NAD(P)H Oxidases Regulate HIF-2α Protein Expression*

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Karen Block1†, Yves Gorin‡, Paul Hoover‡, Paul Williams5, Tomasz Chelmicki‡, Robert A. Clark‡, Toshiyuki Yoneda5, and Hanna E. Abboud1‡

From the Divisions of ¹Nephrology and ²Endocrinology, Department of Medicine, George O’Brien Kidney Research Center, University of Texas Health Science Center, San Antonio, Texas 78229 and the ³South Texas Veterans Health Care System, San Antonio, Texas 78229

Biallelic inactivation of the von Hippel-Lindau tumor suppressor gene (VHL) is linked to the development of hereditary and sporadic renal cell carcinoma (RCC). In the absence of VHL, the α subunits of heterodimeric hypoxia-inducible transcription factors (HIF-1α and HIF-2α) are stabilized. Reactive oxygen species, generated by NAD(P)H oxidases, are involved in signaling cascades of malignant growth. We show that in VHL-deficient cells p22phox, Nox4 protein levels and NADPH-dependent superoxide generation are increased. Reintroduction of VHL into the VHL-deficient cells down-regulates the expression of p22phox and NADPH-dependent superoxide generation. Inhibition of the 26 S proteasome in VHL-expressing cells increased p22phox protein levels, which correlated with an increase of NADPH-dependent superoxide generation. We also show that p22phox co-immunoprecipitates with VHL in vivo. Moreover, p22phox is a target of ubiquitination. Importantly, in VHL-deficient cells, diphenyleneiodonium chloride (DPI), an inhibitor of Nox oxidases, decreased the expression of HIF-2α. Down-regulation of Nox1, Nox4, and p22phox expression by small interfering RNA also decreased HIF-2α protein expression and inhibited Akt and 4E-BP1 phosphorylation, suggesting that a translational mechanism is involved in maintaining HIF-2α in VHL-deficient cells. Colony formation by RCC 786-O in soft agar was markedly inhibited by DPI. Moreover, DPI significantly inhibited RCC 786-O tumor formation in athymic mice. Collectively, the data demonstrate that VHL protein exerts its tumor suppressor action, at least partially, via inhibition of p22phox-based Nox4/Nox1 NADPH oxidase-dependent reactive oxygen species generation.

Epithelial tumors comprise the majority of renal carcinomas in which ~75% are histologically of the clear cell type (RCC).2

EXPERIMENTAL PROCEDURES

Materials—Diphenyleneiodonium (DPI), rotenone, antimycin, l-NAME, MG132, wortmannin, actinomycin D, and cycloheximide were all purchased from Sigma. LY29004 and epoxomicin were purchased from Calbiochem.

Cell Culture, Transfection, Adenovirus Infection, and Preparation of Cell Lysates—Human renal proximal tubular cells (HK2). VHL-deficient cells (RCC 786-O), and VHL-expressing ACHN cells (from American Type Culture Collection) were

References:
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2. Supported by a National Research Service Award fellowship and an award from the San Antonio Cancer Institute. To whom correspondence should be addressed: University of Texas Health Science Center, Dept. of Medicine, Division of Nephrology MC 7882, 7703 Floyd Curl Dr., San Antonio, TX 78229-3900. Tel.: 210-567-4708; Fax: 210-456-4712; E-mail: block@uthscsa.edu.
3. The abbreviations used are: RCC, renal cell carcinoma; l-NAME, Nω-nitro-l-arginine methyl ester; siRNA, small interfering RNA; VHL, von Hippel-Lindau; HIF, hypoxia-inducible transcription factor; DPI, diphenyleneiodonium chloride; DCF-DA, 2’7’-dichlorodihydrofluorescein diacetate; Nox, NAD(P)H oxidase; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; RT, reverse transcription; GFP, green fluorescent protein; NOS, nitric-oxide synthase; DN, dominant negative.
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maintained in RPMI 1640 (HK2) cells or in Dulbecco’s modified Eagle’s medium (RCC 786-O and ACHN) (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin sulfate at 37 °C with 5% CO₂. Dominant-negative Akt pCMV6-HA-Akt described below. For Western blot analysis, cells were lysed in a prepared for lucigenin-enhanced chemiluminescence as instructions. 48 h post-transfection, cell homogenates were rotated at 4 °C for 1 h. Insoluble debris was removed by centrifugation at 10,000 × g for 15 min at 4 °C. Protein concentration was determined by the Bradford assay (Bio-Rad).

Small interfering RNAs (siRNAs), designed by Dharmacon, were transfected at 200 nm in a double transfection using X-treme Gene. Briefly, RCC 786-O cells were plated in antibiotic-free medium to obtain ~40% confluency on the day of transfection. 200 nm scrambled control or specific siRNA was added to the cells. 24 h later, the medium was aspirated, and fresh medium minus antibiotics was added to the cells. The transfection was repeated, and 48 h later the cells were harvested for Western blot analysis.

Antibodies and Immunoblotting—Anti-Nox4 antibody was generated as described previously (15). Anti-HIF-2α and GAPDH were from Novus Biologicals, and anti-actin, anti-FLAG (M2), and β-tubulin were from Sigma. Nox1 and p22(phox) were from Santa Cruz Biotechnology, pAkt and p4E-BP1 from Cell Signaling, and anti-HA from Roche Applied Science. Between 25 and 70 μg of total protein was typically analyzed by immunoblotting. After exposure with the indicated primary antibody, the immunoblots were washed and incubated with goat anti-rabbit/mouse-coupled horseradish peroxidase (Bio-Rad) followed by chemiluminescence using ECL reagent (Amersham Biosciences).

Immunoprecipitation-Western—Assays were conducted using whole cell lysates generated with radioimmunoprecipitation assay buffer for immunoprecipitations, the amount of lystate used was normalized to an equal amount of total protein, as determined by Bradford analysis (Bio-Rad) or direct Western blot of the protein of interest, and ranged between 200 and 500 μg depending on expression levels. The lysates were immunoprecipitated using 8.8 μg of FLAG M2 antibody (Sigma) or 2 μg of HA antibody (Roche Applied Science). The immunoprecipitates were bound to protein A- or G-Sepharose (Amersham Biosciences), washed with radioimmunoprecipitation assay buffer, boiled, and analyzed by SDS-PAGE. Immunoblots were performed on the immunoprecipitated material or on 30 μg of total cell lysate per gel lane.

Measurement of Intracellular ROS Production—Intracellular ROS were measured as described previously (16). Briefly, subconfluent cells were washed with Hanks’ balanced salt solution without phenol red and then incubated for 15 min in the dark at 37 °C with the same solution containing the peroxide-sensitive fluorophore 2,7-dichlorofluorescin diacetate (DCF-DA; Molecular Probes) at 5 μmol/liter. DCF-DA fluorescence was detected at excitation and emission wavelengths of 488 and 520 nm, respectively, as measured with a multiwell fluorescence plate reader (Wallac 1420 Victor3, PerkinElmer Life Sciences).

NADPH Oxidase Assay—NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method as described (14). Briefly, cultured cells or tumor tissue was homogenized in lysis buffer (20 mM KH₂PO₄, pH 7.0, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin) by using a Dounce homogenizer (100 strokes on ice). Homogenates were centrifuged at 800 × g at 4 °C for 10 min to remove the unbroken cells and debris, and aliquots were used immediately. To start the assay, 100-μl aliquots of homogenates were added to 900 μl of 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 μM NADPH. Photon emission in terms of relative light units was measured in a luminometer every 30 s for 5 min. There was no measurable activity in the absence of NADPH. Superoxide anion production was expressed as relative chemiluminescence (light) units (RLU)/mg protein. Protein content was measured using the Bio-Rad protein assay reagent. S.E. was calculated for each set of samples.

RT-PCR—Primers were designed as described previously (17): hNox1, top, 5’-GTACCATACTGGTGCAGAGCAGC-3’, and bottom, 5’-CAGACCTGAATCTGGTGA-CAGCA-3’; hNox2, top, 5’-GGAGTTTCAAGATGGCG-TGAAACTA-3’, and bottom, 5’-GCCAGACTCAGT-TGGAGATGCT-3’; hNox3, top, 5’-GGATCGGAGTCCT-CCTTGCGTC-3’, and bottom, 5’-ATGAAACCTTCTGG-GTCAGCTGA-3’; hNox4, top, 5’-CTCGCAGGGAATCTTCACTCCTTGAGTC-3’, and bottom, 5’-AGAGGAAACAGCA-ATCGCCTTAG-3’; hNox5, top, 5’-ATCAAGGGCGCCCCC- TTTTTTTCAC-3’, and bottom, 5’-CTCTTTTCACCTC-CTCGACAGC-3’. Total RNA was isolated from the indicated cell cultures, and the RT-PCR reaction was carried out using the SuperScript One-Step RT-PCR kit (Invitrogen). The PCR conditions were as follows: 50 °C for 15 min; 95 °C for 1.5 min; 28 cycles of 95 °C for 30 s, 60 °C for 20 s, 72 °C for 45 s; 72 °C for 5 min, 4 °C. End products were resolved on a 2.0% agarose gel.

Soft Agar Growth Assays—Anchorage-independent growth was determined by the ability of cells to form colonies in soft agar. RCC 786-O cells were treated for 24 h in buffer alone (Me₃SO) or DPI (10 μM). After 24 h treatment, viable cells of both buffer- and DPI-treated (11,000 cells/plate) were grown in 0.3% agar on a cushion of 0.6% agar containing Me₃SO (buffer) or DPI (10 μM) in 6-well plates. RCC 786-O cell growth was allowed to proceed for ~25 days. Colonies were stained using a cell stain solution (Chemicon).

Tumorigenesis Assays—Nude mouse xenograft assays were performed as described (18) with minor modifications. Briefly,
ROS Generation Is Increased in VHL-deficient RCC 786-O Cells as Compared with HK2 Renal Proximal Tubule Cells—Superoxide anion generation in the VHL-deficient RCC 786-O cells was confirmed using the peroxide-sensitive fluorescence probe DCF-DA (Fig. 1C). These results indicate that VHL deficiency is associated with increased NADPH-dependent superoxide generation. To confirm a role for VHL in the regulation of ROS production, wild-type VHL-FLAG or empty vector were introduced into the VHL-deficient RCC 786-O cells. Anti-FLAG immunoblotting of cell lysates showed significant expression of VHL protein in VHL-transfected cells (Fig. 1D, compare lanes 2 and 3). ROS generation was measured in parallel by NADPH-dependent lucigenin-enhanced chemiluminescence. NADPH-dependent superoxide generation was suppressed in RCC 786-O cells transiently transfected with VHL-FLAG as compared with vector-transfected cells (Fig. 1E).

RCC 786-O cells constitutively express HIF-2α but do not express HIF-1α (19). We analyzed HIF-2α protein expression in lysates from 786-O cells transfected with VHL-FLAG or control vector-transfected cells. Fig. 1F shows a marked decrease in HIF-2α protein expression in VHL-FLAG-transfected RCC 786-O cells compared with the vector-transfected cells. Taken together, these data demonstrate that VHL inhibits NADPH-dependent ROS generation and down-regulates HIF-2α protein expression.

**RESULTS**

ROS Generation Is Increased in VHL-deficient RCC 786-O Cells as Compared with HK2 Renal Proximal Tubule Cells—Superoxide anion (O$_2^-$) production in renal cancer cell lines was measured as lucigenin-enhanced chemiluminescence using NADPH as the substrate. Fig. 1, A and B, shows that the rate of superoxide formation in the VHL negative renal carcinoma cell line RCC 786-O is higher than in the HK2 cells or VHL positive renal carcinoma ACHN cells. The increased ROS generation in RCC 786-O cells was confirmed using the peroxide-sensitive fluorescence probe DCF-DA (Fig. 1C). These results indicate that VHL deficiency is associated with increased NADPH-dependent superoxide generation. To confirm a role for VHL in the regulation of ROS production, wild-type VHL-FLAG or empty vector were introduced into the VHL-deficient RCC 786-O cells. Anti-FLAG immunoblotting of cell lysates showed significant expression of VHL protein in VHL-transfected cells (Fig. 1D, compare lanes 2 and 3). ROS generation was measured in parallel by NADPH-dependent lucigenin-enhanced chemiluminescence. NADPH-dependent superoxide generation was suppressed in RCC 786-O cells transiently transfected with VHL-FLAG as compared with vector-transfected cells (Fig. 1E).

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FIGURE 2. HIF-2α protein expression is down-regulated by a flavoprotein oxidase inhibitor, DPI, in VHL-deficient cells. A, RCC 786-O cells were treated with DPI, a flavoprotein inhibitor, for the indicated times (t = 0–8 h). Equivalent amounts of cell lysates were analyzed by Western blot for HIF-2α expression. HK2 cells were used as a negative control. B, mitochondrial complex I inhibitor rotenone (1 μM), mitochondrial complex III inhibitor antimycin (1 μM), flavoprotein-dependent oxidase inhibitor DPI (10 μM), or the NOS inhibitor L-NAME (1 mM), was added to RCC 786-O for 24 h. Western blot analysis for HIF-2α protein expression was performed on equivalent amounts of cell lysates. Actin was used as a loading control.

inhibitor. Fig. 2B shows that inhibitors of mitochondria, rotenone and antimycin, and the NOS inhibitor L-NAME did not affect HIF-2α protein expression after a 24-h incubation in RCC 786-O cells. On the other hand, DPI did cause down-regulation of HIF-2α protein expression. These data indicate that the activity of NAD(P)H oxidase(s) is required for HIF-2α protein expression in VHL-deficient cells.

VHL Deficiency Is Associated with Up-regulation of NAD(P)H Oxidases—To determine which NAD(P)H oxidase isoforms are expressed in renal tubular epithelial cells and RCC cells, mRNAs were analyzed by RT-PCR, and the end products were resolved on an agarose gel. Both Nox1 and Nox4 mRNAs are expressed in HK2 cells as well as in RCC 786-O cells, whereas Nox2, Nox3, and Nox5 mRNAs were not detected (Fig. 3A). Expression of Nox1 mRNA is comparable in RCC 786-O and HK2 cells. However, Nox4 mRNA levels were higher in RCC 786-O cells compared with HK2 cells (Fig. 3A, lower panel versus upper panel). The steady state protein levels of these oxidases were also determined by Western analysis. Consistent with the RT-PCR results, Nox4 protein expression is higher in VHL-deficient RCC 786-O cells than in HK2 cells (Fig. 3B, Nox4). We did not detect any difference in protein expression of Nox1 in HK2 versus RCC 786-O cells by Western analysis (data not shown). We next analyzed the expression of p22phox, the membrane-bound subunit that binds to and potentiates the activities of both Nox1 and Nox4. Strikingly, p22phox levels were higher in the RCC 786-O VHL-deficient cell line compared with HK2 cells (Fig. 3B). It is likely that the increased ROS generation in VHL-deficient cells is due to the increased expression of p22phox subunit required for activation of Nox1 and Nox4. To examine the involvement of VHL in regulation of p22phox, VHL was transfected into VHL-deficient cells. Introduction of VHL resulted in a decrease in p22phox levels compared with vector-transfected RCC 786-O cells (Fig. 3C).

Proteasome Inhibitors Increase NADPH Oxidase Activity and Increase the Expression of p22phox—VHL is part of an E3 ubiquitin ligase that regulates protein degradation via the 26 S proteasome. We next determined whether p22phox protein expression was regulated through the 26 S proteasome. HK2 cells were treated with increasing amounts of proteasome inhibitor epoxomicin or with buffer alone (–) for 24 h. Cell lysates were analyzed by Western analysis for p22phox protein levels. GAPDH was used as a loading control. C, HK2 cells were treated with increasing amounts of proteasome inhibitor MG132 for 24 h or with buffer alone (–). Equivalent amounts of total protein from cell lysates were evaluated by Western analysis for p22phox protein levels. GAPDH was used as a loading control.

FIGURE 3. Nox1 and Nox4 are expressed in HK2 and RCC 786-O cells. A, RT-PCR analysis of NAD(P)H oxidase isoforms was carried out in RNA isolated from HK2 or RCC 786-O cells using specific human Nox PCR primers (Nox1, Nox2, Nox3, Nox4, Nox5) or GAPDH as control. B, HK2 and RCC 786-O cell lysates were evaluated by Western analysis for Nox4 or p22phox protein levels. Tubulin was used as a loading control. C, lysates of RCC 786-O cells transfected with vector alone or VHL-FLAG were analyzed by Western blot analysis for p22phox expression. GAPDH was used as a loading control. Detection of VHL-FLAG transfection was evaluated by Western blot analysis with FLAG antibody.

FIGURE 4. p22phox is stabilized in the presence of proteasome inhibitors, which correlates increased NADPH-dependent superoxide generation. A, homogenates were prepared from buffer (–) or MG132 5 μM (+)-treated HK2 cells and examined for NADPH-dependent superoxide anion generation using the lucigenin assay. B, HK2 cells were treated with increasing amounts of proteasome inhibitor epoxomicin or with buffer alone (–) for 24 h. Cell lysates were analyzed by Western analysis for p22phox protein levels. GAPDH was used as a loading control. C, HK2 cells were treated with increasing amounts of proteasome inhibitor MG132 for 24 h or with buffer alone (–). Equivalent amounts of total protein from cell lysates were evaluated by Western analysis for p22phox protein levels. GAPDH was used as a loading control.

p22phox Interacts with VHL in Vivo—To examine whether p22phox and VHL can interact in vivo, 293 cells were transfected...
**FIGURE 5.** p22\textsuperscript{phox} and VHL interact in vivo. A, human 293 cells were co-transfected with vector control or mammalian expression plasmids encoding VHL-FLAG tag and C-terminal HA-tagged p22\textsuperscript{phox} (p22\textsuperscript{phox}-HA). Equivalent amounts of total protein were immunoprecipitated (IP) from transfected cell lysates (vector control or p22\textsuperscript{phox}-HA) with anti HA followed by Western blot analysis (IB) with FLAG antibody. In the input lane, 15% of the total cell lysate were loaded. The solid arrow indicates the protein band corresponding to VHL-FLAG. B, the same blot as used for A was probed with an antibody against p22\textsuperscript{phox} (anti-p22\textsuperscript{phox}). Note that the p22\textsuperscript{phox} antiserum immunoreacts with a band of -22 kDa (p22\textsuperscript{phox}-HA) as well as higher molecular mass species. C, the same blot as used for A was probed with an antibody against Ubiquitin (Ub). Note that the ubiquitin antiserum correlates with p22\textsuperscript{phox} in vivo. D, human 293 cells were co-transfected as described in A, and equivalent amounts of total protein were immunoprecipitated from transfected cell lysates (IP; vector control or p22\textsuperscript{phox}-HA) with anti-FLAG followed by Western blot analysis with anti p22\textsuperscript{phox} antibody. Input shows the total cell lysate analyzed by Western bloting alone.

**FIGURE 6.** p22\textsuperscript{phox} is a pivotal component of the Nox oxidase that regulates HIF-2\(\alpha\) protein accumulation. A, lysates of RCC 786-O cells transfected with scrambled control (Scr), small interfering RNA to Nox1 (siNox1), small interfering RNA to Nox4 (siNox4), or small interfering RNA to Nox1 and Nox4 (siNox1 + siNox4) were analyzed by Western blot with Nox1 or Nox4 antibodies. B, the same cell lysates as in A were analyzed by Western analysis for HIF-2\(\alpha\) protein expression or a loading control, actin. C, RCC 786-O cells transfected with scrambled control or small interfering RNA or p22\textsuperscript{phox} (sip22\textsuperscript{phox}) were analyzed by Western blot with p22\textsuperscript{phox} antibody or actin control. D, RCC 786-O cell lysates (Scr or sip22\textsuperscript{phox}), as in C, were analyzed by Western analysis for HIF-2\(\alpha\) protein expression or a loading control, actin.

with HA-tagged p22\textsuperscript{phox} (p22\textsuperscript{phox}-HA) and FLAG-tagged VHL (VHL-FLAG) followed by immunoprecipitation with HA antibodies and immunoblot analysis with FLAG antibodies. The data show that VHL-FLAG co-precipitate with p22\textsuperscript{phox}-HA (Fig. 5A). Importantly, when the same blot was probed for p22\textsuperscript{phox}, an immunoreactive band was detected at -23 kDa, representing p22\textsuperscript{phox}-HA. In addition, several higher molecular mass species of p22\textsuperscript{phox} were observed (Fig. 5B). This suggested a post-translational modification of p22\textsuperscript{phox}. To examine whether the higher molecular mass species could be an ubiquitinated form of p22\textsuperscript{phox}, we probed the blot for ubiquitin. Fig. 5C shows that anti-ubiquitin immunoreacts with the higher molecular mass species, which matches the pattern observed with anti-p22\textsuperscript{phox}. This suggests that p22\textsuperscript{phox} is modified by ubiquitin in vivo. In the reciprocal experiment, antibodies against FLAG were used for immunoprecipitation of VHL followed by immunoblot analysis for p22\textsuperscript{phox}. Western analysis of the immunoprecipitate from the transfected cell lysates indicates a p22\textsuperscript{phox}-immunoreactive band representing HA-tagged p22\textsuperscript{phox} (Fig. 5D). These data demonstrate that p22\textsuperscript{phox} and VHL interact in vivo.

**p22\textsuperscript{phox} and Nox Oxidases Regulate HIF-2\(\alpha\) Protein Accumulation**—To examine the contribution of Nox oxidase components to HIF-2\(\alpha\) expression, we employed knockdown strategy. siRNAs to hNox1, hNox4, and hp22\textsuperscript{phox} were transiently transfected into RCC 786-O cells. Successful knockdown of Nox1 and Nox4 (Fig. 6A) and p22\textsuperscript{phox} (Fig. 6C) was evaluated by Western analysis. HIF-2\(\alpha\) protein levels were decreased in siNox1 as well as in siNox4 knockdown cell lysates compared with scrambled control cell lysates. In cells transfected with both siNox1 and siNox4, we also observed a decrease in HIF-2\(\alpha\) protein levels (Fig. 6B). Importantly, knockdown of p22\textsuperscript{phox} alone significantly decreased HIF-2\(\alpha\) protein levels (Fig. 6D). These data suggest that Nox oxidases, including the subunit p22\textsuperscript{phox}, are important mediators that contribute to HIF-2\(\alpha\) protein expression in VHL-deficient cells.

**Nox Oxidases Increase HIF-2\(\alpha\) Protein Accumulation through a Translational Mechanism**—We determined the potential mechanisms of Nox-dependent HIF-2\(\alpha\) protein accumulation in VHL-deficient cells. Cycloheximide, an inhibitor of protein translation, but not actinomycin D, an inhibitor of transcription, decreased HIF-2\(\alpha\) protein (Fig. 7A). To further investigate a translational pathway in HIF-2\(\alpha\) protein regulation, we evaluated a known pathway linked to translational regulation often activated in cancer, the PI3K/Akt pathway. PI3K/Akt-dependent signaling pathway culminates in phosphorylation of components essential for the cap-dependent translational machinery such as 4E-BP1. Incubation of RCC 786-O cells with the PI3K inhibitor LY29004 blocks HIF-2\(\alpha\) protein accumulation (Fig. 7A, left panel), indicating that PI3K activity is required for HIF-2\(\alpha\) protein expression. Inhibition of PI3K was also associated with decreased phosphorylation of Akt and 4E-BP1 (Fig. 7B). The role of Akt in HIF-2\(\alpha\) expression was examined by...
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FIGURE 7. p22phox-based Nox oxidase regulates HIF-2α protein accumulation through a translational mechanism. A, left panel. RCC 786-O cells were pretreated for 8 h with: buffer alone (−); an inhibitor of transcription, actinomycin D; an inhibitor of PI3K (LY294002); an inhibitor of translation, cycloheximide (CHX); or DPI. Equivalent amounts of cell lysates were analyzed by Western blot analysis for HIF-2α expression. Right panel. RCC 786-O cells were infected with adenovirus to GFP alone or HA-DN-Akt. Equivalent amounts of cell lysates were analyzed by Western blot analysis for HIF-2α expression. Expression of DN-Akt was evaluated by Western analysis for HA. B, left panel. lysates from A, upper left panel, were analyzed for phosphorylation of the known downstream targets of PI3K, pAkt and p4E-BP1. Right panel, lysates from A, upper right panel, were analyzed for the phosphorylation of a known downstream target of Akt, p4E-BP1. Expression of DN-Akt was evaluated by Western analysis for HA. C, equivalent amounts of total protein from RCC 786-O cells transfected with scrambled control, small interfering RNA to Nox1 (siNox1), small interfering RNA to Nox4 (siNox4), or small interfering RNA to p22phox (sip22phox) were analyzed by Western blot (left, immunoblot) for pAkt or D, p4E-BP1 antibody or GAPDH loading control.

FIGURE 8. DPI blocks RCC 786-O growth in vitro. A, colony formation in agar gel was used as an in vitro assay for anchorage-independent growth, a property of transformed cells. Colony formation by RCC 786-O cells in soft agar medium was evaluated in the presence (+) or in the absence (−) of DPI as described under Experimental Procedures. Following incubation, only colonies stained with cell stain solution (Chemicon) were counted and expressed as percent of control. B, enumeration of colonies. C, DPI inhibits RCC 786-O cell growth. Cellular proliferation was measured by plating an equal density of RCC 786-O cells at subconfluence in serum-containing medium. Cells were treated with vehicle alone (DMSO, dimethyl sulfoxide) or DPI. At various time points (in days), the cells were counted (Cell number). The results are expressed as the means ± S.E. **, p < 0.01 compared with control, dimethyl sulfoxide.

infecting RCC 786-O cells with an adenovirus vector expressing a dominant-negative mutant of Akt (Ad DN-Akt). GFP adenovirus was infected as a control. Expression of Ad DN-Akt inhibited 4E-BP1 phosphorylation and blocked HIF-2α protein accumulation similar to the effect of inhibition of PI3K and protein synthesis (Fig. 7, A and B, CHX). Cycloheximide, LY29004, and wortmannin had no effect on NADPH oxidase activity, suggesting that ROS generation is upstream of the translational pathway (data not shown).

To elucidate the role of Akt as a potential mechanism by which p22phox-based Nox oxidases regulate HIF-2α protein accumulation, siRNA-mediated knockdown of p22phox, Nox1, and Nox4 was performed in RCC 786-O cells, and the phosphorylation of Akt was evaluated by Western blot analysis. Knockdown of p22phox was as efficient as either Nox alone or in combination (siNox1 + siNox4) in decreasing the phosphorylation of Akt (Fig. 7C). Importantly, the phosphorylation of the downstream target of PI3K/Akt pathway, 4E-BP1, was also efficiently inhibited by the down-regulation of p22phox (Fig. 7D). These data suggest that p22phox is a pivotal component of the Nox oxidase that regulate the PI3K/Akt/4E-BP1 signaling cascade leading to enhanced translation of HIF-2α protein expression in VHL-deficient cells. However, other mechanisms of HIF-2α regulation cannot be excluded.

**DPI Blocks RCC 786-O Colony Formation in Soft Agar—Because HIF-2α expression is maintained in cells with increased flavoprotein oxidase-dependent ROS generation, we determined the effect of inhibition of ROS generation on tumor cell growth in vitro. Colony formation in agar gel was used as an in vitro assay for anchorage-independent growth. Colony formation by RCC 786-O cells in soft agar medium was assessed in the presence or absence of DPI. Fig. 8A shows RCC 786-O cells forming colonies in soft agar (left panel), which was inhibited in the presence of DPI (right panel). The number of colonies were counted and normalized as percent of control (Fig. 8B). Moreover, we found that cellular proliferation was markedly decreased in DPI-treated cells compared with cells treated with vehicle alone (Fig. 8C). Taken together, these data indicate that inhibition of ROS generation results in down-regulation of HIF-2α protein expression in RCC cells in the absence of VHL and is associated with a decrease in the growth of tumor cells.

**DPI Blocks RCC 786-O Tumor Growth in Vivo—**We next examined the ability of DPI to block tumor formation in an in vivo model. RCC 786-O cells were administered subcuta-
This study demonstrates that VHL deficiency increases the expression and activity of NAD(P)H oxidases. ROS generation through p22phox and activation of Nox1 and Nox4 help maintain HIF-2α expression and thereby contribute to renal carcinogenesis. Nox oxidases represent targets for treatment of renal cell carcinoma. Different approaches were used to detect ROS generation in normal renal epithelial cells (HK2), VHL(+/+) renal carcinoma cells (ACHN), and VHL(−/−) RCC 786-O cell lines. NAD(P)H oxidase activity and ROS production in the VHL-deficient renal cancer cell line RCC 786-O is much higher than in normal renal epithelial HK2 cells or VHL(+/+) ACHN cells. Importantly, we have shown that reintroducing VHL into the VHL-deficient cells reduces ROS production and decreases p22phox protein levels. To our knowledge, this is the first evidence that VHL regulates NADPH-dependent ROS generation.

VHL is mutated in 80% of sporadic renal carcinomas, and as a result, HIF transcription factors are constitutively expressed in an oxygen-independent manner. HIF expression in VHL-deficient cells leads to the up-regulation of genes involved in renal carcinogenesis. In nude mice, down-regulation of HIF-2α is sufficient to impair the growth of tumors (18, 20). Because HIF-2α is a critical downstream target of VHL with respect to suppression of renal carcinogenesis, it is important to identify the pathways involved in the regulation of HIF-2α protein expression. VHL-deficient cells provide a unique opportunity to discover and evaluate these upstream mediators. The increased ROS generation in VHL-deficient cells, maintains the expression of HIF-2α protein levels, because inhibition of ROS generation by the flavoprotein inhibitor, DPI, results in a decrease of HIF-2α protein levels in RCC 786-O cells. Similar results were obtained in another VHL-deficient cell line that expresses HIF-2α (A498; data not shown). Taken together, our data suggest a novel association between NADPH-dependent ROS generation and HIF-2α protein expression. We have extensively characterized the source of enzymatic activity involved in the expression of HIF-2α. Flavoprotein-containing ROS-generating enzymes include NOS, complexes of the mitochondrial respiratory chain, and the NAD(P)H oxidases of the Nox family. Previous studies have implicated the generation of ROS by mitochondria or NAD(P)H oxidases as mediators of HIF-1α protein stabilization and activation (8, 21). Our data strongly support a role for the Nox proteins in maintaining HIF-2α expression based on several observations. Inhibitors of complex I and III of the mitochondrial respiratory chain or inhibition of NOS had no effect on HIF-2α protein expression. DPI is often used to delineate the role of the Nox family of flavoproteins. Indeed, DPI elicited a marked decrease of HIF-2α expression in RCC 786-O cells. We have identified Nox1 and Nox4 as the major Nox catalytic sources of superoxide anion production. Nox4 and p22phox (a known interacting protein with Nox1 and Nox4) are highly expressed in VHL-deficient cells compared with the HK2 cells. The levels of Nox4 and p22phox are also elevated in another VHL-deficient cell line (A498; data not shown). It has been shown previously that a subset of brain tumor cell lines derived from human glioblastomas aberrantly express Nox4 (17). It is interesting to note that VHL is often mutated in glioblastomas (22). p22phox is an integral membrane protein that directly binds to and is required for the activation of both Nox1 and Nox4 (23, 29). In particular, Nox1 activity is dependent on a stable complex formation with p22phox and other regulatory subunits (23–29). Nox4, on the other hand, produces a significant amount of superoxide in a constitutive manner; this production is increased upon its association with p22phox. Knockdown of endogenous p22phox by RNAi results in reduced Nox4 activity (23, 29). Our data show a decrease in p22phox protein levels and NADPH-dependent ROS generation upon reintroduction of VHL into VHL-deficient cells. The regulation of p22phox is currently unknown. We show a role for the 26 S proteasome in p22phox regulation, because proteasomal inhibitors increase p22phox protein levels and NADPH-dependent superoxide generation. We also show for the first time that p22phox binds VHL in vivo. Moreover, we provide evidence that p22phox is a target for ubiquitination, providing a potential mechanism of p22phox regulation in VHL-deficient cells. We hypothesize that VHL binding to p22phox inhibits the activation of the Nox oxidase complex and leads to destabilization of p22phox protein.

The data also demonstrate an important role for specific Nox oxidases in downstream HIF-2α protein accumulation. In RCC 786-O cells transiently transfected with siRNA to Nox4, we observed a small but significant decrease in HIF-2α protein levels. Knockdown of Nox1 produces a clear inhibition of HIF-2α protein levels. Interestingly, it has been shown that up-
regulation of Nox1 activity by hypoxia enhances HIF-1α-dependent gene expression via increased ROS (21). However, our data show that even down-regulation of p22phox alone can markedly decrease the protein expression of HIF-2α. The mechanism by which ROS generation maintains HIF-2α protein expression in VHL-deficient cells requires p22phox-based Nox1 and Nox4 regulation of the PI3K/Akt pathway, culminating in the phosphorylation of 4E-BP1 for ongoing protein translation. Similar regulation of HIF-1α has been demonstrated. For example, attenuation of PI3K/Akt decreased HIF-1α expression and activation of NAD(P)H oxidases (31). We determined the functional consequence of inhibition of ROS generation in VHL-deficient renal cell carcinoma (RCC4) without altering the steady state of HIF-1α mRNA (30). Additionally, it has been shown that maintenance of the nonhypoxic ROS-dependent increase of HIF-1α protein expression by angiotensin II relies on ongoing protein translation in a p22phox/PI3K-dependent manner (31). We determined the functional consequences of inhibition of ROS generation. Colony formation by RCC 786-O in soft agar was markedly inhibited by DPI. To determine the relevance of our in vitro observations to tumor growth in vivo, athymic mice harboring RCC 786-O tumors were treated with DPI or vehicle. DPI-treated animals had a significant decrease in tumor size. Our data indicate that increased expression and activation of NAD(P)H oxidases result in enhanced ROS generation in VHL-deficient cells. Specific catalytic NAD(P)H oxidases Nox1 and Nox4 and their regulatory subunit p22phox are up-regulated and activated by VHL deficiency, leading to increased HIF-2α expression and tumorigenesis in vitro and in vivo. Thus, pharmacological inhibition of flavoprotein oxidases, and in particular the blockade of p22phox binding to these oxidases, represents an alternative approach to the down-regulation of HIF-2α expression and its target pathways involved in renal carcinogenesis.

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