The von Hippel–Lindau tumour-suppressor protein interaction with protein kinase C δ

Xavier ITURRIOZ^{*1}, Joanne DURGAN*, Véronique CALLEJA‡, Banafshé LARIJANI‡, Heiwa OKUDA§, Richard WHELAN* and Peter J. PARKER^{*2}

^{*}Protein Phosphorylation Laboratory, Cancer Research UK London Research Institute, 44 Lincoln's Inn Fields, London, WC2A 3PX, U.K., [‡]Cell Biophysics Laboratory, Cancer Research UK London Research Institute, 44 Lincoln's Inn Fields, London, WC2A 3PX, U.K., and [§]Department of Urology, Kochi Medical School, Kochi 783-8505, Japan

The VHL (von Hippel–Lindau) tumour-suppressor protein forms a multi-protein complex [VCB (pVHL–elongin C–elongin B)–Cul-2 (Cullin-2)] with elongin C, elongin B, Cul-2 and Rbx1, acting as a ubiquitin-ligase (E3) and directing proteasome-dependent degradation of targeted proteins. The α -subunit of Hif1 α (hypoxia-inducible factor 1 α) is the principal substrate for the VCB–Cul-2 complex; however, other substrates such as aPKC (atypical protein kinase C) have been reported. In the present study, we show with FRET (fluorescence resonance energy transfer) analysis measured by FLIM (fluorescence lifetime imaging microscopy) that PKC δ and pVHL (VHL protein) interact directly in cells. This occurs through the catalytic domain of PKC δ (residues 432–508), which appears to interact with two regions of pVHL, residues 113–122 and 130–154. Despite this robust inter-

action, analysis of the PMA-induced proteasome-dependent degradation of PKC δ in different RCC (renal cell carcinoma) lines (RCC4, UMRC2 and 786 O) shows that there is no correlation between the degradation of PKC δ and the presence of active pVHL. Thus, in contrast with aPKC, PKC δ is not a conventional substrate of the ubiquitin-ligase complex, VCB–Cul-2, and the observed interaction between these two proteins must underlie a distinct signalling output.

Key words: fluorescence lifetime imaging microscopy (FLIM), fluorescence resonance energy transfer (FRET), protein complex, protein kinase C (PKC), renal cell carcinoma (RCC), von Hippel–Lindau protein (pVHL).

INTRODUCTION

The VHL (von Hippel–Lindau) tumour-suppressor gene is responsible for inherited familial VHL cancer syndrome, and mutations of the VHL gene, accompanied by loss of heterozygosity, are also found in 70–80% of sporadic clear cell RCCs (renal cell carcinomas) and haemangioblastomas [1]. pVHL (VHL protein) has been shown to associate with elongin C, elongin B, Cul-2 (Cullin-2) and the ring finger protein, Rbx1, to form the VCB (pVHL–elongin C–elongin B)–Cul-2 complex. pVHL has been extensively studied, and its most well understood function to date is as a ubiquitin-ligase (E3) specific for Hif1 α (hypoxia-inducible factor-1 α), the regulatory subunit of the transcription factor Hif1 (for a review, see [2]). During normoxia, Hif1 α is degraded via the ubiquitin–proteasome pathway by an oxygen-dependent mechanism controlled by proline hydroxylases [3–5]. As a consequence of inactivation of the VHL gene, Hif1 α expression is stabilized and the expression level of VEGF (vascular endothelial growth factor) is increased with subsequent stimulation of angiogenesis. Whereas Hif1 α is the best characterized substrate of pVHL, it is predicted that it is the spectrum of such substrates which underlies the distinctive clinical phenotypes associated with specific VHL mutations.

PKC (protein kinase C) δ belongs to a superfamily of lipid-activated serine/threonine kinases, comprising at least ten different proteins involved in a diverse range of intracellular functions. PKCs are divided into three distinct subfamilies (classical, novel

and atypical) based on their mechanism of activation, PKC δ belongs to the novel subfamily (for a review, see [6]). Activation of classical and novel PKC isoforms induced by the phorbol ester class of tumour promoters (such as PMA), typically leads to their activation and eventual down-regulation; the complex effects of these agents being defined in part by this acute-gain- and chronic-loss-of-function response. This down-regulation of the activity of PKCs, has been reported to be mediated by a ubiquitin–proteasome-dependent pathway(s); ubiquitination and degradation of PKC α , δ and ϵ have been described in rat 3Y1 cells upon chronic activation by phorbol esters [7]. Similarly, ubiquitination of activated PKC ζ in HeLa cells [8] and of activated PKC η in baby hamster kidney cells [9] has been observed. More recently, we have shown that PMA- and cell-cycle-induced degradation of PKC δ in NIH3T3 cells is mediated by polyubiquitination of the protein and is dependent on phosphorylation of the activation loop of PKC δ (Thr⁵⁰⁵) [10].

PKC isoforms, in particular PKC δ and PKC ζ , have been shown to bind pVHL and also to up-regulate VEGF expression by activating MAPK (mitogen-activated protein kinase) in cells that lack pVHL activity [11]. pVHL has been shown to ubiquitinate PKC λ/ι , and is to date the only ubiquitin-ligase described that shows specificity for a member of the PKC family [12]. With respect to PKC δ , evidence has previously been presented indicating that pVHL is able to inhibit IGF-I (insulin-like growth factor-I)-mediated cell signalling by directly blocking the association of PKC δ with the IGF-IR (IGF-I receptor) [13]. Moreover, PKC δ

Abbreviations used: aPKC, atypical PKC; BIM-I, bisindolylmaleimide-I; CD, catalytic domain; Cul-2, Cullin-2; Cy3, indocarbocyanine; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; GST, glutathione S-transferase; HA, haemagglutinin; Hif, hypoxia-inducible factor; IGF-IR, insulin-like growth factor-I receptor; NP40, Nonidet P40; ORF, open reading frame; PFA, paraformaldehyde; PKC, protein kinase C; pVHL, VHL protein; RD, regulatory domain; RT, reverse transcriptase; VA, vector alone; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau; VCB pVHL–elongin C–elongin B; RCC, renal cell carcinoma; wt, wild-type.

¹ Present address: INSERM U691–Collège de France, 11 place Marcelin Berthelot, 75231 Paris, France

² To whom correspondence should be addressed (email peter.parker@cancer.org.uk).

has been shown to be activated upon hypoxia and to relay hypoxic signals to Hif1 and heat-shock transcription factor in endothelial cells [14].

The responses described above provide evidence that PKC δ has a relationship with pVHL, and this is of potential importance in that it may be informative of the phenotypic diversity of pVHL mutations. However, whether pVHL exerts its action towards PKC δ via its ubiquitin-ligase function and/or, as alluded to above, a modulatory function towards this protein kinase remains unresolved. In order to probe the pVHL/PKC δ relationship, we have demonstrated the interaction of these proteins in intact cells by employing FRET (fluorescence resonance energy transfer) analysis. We have further mapped this interaction to the CD (catalytic domain) of PKC δ and have extended these studies to determine the pattern of interactions for a series of pVHL mutations found in human tumours. The evidence indicates that PKC δ interacts with pVHL via two regions of pVHL. Interactions display a characteristic pattern of behaviour indicative of a role in pVHL mutant-specific phenotypes. To determine whether this pattern of interaction correlates with activation-induced PKC δ down-regulation, we have investigated a series of RCCs that express either WT (wild-type) or mutant pVHL. The results show that pVHL does not directly promote the degradation of PKC δ nor does it inhibit catalytic activity in cells, indicating that distinctive signalling role(s) for the cellular complex characterize this interaction.

EXPERIMENTAL

Antibodies

Anti-PKC δ specific antibodies were purchased from Santa Cruz (rabbit polyclonal C-20). Antibodies against PKC ϵ and actin were obtained from Santa Cruz (rabbit anti-PKC ϵ C-20; goat anti-actin). Anti-Hif1 α and anti-PKC ζ -specific antibodies were from BD Transduction Laboratories (mouse monoclonal antibodies). A rabbit anti-PKC ζ N-terminal antibody was generously provided by Dr T. Sacktor (Department of Physiology and Pharmacology, State University of New York Downstate Medical Center, Brooklyn, New York, NY, U.S.A.). A mouse anti-(α tubulin) antibody was purchased from Sigma-Aldrich. Polyclonal antisera to the Ser²⁹⁹ phosphorylation site in PKC δ were raised in rabbits (J. Durgan and P. J. Parker, unpublished work).

Cell culture and treatments

RCC4, UMRC2 and 786 O cell lines were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) FBS (foetal bovine serum), 50 units/ml penicillin/50 μ g/ml streptomycin and 0.5 mg/ml G418. COS7 and HEK-293T [where T stands for large T-antigen of SV40 (simian virus 40)] cells were maintained in DMEM containing 10% (v/v) FBS, 50 units/ml penicillin/50 μ g/ml streptomycin. All the cells were cultured at 37°C, 5% CO₂. COS7 and HEK-293T cells were transfected with LipofectamineTM 2000 (Invitrogen) according to the manufacturer's instructions.

For the steady-state experiments, the RCC4 line [VA (vector alone) and VHL] were seeded at 25% and 100% confluency and maintained for 48 h before harvesting of the cells in sample buffer [100 mM Tris/HCl (pH 6.8), 4% (w/v) SDS and 20% (v/v) glycerol]. The amount of protein was determined by bicinchoninic acid assay (Pierce) and protein levels were adjusted to 0.1 μ g/ μ l by diluting the sample with water, DTT (dithiothreitol; 1M) and Bromophenol Blue.

For all the drug treatment experiments RCC4, UMRC2 and 786 O cells (VA and VHL) were seeded at 100% confluency,

maintained for 24 h to recover, and starved overnight in DMEM containing 0.5% FBS. The cells were then treated with 400 nM PMA (Sigma) for various times before harvesting of all samples in SB2 $\times$ (sample buffer 2 $\times$). For the treatments with drugs such as the protease inhibitor, MG132 (25 μ M), calyculin A (10 μ M), BIM-I (bisindolylmaleimide I; 10 μ M), the PKC inhibitors Gö 6976 (1 μ M) and rottlerin (5 μ M) (Calbiochem), RCC4 lines were pre-treated for 30 min prior to stimulation with PMA and were harvested as described above. Cell extracts were resolved by SDS/PAGE and proteins were analysed by Western-blot analysis using specific antibodies.

Plasmid constructs

The VHL ORF (open reading frame) was introduced into pEYFP (enhanced yellow fluorescent protein)-C1 or pEGFP (enhanced green fluorescent protein)-N1 (BD Clontech). pVHL-WT-YFP and pVHL-WT-GFP constructs were made by amplifying the VHL ORF by PCR, using the flanking primers, A, 5'-cggaattct-atgccccggaggcgaggagaac-3' (EcoRI site); B, 5'-gcgggatctcaatct-cccacgttgatgt-3' (BamHI site), and C, 5'-cggaattctcataatgcccc-ggaggcgaggagaac-3' (EcoRI site); D, 5'-ccggatccgcacatctccatcc-gttgatgtgc-3' (BamHI site) respectively. pVHL truncations and deletions were generated using the forward primer, C, described above (pVHL-WT-GFP) and the following primers: pVHL122-GFP, 5'-gcgggatccgcacatctctgaagagccaaggtg-3' (BamHI site); pVHL113-GFP, 5'-gcgggatccgcctcgtagctgtgtagtgcgcg-3' (BamHI site).

For the pVHL Δ 87-130-GFP construct, pVHL Δ 87-130-T7-T7-His₆-pVHL Δ 87-130 was used as the PCR template with the pVHL-WT-GFP flanking primers, C and D. For pVHL point mutations, site-directed mutagenesis was carried out with two PCR steps. The forward primer, 5'-catgacctatgggactttcctac-3' and the reverse primer, 5'-gctgtgtagtgtactccagc-3' were used with the following specific complementary primers for each mutation (the underlined bases encode the new amino acid residue): pVHL-Y98H-GFP, 5'-ggcgagccgcagccccccaacgctgccgctgg-3' and 5'-ccaggcgccgagcgttggtgggctgcggctcgcc-3'; pVHL-C162W-GFP, 5'-ctctgaagagcgtatggctccaggtgtccg-3' and 5'-cggacaacctggagcca-tcgtctttcagag-3'; pVHL-R167W-GFP, 5'-gcctccaggtgtgtctggagc-catgtaagcctg-3' and 5'-caggcttgactaggtccagacaacctggagc-3'.

PKC δ -GST (glutathione S-transferase) constructs were made using the following oligonucleotides as specific flanking primers for PCR: full-length-PKC δ -GST, 5'-gataataccgggagtgaccggtt-ctctgcgc-3' (XmaI site) and 5'-ctaagcaggtaccgttcagggaattgctca-tatttgg-3' (KpnI site); PKC δ -RD (regulatory domain)-GST, 5'-gataataccgggagtgaccggttctctgcgc-3' (XmaI site) and 5'-gataaaggtaccgcccgttgctccccctccag-3' (KpnI site); PKC δ -CD-GST, 5'-gataataccgggagtgactcttgctgaggccttgaacc-3' (XmaI site) and 5'-ctaagcaggtaccgttcagggaattgctcatatttgg-3' (KpnI site). The PCR products were digested with XmaI and KpnI restriction enzymes and were cloned into pMT2SM-GST that was similarly digested.

Truncations of the CD of PKC δ were introduced by PCR, using the following oligonucleotides as forward primers: CD 376, 5'-gataataccgggagtgactacctaagaaggcagctgtg-3' (XmaI site); CD 432, 5'-gataataccgggagtgaaagccgcttcgaactctacc-3' (XmaI site); CD 508, 5'-gataataccgggagtgactcctgactacatcgccctg-3' (XmaI site) and CD 616, 5'-gataataccgggagtgccgccccttaagcccaagtgaatcc-3' (XmaI site). The reverse primer was 5'-ctaagcaggtaccggttccagggaattgctcatatttgg-3' (KpnI site).

Construction of GFP-PKC δ -WT, T505A and K376M was described previously [10] GFP-PKC δ A147D was made by site-directed mutagenesis with PCR using the oligonucleotides, 5'-cggaattcatggcaccgttctctgcgc-3' (EcoRI site) and 5'-gggggtacctattccagggaattgctcata-3' (KpnI site), as flanking primers and the

oligonucleotides, 5'-cccaactatgaaccgccgtggagacattaacaggccaag-attcac-3' and 5'-gtgaatcttgccctgtttaatgtctccacggcgggtcatagtggg-3', as complementary mutagenesis primers.

RNA extraction, RT (reverse transcriptase)-PCR and sub-cloning of VHL mutants expressed in the RCC4 VA cell line

RNA extraction from RCC4 VA cells was performed with the Qiagen RNeasy system according to the manufacturer's instructions. Reverse transcription and PCR was performed using Sigma's SuperScript One-Step RT-PCR with a Platinum Taq System with the primers, C and D. The PCR product was subcloned into pEGFP-N1.

Pull-down and co-immunoprecipitation experiments

Post-transfection (24 h), cells were harvested in 500 μ l of lysis buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.5 % NP40 (Nonidet P40) or 0.2 % CHAPS, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 2 mM orthovanadate and 1 protease inhibitors complete tablet (Roche)]. After 30 min of incubation on ice and centrifugation at 14000 g for 10 min the supernatants were subjected to immunoprecipitation with a rabbit anti-GFP antibody (0.5 μ l per immunoprecipitation) (BD Clontech) and Protein A–Sepharese, or pull-down with glutathione–Sepharese (15 μ l per pull-down) (Amersham) and were incubated for 2 h at 4°C. The beads were washed five times with 1 ml of lysis buffer and the final bead pellets were resuspended in 40 μ l of SB2 $\times$, boiled and resolved by SDS/PAGE and Western blotting. Immunoreactivity was analysed by chemiluminescence using the ECL[®] system (Amersham).

In vivo polyubiquitination assay

Post-transfection (24 h), HEK-293T cells were pre-treated for 30 min with medium containing 25 μ M MG132, and were subsequently stimulated with 400 nM PMA for 30, 60 and 120 min. Finally, cells were lysed in 500 μ l of lysis buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 % NP 40, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 2 mM orthovanadate, one Complete protease inhibitor tablet (Roche), 10 mM N-ethylmaleimide and 50 μ M ALLN (*N*-acetyl-L-leucyl-L-leucylnorleucinal)]. After 30 min of incubation on ice and centrifugation at 14000 g for 10 min the supernatants were subjected to immunoprecipitation with a mouse anti-HA (haemagglutinin) antibody (3 μ g per immunoprecipitation) and Protein A–Sepharese, and were incubated for 2 h at 4°C. The beads were finally washed five times with lysis buffer and the immunoprecipitated proteins were recovered by adding 40 μ l of SB2 $\times$ to the last bead pellets and by boiling the beads. The proteins were then resolved by SDS/PAGE and Western-blot analysis.

Immunofluorescence and FRET

COS7 cells were transfected with YFP–VHL and PKC δ –GST constructs on coverslips. Post-transfection (24 h) the coverslips were fixed with 2 % PFA (paraformaldehyde) in PBS and after permeabilization and blocking with 0.1 % Triton X-100/1 % BSA in PBS, the coverslips were incubated with the primary rabbit anti-GST antibody (Santa Cruz) (1:250 in PBS/1 % BSA) for 1 h, washed with PBS and incubated for 45 min with a Cy3 (cytidine 3)-conjugated anti-rabbit (1:500) secondary antibody (Dako). Finally, coverslips were mounted on to slides with Mowiol and examined using a confocal laser scanning microscope (LSM 510, Carl Zeiss Inc.) equipped with krypton/argon lasers and with a 1.4 numerical aperture, 63 $\times$ Plan-APOCHROMAT oil-immersion objective. Double-labelled images (1024 pixels $\times$ 1024 pixels)

were analysed in sequential scanning mode by exciting YFP at 488 nm and Cy3 at 543 nm. For FRET experiments, COS7 cells were co-transfected with a 3:1 ratio of Myc-empty vector/VHL–WT–GFP or PKC δ –Myc/VHL–WT–GFP. Post-transfection (24 h) the cells were fixed for 10 min with 4 % PFA in PBS. The cells were then permeabilized with 0.2 % Triton X-100 in PBS for 5 min, autofluorescence of the cells was quenched with 1 mg/ml sodium borohydrate in PBS for 5 min and cells were blocked with 1 % BSA in PBS for 5 min. The cells were then subjected to immunofluorescence (1 h) staining with a mouse anti-Myc antibody (9E10; CR-UK) previously labelled with Cy3. Then coverslips with cells were mounted with Mowiol containing DABCO (1,4-diazabicyclo[2.2.2]octane).

A detailed description of the FRET analysis monitored by FLIM (fluorescence lifetime imaging microscopy) can be found elsewhere [15,16]. We monitored lifetime in the frequency (phase) domain; phase methods provide an average lifetime whereby sinusoidally modulated light is used to excite the sample. The lag in the emitted fluorescence signal permits measurement of phase (τ_p) and modulation depth (τ_m) of the fluorescence. The lifetime, τ , is the average of phase-shift and relative modulation depth $\frac{(\tau_m + \tau_p)}{2}$ of the emitted fluorescence signal. All images were taken using a Zeiss Plan-APOCHROMAT 100 $\times$, 1.4 numerical aperture, phase 3 oil-immersion objective, with images recorded at a modulation frequency of 80.218 MHz. The donor (pVHL–GFP) was excited using the 488 nm line of an argon/krypton laser and the resultant fluorescence separated using a combination of the dichroic beam splitter (Q 505 LP; Chroma Technology Corp.) and interference filter (HQ510/20; Chroma Technology Corp.).

RESULTS AND DISCUSSION

Previous studies have shown, by co-immunoprecipitation, that pVHL interacts with PKC δ [11]. This observation was initially confirmed via co-immunoprecipitation of pVHL–HA from the RCC line, 786-0 VHL, and immunoblotting of endogenous PKC δ (see Supplementary Figure at <http://www.BiochemJ.org/bj/397/bj3970109add.htm>) and was extended to determine whether the proteins directly interact in intact cells. Shiao et al. [17] have shown that pVHL localizes to mitochondria in rat clear cell kidney tumours and that altered pVHL leads to malfunction of this organelle. As previously described, the fusion of YFP to the N-terminus of pVHL–WT (Figure 1A) leads to its accumulation in structures surrounding the nucleus (Figure 1B; pVHL–YFP images, green channel); a similar distribution was observed with a dsRed (red fluorescent protein from the cerial *Discosoma* sp.)–pVHL construct (results not shown). We have exploited this localization property to determine whether this form of pVHL is able to recruit different PKC δ GST-tagged constructs into these structures. Expression of the full-length, RD or CD of PKC δ in COS7 cells, shows a diffuse cytoplasmic pattern of staining (Figure 1B, red channel). However, when co-expressed with YFP–pVHL the full-length PKC δ and the CD co-localize with the structures formed by YFP–pVHL (Figure 1B, top and bottom images). By contrast, the co-expressed RD of PKC δ does not co-localize with pVHL–YFP (Figure 1B, middle images) demonstrating that the recruitment is specific and not a function of protein co-expression.

Direct pVHL/PKC δ interaction in cells was monitored by FRET, as measured by FLIM. This technique determines the lifetime of the donor (pVHL–GFP) such that when the donor interacts with the acceptor (Myc–PKC δ , immunostained with an anti-Myc antibody previously labelled with the acceptor, Cy3) the lifetime of the donor decreases (see [15,16] for details of the methods

Figure 1 Interaction of PKC δ with pVHL in cells

(A) Schematic representation of the YFP-pVHL construct. (B) Immunofluorescence analysis of COS7 cells co-transfected with YFP-pVHL and three different PKC δ -GST constructs (as described in the Experimental section). Confocal images show an optical section in the equatorial plane of the cells. (C) COS7 cells were co-transfected with pVHL-GFP/Myc-empty vector or pVHL-GFP/Myc-PKC δ . Myc expression was detected with an anti-myc monoclonal antibody directly labelled with Cy3, and the interaction of pVHL-GFP with Myc-PKC δ was analysed by FRET and measured by FLIM. The images at the top correspond to the image intensity of the donor and the images at the bottom show the lifetime (τ) of GFP, as indicated with pseudocolour scales from red (1.8 ns) to blue (2.5 ns). (D) Two-dimensional histograms of (τ_p) and (τ_m) lifetimes obtained in the frequency domain, illustrating the variation in lifetime for three independent experiments. pVHL-GFP/Myc-empty vector (white), VHL-GFP/Myc-PKC δ (red).

Figure 2 Characterization of PKC δ interaction with pVHL

(A) Schematic representation of PKC δ -GST constructs, T7-pVHL-WT and pVHL-WT-GFP fusion proteins used in pull-down experiments. (B) COS7 cells were co-transfected with GST alone (—), PKC δ full-length (FL), RD or CD GST fusion proteins and pVHL-WT-GFP. The interaction with pVHL-WT-GFP is indicated in the central panel. IB, immunoblot. (C) Schematic representation of PKC δ CD GST-tagged mutations and truncations. Lys³⁷⁶ and Thr⁵⁰⁵ correspond to the ATP binding site and the T-loop site respectively. The results of three different pull-down experiments are summarized on the right-hand side. (D) COS 7 cells were co-transfected with truncation mutant constructs of the PKC δ CD and pVHL-WT-GFP. Cell extracts were subjected to a pull-down experiment on GST beads, and protein complexes were resolved by SDS/PAGE and Western-blot analysis with an anti-GST antibody against the PKC δ truncation mutants and an anti-GFP antibody for pVHL recovery. IB, immunoblot.

employed in the present study). Figure 1(C) shows the image intensity corresponding to the fluorescence intensity of the donor and the τ map, which corresponds to the lifetime, τ , of the donor (pVHL-GFP). When pVHL-GFP is co-expressed with an Myc-empty vector and co-stained with an anti-Myc-Cy3 antibody, the lifetime of the donor corresponds to the lifetime of the GFP. However when co-expressed with Myc-PKC δ the observed lifetime decreases significantly, demonstrating the existence of FRET between the donor and the anti-Myc-Cy3 acceptor. In Figure 1(D) the cumulative lifetimes of all pixels recorded for the pVHL-GFP/Myc-empty vector and pVHL-GFP/Myc-PKC δ are presented. This two-dimensional histogram shows the lifetimes measured by the two parameters assessed by FLIM (phase and modulation); the concomitant decrease in both indicates a significant decrease in GFP lifetime of the donor due to FRET. This acceptor-dependent lifetime decrease indicates a close interaction between pVHL and PKC δ in the cytoplasm of intact cells.

To determine the domain of interaction of these two proteins, several deletion constructs were employed (Figure 2). We found that for PKC δ , the full-length protein and the CD interact strongly with pVHL-GFP (Figure 2B). Similar results were obtained with a T7-pVHL construct, indicating that neither the tag nor the position influence this behaviour (results not shown). To more precisely dissect out the interaction of PKC δ and pVHL, we further truncated the PKC δ CD (Figure 2C). GST pull-down experiments (as illustrated in Figure 2D) revealed that truncation mutants 376 CD-GST and 432 CD-GST still interact with pVHL; the mutants with shorter truncations of the PKC δ CD, 508 CD-GST and 616 CD-GST, do not pull-down pVHL. Although the expression level of the 508 truncation mutant is low in this experiment, this was not a consistent observation and at equivalent levels of expression with the 376 or 432 truncation mutants there was still no equivalent ability to interact with pVHL. In summary, these results suggest that the domain of interaction of PKC δ with pVHL includes residues between positions 432 and 508.

Figure 3 pVHL interaction with PKC δ mutants

HEK-293T cells stably expressing pVHL-WT-T7 were co-transfected with WT PKC δ , and PKC δ mutants (A147D, K376M and T505A). Cells co-transfected with WT PKC δ were treated with or without 400 nM PMA for 24 h. After 48 h of transfection the cells were lysed and subjected to immunoprecipitation (IP) with a rabbit anti-GFP antibody and protein complexes were resolved by SDS/PAGE and immunoblot (IB) analysis.

As the domain of interaction overlaps the catalytic site of PKC δ and includes the activation loop, we investigated whether proteins with mutations of the catalytic lysine (K376M) or of the activation-loop phosphorylation site T505A were able to interact with pVHL. We also determined the effect of chronic PMA treatment (24 h) on the interaction between WT PKC δ and pVHL, as well as in the interaction between the constitutively active PKC δ mutant (A147D) and pVHL. These studies showed that a modest increase in pVHL recovery was observed upon PMA treatment; however, none of the mutations significantly affected the interaction of PKC δ with pVHL (Figure 3). This indicates that, although the CD is sufficient, PKC δ activity itself is not required for the pVHL interaction.

For comparison of the mode of interaction of PKC δ and Hif1 α with regard to pVHL, a series of deletion/mutation pVHL constructs (Figure 4A) was tested for interaction with PKC δ ; these included deletions of the Hif1 α -interacting domain (residues 113–122) and mutations that prevent interaction with the elongin complex (C162W and R167W). Pull-down experiments (Figures 4B and 4D) revealed that PKC δ binds the 1–122 truncation mutant but not the 1–113 truncation mutant, suggesting that the domain of interaction between pVHL and PKC δ includes residues between positions 113 and 122, as previously reported for the regulatory domain of aPKC [18]. However, the pVHL 87–130 deletion mutant interacts with PKC δ 376 CD–GST, revealing the

Figure 4 Analysis of the interaction of pVHL mutants with PKC δ

(A) Schematic representations of pVHL constructs. (B) COS7 cells were co-transfected with pVHL–GFP constructs and CD 376 PKC δ –GST or GST-empty vector. IB, immunoblot. (C) Schematic representation of pVHL constructs that contained the two defined allelic mutations present in RCC4 VA cells. (D) COS 7 cells were co-transfected with CD 376 PKC δ –GST or GST-empty vector and WT or mutated pVHLs. (B) And (D), cells were then lysed and protein complexes were purified by GST pull-down and analysed by SDS/PAGE and Western-blot analysis.

A **B**

Figure 5 Analysis of PKC δ turnover and phosphorylation by pVHL

(A) Steady-state protein levels of different PKCs in RCC4 VA and RCC4 VHL cells (cell extracts: 4 μ g for Hif1 α , PKC α and PKC ϵ , 2 μ g for PKC ι and ζ , and 1 μ g for PKC δ and actin) (as described in the Experimental section). Densitometric analysis of the steady-state protein levels of PKCs in RCC4 VA and RCC4 VHL cells at different cell densities is described below. (B) The steady-state phosphorylation of PKC δ was determined for the activation loop, Thr⁵⁰⁵, and the C-terminal turn motif, Ser⁶⁴³. The histogram shows densitometric analysis of the steady-state phosphorylation of PKC δ in RCC4 VA and RCC4 VHL for Thr⁵⁰⁵ and Ser⁶⁴³. HD, high density; LD, low density.

existence of an additional point of contact. Interestingly, the three hot-spot mutations for human VHL syndromes, Y98H, C162W and R167W, all interact with PKC δ (Figure 4B). This indicates that mutations which affect the Hif1 α interaction (as

opposed to the elongin C interaction, in particular Δ 87–130 and Y98H), may retain their 'activity' towards PKC δ .

To further characterize the mode of interaction of pVHL with PKC δ , we have isolated the VHL cDNA sequences from RCC4

lines lacking pVHL activity against Hif1 α (RCC4 VA). We have thus demonstrated that two mutant alleles are present, namely S65W and S65W/ Δ 114–154 (Figure 4C). These were sub-cloned into the pEGFP-N1 expression vector and used to determine their ability to bind PKC δ by GST pull-down experiments (Figure 4D). The pVHL S65W mutant interacted with PKC δ , whereas the pVHL S65W/ Δ 114–154 mutant, corresponding to the deletion of exon 2, lost its ability to bind PKC δ . This result alongside the Δ 87–130 deletion data, indicates that the second domain of interaction between PKC δ and pVHL is likely to be located between residues 130 and 154. These results also show that the two domains of interaction (residues 113–122 and 130–154) can act independently. The combination of these two domains may

increase the stability of the complex or have functional activity. Indeed, as noted by Datta et al. [13] the pVHL sequence comprising residues 113–122 contains a pseudo-substrate-like sequence that has the ability to inhibit the kinase activity of PKC δ *in vitro*.

To determine whether the expression level of PKC δ is influenced by pVHL activity, we initially determined the steady-state levels of different PKCs expressed in RCC4 VA or in the same cells re-expressing WT pVHL (RCC4 VHL). Since Lee et al. [19] have shown that pVHL shuttles between the cytoplasm and nucleus during the cell-cycle (being predominantly nuclear in sparsely growing cells and cytosolic in confluent cells) and because the interaction between pVHL and PKC δ occurs in the

Figure 6 For legend see facing page

D **Figure 6** PKC δ downregulation in RCC4 lines

(A) PMA-induced degradation of PKCs in high density RCC4 VA and RCC4 VHL cells. (B) Logarithmic expression of the PMA-induced degradation time courses for different PKCs obtained in RCC4 cell lines. (C) The effect of calyculin (calA; 10 μ M), Bim I (10 μ M), Gö 6976 (1 μ M) and rottlerin (Rott; 5 μ M) on the PMA-induced degradation of PKC α and PKC δ in RCC4 lines. (D) pVHL promotes polyubiquitination of PKC δ . Transfected HEK-293T cells were treated for various times (T) with 400 nM PMA in the presence of MG132 (16 μ M) and cells were then lysed in polyubiquitination buffer and subjected to immunoprecipitation with a rabbit anti-HA antibody (Santa Cruz). Proteins were then resolved by SDS/PAGE and Myc-PKC δ was analysed with an anti-PKC δ (Santa Cruz) antibody. IB, immunoblot.

cytoplasm, we have analysed PKC's expression level under different culture conditions. Thus RCC4 lines were seeded sparsely (20 % confluent) or at high density (100 %). Amongst the PKCs tested, only PKC δ showed a significantly decreased expression level in RCC4 VHL versus RCC4 VA cells; this was only observed when cells were grown at high density (Figure 5A). PKC ϵ , which, like PKC δ , belongs to the novel PKC family, showed the opposite expression profile, without any effect from cell density (Figure 5A). Contrary to expectation, PKC ι and PKC ζ , which have been shown to be polyubiquitinated by pVHL, were modestly up-regulated when WT pVHL is expressed and cells were cultivated at high density (Figure 5A; as described below). As previously shown, Hif1 α expression levels were increased when pVHL activity was lost (Figure 5A). Since Pause et al. [20] have shown that the lack of VHL activity only affects cell-cycle arrest upon serum withdrawal and that cell-cycle exit by contact inhibition remains identical in RCC4 and RCC4 lines that re-express VHL, we can indeed conclude that the effects observed at high density are not a function of a differential ability of these cells to arrest the cell-cycle. This decrease in PKC δ expression level only in high density RCC4 VA cells suggests that pVHL could promote the down-regulation of PKC δ expression under these conditions and that the presence of pVHL in the cytoplasm is necessary for this degradation. It is of note that no modification

of the steady-state phosphorylation of PKC δ at Ser⁶⁴³ and Thr⁵⁰⁵ was observed in RCC4 VA and RCC4 VHL cells at different cell densities (Figure 5B).

PMA-induced degradation was also followed in high-density RCC4 VA and RCC4 VHL cells, and by monitoring PKC α , PKC δ and PKC ϵ levels with Western-blot analysis (Figure 6A). PMA treatment of RCC4 cell lines induced down-regulation of all three PKC isoforms, with almost complete loss occurring after 24 h of treatment. The involvement of the proteasome in this PMA-induced response was indicated by its inhibition upon co-treatment with MG132. The values obtained by densitometric analysis of different sets of experiments ($n = 6$ for PKC δ , and $n = 5$ for PKC α and PKC ϵ) and logarithmic expression of these time courses have been used to determine the rates of degradation (slope of the curves) of PKCs in RCC4 cell lines (Figure 6B). The presence of WT pVHL does not affect the rate of degradation of PKC α [0.103 ± 0.027 versus 0.083 ± 0.044 An (arbitrary absorbance units)/h] and PKC ϵ (0.103 ± 0.035 versus 0.106 ± 0.025 Au/h), but it is associated with a 3-fold ($P < 0.05$) increase in the rate of degradation of PKC δ (0.085 ± 0.034 versus 0.306 ± 0.032 Au/h). These results suggest that pVHL might promote the degradation of PKC δ and that this degradation is under the control of the ubiquitin-proteasome system. *In vivo* polyubiquitination assays of PKC δ co-expressed with pVHL and

Figure 7 Analysis of PKC δ degradation in UMRC2 and 786 O cell lines with or without pVHL

(A) Steady-state protein levels of different protein kinase Cs in UMRC2 and 786 O cells. Steady-state protein levels of different PKCs in confluent UMRC2 and 786 O cell lines (cell extracts: 80 μ g for PKC α , 60 μ g for PKC ϵ , 2 μ g for PKC ζ and tubulin and 1 μ g for PKC δ). (B) PMA-induced degradation of PKCs in high density UMRC2 (VA and VHL) and 786 O (VA and VHL) cells.

HA-tagged ubiquitin in HEK-293T cells confirmed the ubiquitin-ligase activity of pVHL towards PKC δ under overexpression conditions (Figure 6D).

We have shown previously that the down-regulation of PKC δ expression during cell-cycle progression is dependent on the phosphorylation of the activation loop at Thr⁵⁰⁵ and can be promoted by calyculin A, a PP2A (protein phosphatase 2A) selective inhibitor, and inhibited by the PKC inhibitor BIM-1 [10]. This pattern of behaviour is only partially retained in the RCC4 lines (Figure 6C). Co-treatment of RCC4 lines with calyculin A does

not inhibit PKC δ degradation, whereas inhibition of PKC δ with BIM-1 or rottlerin, but not the more PKC α -selective, Gö 6976, blocks PMA-induced degradation of PKC δ in both cell lines. It should be noted that Gö 6976 was effective in blocking PMA-induced PKC α degradation. The results indicate that, although the presence of WT pVHL is associated with increased PMA-induced degradation of PKC δ , the degradation of PKC δ in these RCCs requires functional kinase activity, clearly contrasting with the requirement for pVHL–PKC δ interaction (as described above).

The distinction between PMA-induced PKC δ down-regulation and the PKC δ /pVHL interaction raised the question as to whether the effects observed either in RCC4 or RCC4 VHL cells are due to clonal variation or reflect an independent function for the interaction. Since direct siRNA (small interfering RNA) knock-down of pVHL expression in the RCC4 lines has not been possible (results not shown), we investigated this issue by analysis of two other RCC lines that retain or lack pVHL activity. Specifically the UMRC2 (VA and VHL) and the 786 O (VA and VHL) lines were employed to determine the steady-state PKC δ level and also its degradation behaviour in these cell lines (Figure 7). UMRC2 VA has weak expression of a mutated form of pVHL, whereas the 786 O VA cell line lacks detectable pVHL expression. In contrast with the results obtained using the RCC4 lines, the presence of pVHL in UMRC2 or 786 O cell lines does not change the steady-state level of PKC δ (the concentration being assessed as a function of tubulin expression). In comparison with the steady-state behaviour of aPKCs, we noted that the effect of pVHL expression does not correlate with the overall level of protein, but rather with the relative accumulation of the dephosphorylated form of the protein. The low abundance of the shifted phosphorylated aPKC form makes it impossible to determine whether the loss of this more slowly migrating phosphorylated form is directly related to the increase of the faster migrating form or not, i.e. whether the apparent effect of pVHL is due to degradation of the phosphorylated form as proposed elsewhere [21] or dephosphorylation of the pVHL-associated kinase.

For PMA-induced degradation of PKC δ , this is significantly increased in UMRC2 VA cells by comparison with the UMRC2 VHL; there is a modest and equivalent extent of PMA-induced degradation in the 786 O VA and VHL cells (Figure 7B). These results demonstrate that there is no correlation between the presence of active pVHL and the induced degradation of PKC δ in these RCC lines. It is evident that pVHL is not required for the activation-induced down-regulation of PKC δ and that the differences in PKC δ degradation observed between these RCC lines are most likely due to clonal variation. Similarly a comparison of the levels of PKC δ in the three pairs of RCC lines indicated that there is also no correlation between PKC δ steady-state levels and VHL expression.

The lack of a requirement for pVHL in the PMA-induced down-regulation and steady-state turnover of PKC δ juxtaposed with the clear demonstration of complex-formation in cells, supports the notion that through this interaction pVHL influences PKC δ activity. Two different studies have indeed reported such functions. Pal et al. [11] have demonstrated that pVHL can block translocation of PKC ζ and PKC δ to cell membranes, blocking activation and preventing a signalling step that leads to VPF/VEGF expression. Datta et al. [13] have shown that PKC δ kinase activity *in vitro* can be inhibited by pVHL-derived peptides and that in the context of IGF-I signalling, pVHL can inhibit the association of PKC δ with IGF-IR, decreasing the invasiveness of the cells expressing pVHL. These effects of complex-formation on PKC δ function/activity indicate that the key role of the interaction is in tempering the immediate functional output of PKC δ rather than the more characteristic function of pVHL i.e., the induced degradation of the pVHL-associated protein. To assess whether this is due to a direct effect on PKC δ activity effected through the defined *in vitro* inhibitory effect of pVHL [13], we monitored PKC δ activity in intact cells by employing a novel autophosphorylation site (J. Durgan and P. J. Parker, unpublished work). PMA-induced phosphorylation of PKC δ at Ser²⁹⁹ was not significantly affected by the presence or absence of pVHL (Figure 8). The lack of an effect of pVHL on PKC δ activity juxtaposed with its lack of involvement in the PKC δ down-regulation pathway indicates that

Figure 8 pVHL does not inhibit PKC δ autophosphorylation in 786 O cells

Cells were allowed to approach confluency and then switched to 0.5% FBS in DMEM overnight. Cells were then either treated with vehicle (0.1% ethanol) or PMA (1 μ M) for 15 min. Extracts were prepared and blotted for PKC δ protein (upper panel) for the induced autophosphorylation of Ser²⁹⁹ in the presence of the dephosphopeptide to suppress any recognition of the dephosphorylated site (middle panel), or both dephospho- and phosphopeptides to demonstrate the specificity of immunodetection (lower panel). This is representative of two similar experiments. The asterisks denote non-specific immunoreactivity. P, phospho; comp., P-antigen competition.

its functional effect is likely to be directed at a specific substrate(s), perhaps mediated through localization.

PKC δ has been implicated in cell-cycle regulation and apoptosis in many different cell types. The extent to which these properties contribute to the pattern of pVHL-associated disease progression remains to be determined. However, it is now clear that this will not be reflected simply in the altered turnover of PKC δ , nor through direct inhibition, but rather through altered localization and/or selective substrate targeting. This distinction is important, as it determines the nature of the parameters required for an assessment of whether PKC δ contributes to VHL disease sub-classes in the clinical setting.

We thank Dr Peter Ratcliffe and Dr Norma Masson (The Henry Wellcome Building of Genomic Medicine, Roosevelt Drive, Oxford OX3 7BN, U.K.) for providing RCC4, UMRC2 and 786 O cell lines.

REFERENCES

- Barry, R. E. and Krek, W. (2004) The von Hippel-Lindau tumour suppressor: a multi-faceted inhibitor of tumorigenesis. *Trends Mol. Med.* **10**, 466–472
- Kim, W. Y. and Kaelin, W. G. (2004) Role of VHL gene mutation in human cancer. *J. Clin. Oncol.* **22**, 4991–5004
- Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, W. G. (2001) HIF-1 α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science* **292**, 464–468
- Yu, F., White, S. B., Zhao, Q. and Lee, F. S. (2001) HIF-1 α binding to VHL is regulated by stimulus-sensitive proline hydroxylation. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 9630–9635
- Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J. et al. (2001) Targeting of HIF-1 α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science* **292**, 468–472
- Mellor, H. and Parker, P. J. (1998) The extended protein kinase C superfamily. *Biochem. J.* **332**, 281–292
- Lu, Z., Liu, D., Hornia, A., Devonish, W., Pagano, M. and Foster, D. A. (1998) Activation of protein kinase C triggers its ubiquitination and degradation. *Mol. Cell Biol.* **18**, 839–845
- Smith, L., Chen, L., Reyland, M. E., DeVries, T. A., Talanian, R. V., Omura, S. and Smith, J. B. (2000) Activation of atypical protein kinase C ζ by caspase processing and degradation by the ubiquitin-proteasome system. *J. Biol. Chem.* **275**, 40620–40627

- 9 Kang, B. S., French, O. G., Sando, J. J. and Hahn, C. S. (2000) Activation-dependent degradation of protein kinase C η . *Oncogene* **19**, 4263–4272
- 10 Srivastava, J., Procyk, K. J., Iturrioz, X. and Parker, P. J. (2002) Phosphorylation is required for PMA- and cell-cycle-induced degradation of protein kinase C δ . *Biochem. J.* **368**, 349–355
- 11 Pal, S., Claffey, K. P., Dvorak, H. F. and Mukhopadhyay, D. (1997) The von Hippel-Lindau gene product inhibits vascular permeability factor/vascular endothelial growth factor expression in renal cell carcinoma by blocking protein kinase C pathways. *J. Biol. Chem.* **272**, 27509–27512
- 12 Okuda, H., Saitoh, K., Hirai, S., Iwai, K., Takaki, Y., Baba, M., Minato, N., Ohno, S. and Shuin, T. (2001) The von Hippel-Lindau tumor suppressor protein mediates ubiquitination of activated atypical protein kinase C. *J. Biol. Chem.* **276**, 43611–43617
- 13 Datta, K., Nambudripad, R., Pal, S., Zhou, M., Cohen, H. T. and Mukhopadhyay, D. (2000) Inhibition of insulin-like growth factor-I-mediated cell signaling by the von Hippel-Lindau gene product in renal cancer. *J. Biol. Chem.* **275**, 20700–20706
- 14 Baek, S. H., Lee, U. Y., Park, E. M., Han, M. Y., Lee, Y. S. and Park, Y. M. (2001) Role of protein kinase C δ in transmitting hypoxia signal to HSF and HIF-1. *J. Cell Physiol.* **188**, 223–235
- 15 Squire, A. and Bastiaens, P. I. (1999) Three dimensional image restoration in fluorescence lifetime imaging microscopy. *J. Microsc.* **193**, 36–49
- 16 Larijani, B., Allen-Baume, V., Morgan, C. P., Li, M. and Cockcroft, S. (2003) EGF regulation of PIP dynamics is blocked by inhibitors of phospholipase C and of the Ras-MAP kinase pathway. *Curr. Biol.* **13**, 78–84
- 17 Shiao, Y. H., Resau, J. H., Nagashima, K., Anderson, L. M. and Ramakrishna, G. (2000) The von Hippel-Lindau tumor suppressor targets to mitochondria. *Cancer Res.* **60**, 2816–2819
- 18 Okuda, H., Hirai, S., Takaki, Y., Kamada, M., Baba, M., Sakai, N., Kishida, T., Kaneko, S., Yao, M., Ohno, S. and Shuin, T. (1999) Direct interaction of the β -domain of VHL tumor suppressor protein with the regulatory domain of atypical PKC isotypes. *Biochim. Biophys. Res. Commun.* **263**, 491–497
- 19 Lee, S., Chen, D. Y., Humphrey, J. S., Gnarr, J. R., Linehan, W. M. and Klausner, R. D. (1996) Nuclear/cytoplasmic localization of the von Hippel-Lindau tumor suppressor gene product is determined by cell density. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1770–1775
- 20 Pause, A., Lee, S., Lonergan, K. M. and Klausner, R. D. (1998) The von Hippel-Lindau tumor suppressor gene is required for cell cycle exit upon serum withdrawal. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 993–998
- 21 Lee, S., Nakamura, E., Yang, H., Wei, W., Linggi, M. S., Sajan, M. P., Farese, R. V., Freeman, R. S., Carter, B. D., Kaelin, Jr, W. G. and Schlisio, S. (2005) Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: developmental culling and cancer. *Cancer Cell* **8**, 155–167

Received 6 March 2006/25 April 2006; accepted 2 May 2006

Published as BJ Immediate Publication 2 May 2006, doi:10.1042/BJ20060354