The von Hippel-Lindau Tumor Suppressor Targets to Mitochondria

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Abstract

Subcellular localization of von Hippel-Lindau (VHL) tumor suppressor may clarify its role in tumorigenesis. In rat kidney, we observed a granular cytoplasmic immunostaining of VHL, as seen in human tissues. The green fluorescent protein (GFP)-tagged VHL also appeared as cytoplasmic granules in vitro and was colocalized with a mitochondrion-selective dye. Immunogold electron microscopy localized VHL specifically to the mitochondrion. Mitochondria retaining GFP-VHL fusion protein, mimicking an insertional VHL mutant, displayed abnormal phenotypes. Among these, small mitochondria have been observed in clear cell renal carcinomas known to have frequent VHL alterations. Thus, VHL may contribute to tumorigenesis through mitochondria-based action.

Introduction

Diverse effects of VHL include down-regulation of angiogenic factors, such as VEGF1 and TGF-β1, and proteolysis of hypoxia-inducible factor 1 under normoxia condition (1-3). It also has been shown that VHL acts as a ubiquitin ligase (4), suggesting that VHL participates in proteolysis via ubiquitination and may in this way regulate the levels of VEGF, TGF-β1, and hypoxia-inducible factor 1. The VHL gene is frequently mutated in human renal cell carcinomas, especially with clear cell phenotype (5). These various phenomena would be integrated by the presence of VHL in the mitochondria, because VEGF and TGF-β1 have been localized predominately in the mitochondrion (6, 7), and ubiquitination-associated enzymes have also been observed in this organelle (8, 9). Furthermore, the mitochondria play a key role in glucose and lipid metabolism (10), and alteration in these processes as a result of abnormal VHL could lead to accumulation of glycogen and lipid in the cytosol, as seen in clear cell renal carcinomas (11). Here, we demonstrate that VHL protein is in fact localized in the mitochondria, and altered VHL leads to pathology of this organelle.

Materials and Methods

Immunohistochemistry. After blocking of endogenous peroxidase activity with normal horse serum, 5 μm of dewaxed, formalin-fixed Fischer 344 rat kidney tissue sections were incubated at room temperature for 30 min with a 1:500 dilution of the monoclonal antibody, VHL06102 (12). The human VHL epitope recognized by the antibody shares a 97% identity and a 99% similarity to rat VHL. For signal detection, the avidin-biotin complex procedure (Vector Elite ABC kit) was used according to the manufacturer’s direction (Vector Laboratory Co., Burlingame, CA).

Plasmid Constructions and Transient Transfection. The entire coding region of wild-type rat VHL (GenBank U14746), which is flanked by HindIII and BamHI recognition sequences and by the Kozak GCCACC motif preceding the start codon, was generated by PCR. The PCR products were cloned using a TA Cloning Kit (Invitrogen, San Diego, CA), and the sequence of the insert was confirmed by sequencing both DNA strands. The insert was then subcloned into a pEGFP-N1 plasmid (Clontech, Palo Alto, CA) to produce wtVHL, as well as a result of the VHL UGA stop codon preceding the GFP gene, or a pEGFP-C1 (Clontech) to yield GFP-wtVHL fusion protein with wtVHL at the COOH terminal. The native pEGFP-C1 expresses GFP only. The NRK-52E cell line was cultured with 1 μl of DMEM supplemented with 5% fetal bovine serum in a four-well Lab-Tek II chamber slide (Nalge Nunc International, Naperville, IL). Lipofectamine Plus reagent (Life Technologies, Inc., Gaithersburg, MD) was used to transfect the 60-80% confluent NRK-52E cells with 1.2 μg/ml wtVHL, GFP-wtVHL, or GFP plasmid according to the manufacturer’s instruction.

Staining of Organelle-selective Fluorochromes. BODIPY TR ceramide, LysoTracker Red DND-99, and MitoTracker Orange CMXRos fluorochromes (Molecular Probes, Eugene, OR) were applied to the transiently transfected cells following the manufacturer’s directions. In brief, BODIPY of 0.5 μM in 5 mg/ml bovine serum albumin was incubated in the dark with methanol-fixed cells for 1 h at room temperature. Living cells were stained with LysoTracker (1:20,000 dilution) and 200 nM MitoTracker in fresh culture media at 37°C for 2 h and 30 min, respectively, and were then fixed in 10% buffered formalin. Nuclei were counterstained with 2 μg/ml 4′,6-diamidino-2-phenylindole fluorescence (Molecular Probes), and slides were coverslipped with a Vectashield mounting agent (Vector Laboratory). The cells were evaluated using a water immersion ×40 objective lens of a Zeiss 310 confocal microscope equipped with 473-, 488-, and 543-nm laser beams.

Immunogold Electron Microscopy. Cells were cultured in a 60 × 15-mm Permanox culture dish (Miles Laboratory, Naperville, IL) and transiently transfected with wtVHL and GFP-wtVHL plasmids, as described above. The parental NRK-52E and transfected cells were fixed in buffered 4% paraformaldehyde and 0.1% glutaraldehyde for 2 h at 4°C. After 50 mM ammonium chloride treatment and ethanol dehydration, the cells were embedded in a LR gold resin (Polyscience, Warrington, PA) at −20°C for 24 h, as described previously (13). Thin sections of 50-60 nm were cut and were mounted on 300-mesh nickel grids. Normal goat serum was applied to block nonspecific binding, and the sections were incubated at room temperature for 2 h with the VHL06102 monoclonal antibody (1:50 dilution). Colloidal gold-conjugated secondary antibody of 1:100 dilution was used, and the sections were later counterstained with an Ultrastain reagent (Leica, Deerfield, IL). About 100 cells were examined using a transmission electron microscope (Hitachi, Tokyo, Japan) operated at 75 KV.

Results and Discussion

Previously, we reported for the first time that VHL is mutated in rat clear cell kidney tumors (14), indicating that the rat is an alternative biological system for the study of VHL-associated pathogenesis. VHL protein is detected exclusively in the cytoplasm of many human tissues (12, 15). Using the same immunohistochemical technique (12), we observed a punctate or granular staining of VHL in the cytoplasm of adult rat kidney tissues (Fig. 1), similar to that seen in human tissues. This unique staining pattern suggests that VHL protein is present in cytoplasmic organelles.
Under optimal immunohistochemical conditions, VHL was vari-
ably detected in rat kidney sections, with strong expression in 
5% of cells, mainly of proximal tubules (Fig. 1). To increase the number of 
cells that express detectable VHL, normal rat kidney epithelial-like 
cells (NRK-52E) were transfected with plasmids coding for GFP for 
wild-type rat VHL (wtVHL) and for GFP-wtVHL fusion protein. 
The expression of GFP-wtVHL as a green fluorescence was readily 
detected in 20% of the transiently transfected cells. The intense 
green fluorescent signal appeared to be punctate or granular in the 
mitochondria (Fig. 2A), consistent with the pattern observed in rat kidney 
tissues. The green signal was not seen in the nuclei and became 
undetectable in the cytoplasm after >3 days of cell culture. Cell death 
was common for GFP-wtVHL-positive cells. The kidney cells trans-
sected with GFP control plasmid showed a homogeneous distribution 
of green fluorescent signal in both nuclei and cytoplasm (Fig. 2B), and 
the signal was persistent even after two to three cell passages, indicating that GFP is not cytotoxic to the cells. The nonspecific general 
localization of the GFP control demonstrates that the granular pattern 
in GFP-wtVHL-transfected cells is specifically contributed by the 
wtVHL part of the fusion protein.

We further characterized the cytoplasmic granules using BODIPY 
(for the Golgi apparatus and the endoplasmic reticulum), LysoTracker 
(for the lysosome), and MitoTracker (for the mitochondria) fluores-
cent dyes. Strikingly, only the MitoTracker dye was colocalized with 
the green fluorescent granules in GFP-wtVHL-transfected cells (Fig. 
2, C–E). The BODIPY and LysoTracker red fluorescent dyes did not 
colocalize with the green signal (data not shown).

Immunogold electron microscopy was next used to confirm that 
VHL proteins were localized in the mitochondria. Immunogold par-
ticles indicating wtVHL were detected exclusively in the mitochon-
dria (Fig. 3, A and B). The GFP-wtVHL fusion protein, representing 
a frameshift VHL mutant as a result of GFP insertion, showed 
concentrated localization in organelles that appeared to be giant 
mitochondria (Fig. 3C). Fusion of these giant mitochondria were also 
detected (Fig. 3D). In some cells, the sizes of mitochondria were much 
smaller in comparison with neighboring cells. The small mitochondria 
were often observed near the immunogold-positive fragmented 
organelles retaining the features of mitochondrial cristae (Fig. 3E). 
These abnormal mitochondria are unlikely to have been caused by the 
GFP protein, because cytotoxicity was not seen in cells transfected 
with GFP control plasmid, as indicated above. The parental NRK-52E 
cells did not have any detectable immunogold signal (Fig. 3F), 
indicating that the immunogold signal is specific to wtVHL and GFP-
wtVHL. The presence of endogenous VHL in the mitochondria has
been confirmed by fractionation of NRK-52E cells and immunoblotting assay. Together with the reports of nuclear/cytoplasmic trafficking (16–18), it appears that VHL can be recruited by different cellular compartments for specific actions.

The finding of VHL in the mitochondria is provocative and has many implications for phenotypic characteristics of clear cell renal carcinomas, such as possession of small mitochondria, accumulation of glycogen and/or lipid, and angiogenesis (11, 19). There are also implications for oncocytic kidney tumors, in which abnormal mitochondria are readily observed as a subcellular pathology, although mutations in the VHL gene have not yet been found in these tumors (5, 11, 19). Localization of VHL to the mitochondria indicates a fundamental importance of VHL in the organelle. Because the mitochondrion plays an essential role in glucose/lipid metabolism and apoptosis (20) and contains angiogenic factors and enzymes required for proteolysis by ubiquitination, alterations of VHL potentially would initiate many pathological events through mitochondrion-based actions.

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References


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