase in oxygen consumption capacity in 786-0 cells transfected with VHL. Cells were permeabilized with digitonin (0.1 mg/mg protein) and oxygen consumption (pmol/s/mg protein) was monitored after successive additions of substrates and inhibitors of the mitochondrial respiratory chain (10 mM pyruvate + 3 mM malate, 1 mM ADP, 10 mM succinate, 10 μM oligomycin, 1 μM FCCP, 1 μM antimycin A, 5 mM ascorbate, 300 μM TMPD, 500 μM KCN). The graph shows mean values ± SD from four experiments of pyruvate + malate + succinate oxidation stimulated by ADP (state 3 ADP) or FCCP (state 3u FCCP) and KCN-sensitive ascorbate + TMPD oxidation (COX) or inhibited by oligomycin in parental 786-0 cells (white boxes) and WT-8 transfected with wild-type VHL cells (black boxes).

Lack of effect of VHL transfection on the mitochondrial membrane potential of VHL-deficient cells

VHL-deficient 786-0 cells (PRC3 or 786-0 parental) and cells transfected with VHL (WT8, WT10) were permeabilized and treated with different concentrations of digitonin (0–0.5 mg/mg of protein) to optimize the cell permeabilization conditions, as tested with propidium iodide fluorescence and TMRM staining with FACS. A complete permeabilization was obtained with 0.1 mg digitonin/mg of protein that gave a maximal TMRM signal (not shown). Simultaneously to the measurement of TMRM fluorescence, MitoTracker Green (Molecular Probes\textsuperscript{TM}) fluorescence was determined. The latter fluorescence is considered as proportional to the amount of mitochondria, independently of the membrane potential. In this way, the membrane potential could be normalized to the amount of mitochondria in each cell line. The normalized intensities of TMRM fluorescence were not significantly different in VHL-deficient 786-0 cells and in cells expressing wild-type pVHL. In all cases, the high TMRM fluorescence observed in state 4 was collapsed after FCCP addition and was decreased after ADP addition (the ADP decrease was prevented by oligomycin, aurovertin or atracyloside, data not shown) similarly for all types of cells. This indicated that the mitochondria present in VHL-deficient cells were able to maintain a membrane potential similar to that of cells expressing wild-type pVHL.

Increase in OXPHOS protein contents by transfection of VHL in VHL-deficient cells

Total proteins were extracted from VHL-deficient (786-0 parental, PRC3) and VHL-transfected cells (WT8, WT10)
and western blot analysis was performed using antibodies specific for pVHL and for OXPHOS complex subunits. Increasing protein amounts (10–30 μg) were used in each experiment to estimate the proportionality between the signal and the amount of protein for each tested antibody. After transfer, the membranes were stained with Ponceau Red to verify that similar protein amounts were present for each cell type. In Figure 3, the signal observed with the anti-pVHL antibody confirmed that pVHL was only present in VHL-transfected cells. Staining obtained with antibodies against complex III subunits (core II, 13.4 kDa and iron sulfur protein), complex IV subunits (COX II subunit encoded by mitochondrial DNA and COX IV subunit encoded by nuclear DNA) and complex V (F1-ATPase F1 alpha subunit (nuclear origin), ATPase-6 (mtDNA-encoded) and pVHL) were used.

It was checked that respiratory chain subunit contents that were lower in 786-0 cells than in VHL-transfected cells, were also depleted in tumor samples as compared with their normal adjacent renal tissue and that therefore, this depletion was not limited to the 786-0 tumor cell line. Total proteins of CCRC tumor samples and of normal adjacent tissues were extracted and western blot analysis was performed with antibodies directed against different subunits of respiratory chain complexes, and against pVHL. In agreement with the above results, a strong decrease in OXPHOS complex contents of CCRC tumor samples was observed (Figure 4). Subunits of ubiquinol-cytochrome c reductase (core II and 13.4 kDa subunits) and of cytochrome c oxidase (subunit II and subunit IV) were barely detectable in tumors as compared with normal adjacent tissues. In addition, all three CCRC samples tested...
in this experiment were deficient for pVHL but contained similar amounts of adenine nucleotide translocator. It should be noted that the differences in the amounts of OXPHOS proteins between normal and tumor tissues were more pronounced than those observed between 786-0 cells expressing pVHL versus null (compare Figures 3 and 4). However, the protein amount detected by western blot should not be considered as a precise quantification because the signal intensity is only proportional to the amount of proteins in a restricted range and the respiratory chain subunit amounts remaining in the tumor are probably below the respective antibody detection limits.

**Increase in mtDNA level by VHL transfection in VHL-deficient cells**

In CCRCs, the decrease in OXPHOS activities is concomitant to a decrease in mtDNA content (14). We therefore determined the effect of VHL transfection on the amount of mtDNA to know whether an increase in mtDNA could be correlated to OXPHOS stimulation by pVHL observed above by oxypolarography, by enzymatic analysis or by western blot. Total DNA was extracted from 786-0 parental cells or cells transfected with an empty vector (PRC3) or with a vector expressing wild-type pVHL (WT8 and WT10) in order to quantify mtDNA by real time PCR. Nuclear or mitochondrial DNA was amplified in the presence of primers designed for SURF1 or CYTB genes, respectively (Figure 5). The ratio between mitochondrial DNA and nuclear DNA levels appeared about two times higher in cells transfected with VHL (WT8 or WT10) than in VHL-deficient cells (786-0 parental cells or PRC3 cells). Similar data were obtained when comparing VHL-deficient RCC10 cells to the RCC10-53 cells transfected with VHL.

**Capacity of VHL-deficient cells to rely on ATP provided by OXPHOS for their growth**

Studies made by Steinberg et al. (41) have clearly shown that glycolysis is activated in CCRCs. The question was raised to know whether the OXPHOS activity remaining in VHL-deficient 786-0 cells was efficient enough to sustain cell growth and survival. In previous studies using cells exhibiting partial OXPHOS deficiency, growth impairment has been reported when the cells were incubated in a medium containing galactose in place of glucose (28,42,43). Galactose is not efficiently utilized by mammalian cells as a glycolytic substrate and these cells are forced to rely almost exclusively on OXPHOS for ATP production (43). VHL-deficient (786-0 parental) and VHL-transfected (WT8) cells seeded at $15 \times 10^5$ cells/dish were cultured in DMEM supplemented with SVF (10%) and either D-glucose or D-galactose (1-5 g/l). Preliminary experiments showed that optimal growth rate was observed when the sugar concentration was at least 2 g/l. Figure 6A compares the growth curves of VHL-deficient 786-0 cells or 786-0 cells transfected with wild-type VHL in media containing pyruvate and either glucose or galactose. Both VHL-deficient cells and cells transfected with VHL exhibited an exponential growth in the presence of glucose for 4-6 days until cells were confluent. However, in glucose-containing medium, the VHL-deficient cells grew at a slower rate (initial doubling time of 28 h) than the cells transfected with VHL (initial doubling time of 19 h). In contrast, in glucose-depleted galactose-containing medium, the VHL-deficient 786-0 cells failed to divide. The cells expressing pVHL...
(WT10) grew at a reduced rate in glucose-depleted galactose medium, when compared with that observed in glucose medium (initial doubling time of 32 h in galactose instead of 19 h in glucose). VHL-deficient PRC3 cells transfected with a void vector behave similarly as the VHL-deficient 786-0 cells and the VHL-transfected WT8 clone could be maintained in the presence of galactose in a similar manner as that of the WT10 clone (not shown). When pyruvate was removed from the culture medium (Figure 6B), the VHL-deficient cells progressively died in glucose-depleted galactose-containing medium. After 6 days, almost all the VHL-deficient cells grown in the absence of pyruvate in glucose-depleted galactose-containing medium were floating. In contrast, cells transfected with VHL could grow in the presence as well as in the absence of added pyruvate.

**Effect of pVHL expression on the level of mitochondrial transcription factor A involved in mtDNA maintenance and synthesis**

The above data showed that, although the membrane potential was maintained in the absence of pVHL, a decrease in OXPHOS capacity sufficient to prevent the cells from growing in the absence of glucose was observed. This was related to a diminution in the contents of several OXPHOS complex proteins. The lowered level of mtDNA could explain the decreased amount of OXPHOS subunits. Yet, it remains to understand how pVHL could regulate mtDNA synthesis, which is known to be under the control of nuclear genes (44–47). As expected from the mtDNA increase (Figure 5), the protein expression of TFAM was higher in cells containing pVHL than in cells devoid of pVHL (Figure 7). Interestingly, the TFAM protein amount was also decreased in VHL-deficient tumor samples when compared with normal adjacent tissues.

![Fig. 7. Influence of pVHL on TFAM protein expression. The TFAM protein amount present in cell lines expressing pVHL (WT8 and WT10), VHL-deficient (786-0) or transfected with an empty vector (PRC3) and in tumors (T) or in their adjacent normal kidney (N) was analyzed by western blot, as described in Figure 3, using 10 or 20 μg protein loads in the gel electrophoresis and anti-TFAM antibody. The signal obtained on the western blot film was scanned and quantified with the Image Quant software™. The ratio between the signal intensity observed in the studied cells to that observed in the absence of pVHL (PRC3 cells) was calculated in each experiment. The given value represents the mean of six determinations ± SD. For the tumors, the ratio between normal adjacent kidney and tumor was calculated and the given value is the mean ratio obtained for four different tumors±SD. *P <0.05; **P <0.01. No pVHL could be detected in these four tumors.](http://carcin.oxfordjournals.org/)

**Discussion**

The data presented in this report demonstrate that transfection of VHL in VHL-deficient cells increased mitochondrial OXPHOS protein or DNA contents and their functions. Indeed, VHL transfection increased the expression of respiratory chain complexes, stimulated succinate-cytochrome c reductase and cytochrome c oxidase activities, improved the rate of substrate oxidation and restored their ability to grow in the absence of glucose that is at the only expense of ATP provided by mitochondria. Therefore, the absence of VHL gene expression was at least one of the components involved in mitochondrial impairment in VHL-deficient cells.

On the opposite, VHL-transfection in VHL-deficient cells modified neither their mitochondrial membrane potential nor their oligomycin- and aurovertin-sensitive ATPase activity. It means that the mitochondria present in VHL-deficient cells can maintain their membrane potential even though their respiratory chain content was lower than that of cells transfected with VHL. Previous studies had shown that the mitochondrial membrane potential can be maintained in ρ0 cells devoid of mtDNA and hence unable to synthesize ATP, provided that the F1-ATPase and the adenine nucleotide translocator remained active (31,48). The VHL-deficient cells contained similar ATPase activity and amounts of F1-ATPase subunit as cells transfected with VHL (Figure 3). F1-ATPase was also found in all tested tumor samples as an at least partly assembled F0-F1 complex, although its activity could be decreased in some cases (14). Adenine nucleotide translocator was also present in CCRC tumor samples (this work and ref. 49) as well as in renal cancer cell lines (49). The maintenance of this mitochondrial membrane potential is essential for cell survival. Indeed it is necessary in mitochondria for the import of proteins involved in the citric acid cycle and amino acid, fatty acid or nucleic acid metabolism. On the contrary, mitochondrial synthesis of ATP may be suppressed without inducing cell death provided that glycolysis could yield enough ATP to the cytoplasm. It is therefore not surprising that these cancer cells could maintain their mitochondrial membrane potential. The ATPase activity was decreased in most high-grade CCRC tumor biopsies, and the proportion between free F1 and assembled F0-F1 complex was somewhat variable in previously analyzed tumor biopsies (14). Transfection with VHL did not modify the amount of F1 but increased that of subunit 6, as mentioned above. The lower amount of ATPase subunit 6 in VHL-deficient cells than in cells transfected with VHL implies that the whole amount of complex V was lower in VHL-deficient cells. If free F1 compensated for the lack of complex V this could maintain the ATPase activity and hence the membrane potential (31). But free F1 or complex V devoid of subunit 6 should be aurovertin-sensitive and not oligomycin-sensitive. The fact that pVHL did not change the relative sensitivity to aurovertin and to oligomycin suggested that VHL transfection did not significantly change the proportion of active ATP synthase made of intact oligomycin-sensitive F0-F1 complex to that of active ATPase containing F1 or incomplete F0-F1 complex. Previous studies on CCRC biopsies have shown that, unexpectedly, the presence of free F1 was associated to low aurovertin-sensitive ATPase activity (14), which suggests that either the F1-ATPase activity was partly inhibited in these tumors or that the aurovertin-sensitivity was decreased. Moreover, taking into account the fact that dissociation of F1-sector from F0 leads to loss of ATP synthase...
activity but to an increase of ATPase activity up to a factor of 10 (50), the measured ATPase activity may not be directly related to the F1 or F0-F1 amounts and identical rates of ATP hydrolysis in VHL-deficient or VHL-transfected cells may be fortuitous. Further studies will be necessary to understand the mechanisms regulating F0-F1 assembly and activity in these tumor cells. Anyhow, these data suggest that pVHL differentially regulates the biogenesis and/or assembly of F0-F1 and that of respiratory chain complexes.

The differences in the respiratory chain activities and subunit contents observed between VHL-deficient cells and cells transfected with wild-type VHL were not as high as those observed between tumor samples and adjacent renal cortex: the respiratory chain subunit contents were approximately two times higher in VHL containing cells than in VHL-deficient cells (Figure 3) and 5-10-fold higher in renal cortex than in tumor tissue (Figure 4). The succinate-cytochrome c reductase activity measured in normal kidney homogenates were on the average five times higher than that of adjacent tumors (14) and two to three times higher in cells transfected with VHL than in parental tumor cells or in cells transfected with a void vector (Figure 1B). The cytochrome c oxidase activity was also much more reduced when tumors were compared with normal tissue (14) than when VHL-deficient cells were compared with cells transfected with VHL (Figure 2). It should be noticed that the specific activities of these complexes calculated per milligram of protein were comparable in VHL-deficient cultured cells and in patient tumor biopsies. One reason to explain the difference between the high renal cortex activities and the comparatively low activity of cells expressing pVHL could be that, in vivo, the normal proximal renal tubule from which these tumors are originated is irrigated by an abundant oxygen flow and must express a high OXPHOS activity to sustain the energy-demanding pumping activity of the kidney that is not needed in cells cultured ex vivo (51). It has been demonstrated clearly, at least in the heart and muscle, that an increased energy demand stimulates mitochondrial biogenesis (52). Another hypothesis explaining a more pronounced decrease in OXPHOS functioning observed between tumor and adjacent tissue than between VHL-deficient and VHL-transfected cells might be related to the additional modifications of genes that would cooperate with VHL to up-regulate mitochondrial biogenesis. Indeed, in spite of the large incidence of pVHL inactivation in CCRCs, many other chromosomal alterations have been observed in these cancers (reviews in refs 24,25). In the three tumors analyzed in this study, the western blot analysis of tumor tissues revealed a very low content of core II and 13 kDa complex III subunits as well as COX II and COX IV subunits. In a previous paper (14), an average of ~20% residual enzyme activity was observed in such tumors. As mentioned above, the difference between residual complex activities in the tumors (14) and apparent residual amounts of subunits belonging to these complexes (Figure 4) might be due to the restricted range of proportionality between the amount of proteins and the antibody detection limits.

The mitochondrial biogenesis down-regulation induced by the lack of pVHL mainly concerns the OXPHOS complexes and not all mitochondrial activities. Indeed, there was no significant difference in citrate synthase activity between cultured cells containing or devoid of pVHL (Figure 1) although this activity was lowered by a factor of about two in tumor CCRC biopsies as compared to normal adjacent renal cortex (14). Therefore, while the absence of pVHL is correlated with a decrease in the biogenesis of OXPHOS complexes both in cultured cells and in tumors, a VHL-independent mechanism down-regulating citrate synthase activity is responsible for the low citrate synthase activity observed in tumors. Among OXPHOS subunits that have been tested in this study, those encoded by mtDNA, COX II and ATPase 6 were both decreased. Similarly, subunits encoded by nuclear genes were also decreased in the case of complex III and complex IV. On the contrary, the F1-ATPase α subunit expression was not modified. The assembly of this subunit to form the F1 complex or an incomplete F0-F1 sub-complex in mammalian cells does not require the presence of the subunit 6 of mitochondrial origin as F1 can be assembled in ρ0 cells (31,53). On the contrary, a lack of mitochondrial encoded subunits prevents complex IV or complex III from being stably assembled (54,55). Limiting amounts of mitochondrial encoded subunits can therefore explain the decreased activities of these two complexes.

mtDNA content was decreased by a factor of about two when comparing both renal carcinoma to normal kidney or VHL-deficient cells to cells transfected with VHL. Therefore, the VHL-induced OXPHOS complex down-regulation is probably due to this mtDNA decrease, which suggests that VHL was somehow involved in the regulation of mtDNA replication and transcription. The mitochondrial transcription factor A, TFAM was also increased by VHL transfection in parallel to the mtDNA increase. This could be related either to an increased stability of the TFAM protein or to an up-regulation of its synthesis. TFAM is a nuclear-encoded mitochondrial transcription factor, which is imported into the mitochondria, binds to light and heavy strand promoter of mtDNA and is known to be essential for the maintenance and repair of mtDNA (45). TFAM co-localizes with mtDNA in nucleoprotein complexes (38). The stability of TFAM is tightly associated to the presence of mtDNA within the cell since cells permanently (56) or transiently (57) depleted of mtDNA (ρ0 cells) are characterized by the absence of detectable levels of TFAM protein. However, the TFAM transcript is expressed at normal levels in the ρ0 cells (56). Chronic electrical stimulation increases muscle mitochondrial biogenesis (58) through an increase of TFAM and of mitochondrial COX III mRNA expression (59). Similarly, elevated TFAM mRNA expression was matched by a parallel increase in mitochondrial COX I and COX II mRNAs in rat hepatoma cells (60). However, reported studies made either by Serial Analysis of Gene Expression (SAGE) in 786-0 cells transfected or not with VHL (61,62) or by microarray analysis of CRCC biopsies compared with their normal adjacent tissue (63-65) did not put forward prominent changes in the expression of genes encoding OXPHOS complex subunits in mitochondrial DNA or that of nuclear genes encoding factors known to regulate mitochondrial biogenesis such as TFAM, NFR1, NFR2, PGCL1 or PRC (see review, ref. 66). Preliminary experiments using a semi-quantitative RT-PCR analysis initiated in our laboratory did not either suggest important modifications in the expression of these genes. Peroxisome proliferator-activated receptor γ (PPAR-γ) coactivator 1 (PGC1) or PGC1 regulated co-activator 1 (PRC) are considered to orchestrate mitochondrial biogenesis. PGC1 can up-regulate nuclear genes that are required for mitochondrial biogenesis in part through a direct interaction with NRF1 (nuclear respiratory factor 1). PRC that is cell-cycle regulated in cultured cells under conditions where PGC1 is not expressed interacts with NRF1 and its target genes, similarly to PGC1. Since other less well characterized signaling
pathways may also interfere with mitochondrial biogenesis (66), a more extensive study of expression of genes encoding mitochondrial proteins will still be necessary to ascertain whether transcription regulation is involved or not in mitochondrial biogenesis down-regulation of cancers. However, our working hypothesis rather favors mechanisms involving regulation at the level of translation or of protein stability. For example, TFAM is stabilized by its interaction with mtDNA as its transcript level is not changed in 

\[ \text{pH} \] cells while the protein is very low (see above).

The data presented in this work show that VHL-transfection of VHL-deficient CCRC cells rendered the cells able to survive and grow in the absence of glucose. Indeed, replacing glucose by galactose in the culture medium almost completely prevented cell growth, which means that the rate of ATP synthesis provided by the partially deficient mitochondria was not sufficient to sustain growth of pVHL-deficient cells. It has been shown that when glucose is replaced by galactose in OXPHOS-partially deficient cells such as cells from patients (Leber Hereditary Optic Neuropathy, cell death occurs by apoptosis (30). In 786-O cells, no sign of apoptosis could be put forward (data not shown). Gajewski et al. (67), have shown that, in wild-type cells, normal ATP levels were maintained in all cell compartments when the cells were grown in the absence of glucose and presence of pyruvate, which is metabolized in the mitochondria. On the contrary, when cells exhibiting an OXPHOS dysfunction were grown in the absence of glucose and presence of pyruvate, ATP levels were reduced in the mitochondria and to a greater extent, in the nucleus. In our experiments, in the absence of pVHL, a similar depletion of nuclear ATP could occur and therefore, cell division would be stopped by limitation of the high-energy compounds necessary to replicate nuclear DNA. Although HeLa cell growth in galactose induced an increase in mitochondrial oxidative capacity when the treatment was applied for 3 weeks (68), the OXPHOS contents of VHL-deficient cells was probably too low for the cells to overcome the mitochondrial deficiency.

In conclusion, it has been demonstrated for the first time, that the VHL tumor suppressor product up-regulates mitochondrial OXPHOS complex biogenesis and function. Further studies will be needed to understand the mechanisms of this regulation, at the level of gene transcription, of post-transcriptional modifications or of protein turnover.

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