EXPERIMENTAL CELL RESEARCH 314 (2008) 1229–1236

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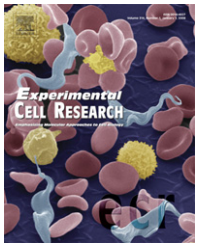

Research Article

Mobility of the von Hippel–Lindau tumour suppressor protein is regulated by kinesin-2

Dorus A. Mans[1], *Martijn P. Lolkema*[1], *Moniek van Beest*, *Laura G. Daenen*, *Emile E. Voest*[1], *Rachel H. Giles*[*,1]

Laboratory of Experimental Oncology, Department of Medical Oncology, University Medical Center Utrecht, Utrecht, The Netherlands

ARTICLE INFORMATION

Article Chronology:
Received 29 June 2007
Revised version received
23 November 2007
Accepted 20 December 2007
Available online 5 January 2008

Keywords:
pVHL
Kinesin-2
FRAP
Mobility fraction
Hemangioblastoma

ABSTRACT

The von Hippel–Lindau tumour suppressor protein (pVHL) participates in many cellular processes including oxygen sensing, microtubule stability and primary cilia regulation. Recently, we identified ATP-dependent motor complex kinesin-2 to endogenously bind the full-length variant of VHL (pVHL30) in primary kidney cells, and mediate its association to microtubules. Here we show that pVHL also endogenously binds the neuronal kinesin-2 complex, which slightly differs from renal kinesin-2. To investigate the role of kinesin-2 in pVHL mobility, we performed fluorescence recovery after photobleaching (FRAP) experiments in neuroblastoma cells. We observe that pVHL30 is a highly mobile cytoplasmic protein, which becomes an immobile centrosomal protein after ATP-depletion in living cells. This response to ATP-depletion is independent of GSK3β-dependent phosphorylation of pVHL30. Furthermore, *VHL* variant alleles with reduced binding to kinesin-2 fail to respond to ATP-depletion. Accordingly, interfering with pVHL30-KIF3A interaction by either overexpressing a dominant negative construct or by reducing endogenous cellular levels of KIF3A by RNAi abolishes pVHL's response to ATP-depletion. From these data we suggest that mobility of a subcellular pool of pVHL is regulated by the ATP-dependent kinesin-2 motor. Kinesin-2 driven mobility of cytoplasmic pVHL might enable pVHL to function as a tumour suppressor.

Introduction

Sporadic renal cell carcinoma (RCC) and central nervous system (CNS) hemangioblastoma are often caused by inactivating mutations/deletions of the *von Hippel–Lindau* (*VHL*) gene encoding the VHL tumour suppressor protein [1]. In the central nervous system, pVHL is expressed in neurons and drives neural differentiation of central nervous system progenitor cells [2]. Furthermore, neuroblastoma cells transduced with exogenous VHL transform into functional neuron-like cells [3].

pVHL localises predominantly to the cytoplasm [4–6], with a small pool shuttling between the nucleus and cytoplasm [7–11]. One report demonstrates that pVHL is also present in mitochondria [12]. Recently pVHL has been shown to decorate microtubules, and that an intact microtubule network is critical for the proper localisation of cytoplasmic pVHL [13]. The interaction of pVHL with microtubules results in increased microtubule stability [5,13] and subsequent primary cilia regulation [14]. Endogenous VHL in the cilium of primary kidney cells has also been described [15].

* *Corresponding author*. Laboratory of Experimental Oncology, Department of Medical Oncology, University Medical Center Utrecht, Heidelberglaan 100, rm F02.126, 3584 CX Utrecht, The Netherlands. Fax: +31 887568479.
E-mail address: r.giles@umcutrecht.nl (R.H. Giles).
[1] These authors contributed equally.

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doi:10.1016/j.yexcr.2007.12.020

Recently we found that full-length pVHL (pVHL30) endogenously interacts with – but is not involved in the degradation of – the anterograde ATP-dependent kinesin-2 microtubule motor subunits KIF3A, KIF3B and KAP3 in primary kidney cells [16]. In the present study we confirm pVHL association with neuronal kinesin-2, which differs from renal kinesin-2 by a single subunit. Because binding of pVHL to kinesin-2 suggests ATP-driven transport in a cell, we applied FRAP-analysis to examine VHL movement. Similar to Mekhail et al. [17] we observe that GFP-labelled pVHL30 is a highly mobile protein under standard conditions. However, we describe altered kinetics of cytoplasmic VHL movement in conditions where kinesin-2 transport is affected. Our data suggest that a significant portion of VHL is transported by kinesin-2 in neural cells.

Materials and methods

Construction of plasmids

GFP-pVHL30, GFP-pVHL19, GFP-pVHL-Y112H, GFP-pVHL-Δ95-123 have been described before [18]. GFP-pVHL-S111N and GFP-pVHL-R167Q were cloned via BglII/EcoR1 into pEGFPc1/2 (Clontech, USA). CFP-pVHL30 was generated by KpnI/ApaI cloning pVHL30 out of pEGFPc1 into pECFPc1 (Clontech). HA-pVHL30 and Myc-HIF1α have been described before [19,20]. GFPc1-pVHL-S68/72A was a gift from Dr. W. Krek. GFP-KIF3A was constructed by cloning KIF3A out of Myc-KIF3A [16] into pEGFPc1 via EcoR1/XhoI. DsRed-γ-tubulin was a gift from Dr. J. Ellenberg. Myc-ΔN-KAP3 has been described before [16]. Murine KIF3A RNAi target sequence (5′-GGTGGTGGTTAGGTGCCGG-3′) was constructed in pTER (gift from Dr. H. Clevers) via BglII/HindIII cloning. All plasmids used have been sequence-verified.

Cell culture

HEK293T and murine neuroblastoma N1E-115 cells were cultured in DMEM supplemented with antibiotics and 5–10% fetal calf serum. Cells were plated 18 h prior to transfection by polyethelenimine (PEI; Polysciences, USA) or standard calcium phosphate transfection, and left for 48 h before the start of experiments. For immunofluorescence and live cell imaging, N1E-115 cells were seeded on gelatin-coated cover slips and incubated in maturation medium (DMEM, 1 μM cAMP, 0.5% FCS, 1% DMSO) 16–24 h prior to imaging. ATP-depletion was obtained by washing the cover slips twice with Dulbecco's phosphate-buffered saline (PBS) and then incubating the cells in PBS containing 5 mM sodium azide (Sigma-Aldrich, USA) and 1 mM 2-deoxy-D-glucose (Merck, Germany) at 37 °C for 30–60 min. ATP-reconstitution of ATP-depleted cells was performed by incubating the cells for 1 h in maturation medium at 37 °C. Stable lines of N1E-115 cells were generated to inducibly express RNAi for KIF3A (KIF3A-pTER construct; clone M7) using the T-Rex protocol (Invitrogen, USA). Positive clones were selected using zeocin (100 μg/ml; Invitrogen) and blasticidin (1 μg/ml; Invitrogen) and tested for inducible expression of the construct using doxycycline (Sigma-Aldrich).

Immunoprecipitations

To study endogenous pVHL-kinesin-2 binding in N1E-115 cells, approximately $1*10^7$ cells were lysed in 500 μl 1% Triton-X lysis buffer (20 mM TRIS pH 8.0, 1% Triton-X-100, 140 mM NaCl, 10% glycerol) containing 'complete' cocktail of protease inhibitors (Roche, Switzerland) at room temperature. Cell remnants were spun down by 10 min centrifugation at room temperature after which cleared lysates were immediately used for immunoprecipitations (IPs). α-VHL (7.5 μg of Ig32; BD-Biosciences, USA) or α-HA (clone 12CA5, hybridoma supernatant; 200 μl) were coupled to protein A/G agarose beads (30 μl; Santa Cruz Biotechnology, USA) in the presence of 1% BSA (Roche) and added to 200–400 μl of cleared lysates. To study GFP-pVHL30 binding to exogenous KIF3A, approximately $1*10^6$ cells were lysed in 400 μl buffer (20 mM TRIS, 1% Triton-X-100, 140 mM NaCl, 10% glycerol, pH 8.0) with protease inhibitors (Roche), then centrifuged 10 min at 13,000 rpm. Mouse monoclonal α-VHL (1.5 μg Ig32; BD-Biosciences) coupled to protein A/G agarose beads (7.5 μl; Santa Cruz Biotechnology), was added to 170 μl cleared lysate. IP reactions were incubated for 4 h at room temperature, washed four times with 1% Triton-X lysis buffer and analysed by western blotting. The IPs were repeated in at least two independent experiments.

Western blotting

Specific protein bands were visualised using the following antibodies: α-VHL (Ig32, 1:500; BD-Biosciences), α-KIF3A (1:500; BD-Biosciences), α-KAP3 (1:500; BD-Biosciences), α-KIF3C (1:500; BD-Biosciences), α-mitogen activated protein (MAP) kinase (1:5000; gift from Dr. O. Kranenburg), α-Myc (1:5; clone 9E10, hybridoma supernatant), α-GFP (1:500; Santa Cruz Biotechnology) and α-β-actin (1:10,000; Abcam, UK). All antibodies were diluted in PBS containing 5% milk and 0.1% Tween-20. Rabbit α-mouse Ig conjugated to horseradish peroxidase (1:20,000; Pierce, USA) or goat α-rabbit Ig conjugated to horseradish peroxidase (1:2000; DAKO, Denmark) was used as secondary antibody after which enhanced chemiluminescence (Roche) was used for detection.

Cell cycle analysis

Doxycycline treated and untreated asynchronous M7 and M34 cells were washed in PBS and fixed overnight in 70% ethanol. Fixed cells were washed in PBS, treated with RNase (1:100; Sigma-Aldrich) and stained with propidiumiodide (1:100; Sigma-Aldrich). Cell cycle analysis was performed on the FACS Calibur (BD-Biosciences).

Live cell imaging

Transfected N1E-115 neuroblastoma cells were visualised on a Zeiss LSM 510 confocal scanning microscope (Carl Zeiss, Germany) fitted with a climate control chamber maintaining humidified 37 °C and 5% CO_2. A circular region of interest with 10 μm radius was photobleached with 80 pulses of approximately 15 mW laser intensity after which the cell was followed by time-lapse imaging (every 0.25 s, total 400 frames). The prebleach fluorescence intensity (F_i) was defined as the mean of 15 images before photobleaching. Total cell fluorescence was measured over time and used to normalise the data as described