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PKCζII is a target for degradation through the tumour suppressor protein pVHL

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Abstract **PKCζII is a rapidly degraded variant of PKCζ that suppresses epithelial cell polarisation. It is shown here that PKCζII is a target for the E3 ligase and tumour suppressor Von Hippel-Lindau protein (pVHL). Deletion studies demonstrate that the C-terminal region is required for the pVHL and proteasome dependent turnover of PKCζII, however it is the N-terminal PB1 domain of PKCζII that is required for pVHL complex formation. Reciprocal deletion studies define the pVHL effector domain as the dominant PKCζII binding site. The results indicate that pVHL recruits PKCζII via its PB1 domain and causes ubiquitination and degradation via the distal C-terminus of PKCζII.**
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Keywords: PKCζ; pVHL; Ubiquitin; Degradation; PB1 domain

1. Introduction

The turnover of key regulators has become increasingly recognised as an important regulatory device. Thus for example the cyclical behaviour of cyclins during progression through the cell cycle is determined by a complex set of events that determine the moments of their destruction in line with the completion of specific cell-cycle associated tasks, such as chromosome duplication, and segregation (reviewed [1]). Such proteolytic processes are involved not only in sequential cascades typified by the cell cycle, but also in the acute intracellular relay of signals. This is well established for the activation of NFκB where degradation of the inhibitory IκB is required for activation [2–4] and also for the induction of hypoxia inducible factor 1α (Hif1α) which is controlled through protein turnover [5].

The mammalian aPKC family belongs to the large family of AGC serine/threonine kinase and is distinguished from the two other subfamilies of protein kinase C (PKC) by its lack of activation by phorbol esters [6]. There are two full length aPKCs, PKCζ and PKCι, and in addition to these two other isoforms of PKCζ have been identified and have been named PKMζ and PKCζII [7,8]. PKMζ is synthesised following transcription initiation of the PKCζ gene from a brain-specific promoter located in an intron and results in the expression of PKCζ catalytic fragment that is constitutively activate [9]. PKCζII is a rapidly degraded variant that comprises an aPKCζ regulatory domain without an associated kinase domain. PKCζII has been shown to interact with Par6 and to be an endogenous inhibitor of tight junction formation playing a regulatory role in the development of cell polarity [7].

The turnover of PKCζII may be mediated by the ubiquitin-proteasome system; ubiquitination of activated PKCζ in HeLa cells has been reported [10]. Interestingly, the E3 ligase pVHL (Von Hippel-Lindau tumour suppressor gene product) has been shown to support poly-ubiquitination of the related PKCι and is to date the only described ubiquitin-ligase showing specificity for a member of PKC family [11]. pVHL interacts with the regulatory domain of PKCι [12], suggesting that PKCζII may interact with pVHL in the same way. By such a mechanism pVHL may be responsible for controlling PKCζII turnover and thereby would have an important function in regulating cell polarity. To explore this predicted function of pVHL we have tested whether pVHL can regulate PKCζII expression levels. Here we define PKCζII as a target for pVHL dependent degradation.

2. Material and methods

2.1. Antibodies and chemicals

Mouse anti-Hif1α and rabbit anti-GFP antibodies were purchased from BD Transduction Laboratories and BD Clontech (AV peptide polyclonal) respectively. Mouse myc (9E10) and GFP (3E1) antibodies were obtained from the CRUK monoclonal facility. Rabbit anti-Flag antibody was obtained from Sigma Aldrich. Horseradish peroxidase-conjugated antibodies were obtained from Amersham. Cy3-conjugated anti-rabbit and anti-mouse were from Dako. MG132 was obtained from Calbiochem.

2.2. Cell culture and transfections

COS7, 293T and MDCK were maintained in DMEM containing 10% FBS, penicillin/streptomycin. All the cells were cultivated at 37 °C, 5% CO_2. Cells were transfected with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

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Abbreviations: PKC, Protein kinase C; pVHL, Von Hippel-Lindau protein; Hif1α, Hypoxia inducible factor 1α

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2.3. Plasmid constructs

pVHL–GFP constructs, pcDNA3 myc-PKCζII full length (FL 376 amino acid) and GFP–PKCζ A119/E were described previously [7,13]. pcDNA3 Hif1α was provided by Dr. N. Masson and Prof. P. Ratcliffe (IMM, Oxford). The 14.3.3β cDNA was kindly provided by Prof. A. Aitken (Edinburgh). Flag-Par6C constructs were generously provided by Prof. T. Pawson (Toronto). Myc-PKCζII truncated constructs, 294, 269 and 236 were obtained by digesting pcDNA3 Myc-PKCζII with *Eco*RV (294), *Xcm*I (269) and *Cla*I (236) and the Myc-PKCζII PB1 domain construct was made by PCR using the oligonucleotides 5′-CCCAAGCTTATGCCGGAGCAGAAGCTGATATCC-GAGGAGGACCTGGCCATGCCCAGCAGGACGGACCCCA-3′ (*Hin*dIII) and 5′-CCGCTCGAGTCATGGGATGCTTGGGAAAA-CATGAATG-3′ (*Xho*I) as forward and reverse primers respectively. The PCR product was digested with *Hin*dIII–*Xho*I and was ligated into the pcDNA3.1 Hygro (Invitrogen) digested by the same restriction enzymes.

2.4. Co-immunoprecipitation experiments

After 24 h of transfection, COS7 cells were washed once with phosphate buffer saline (PBS), harvested in 500 μl of lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% NP40, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 2 mM Orthovanadate, Proteasome inhibitors Complete Tablet (Amersham)). After 30 min of incubation on ice and centrifugation at 10,000 rpm for 10 min the supernatants were subjected to immunoprecipitation (IP) for 2 h at 4 °C, with rabbit anti-GFP (0.5 μl per IP) and Protein A Sepharose or mouse anti-myc (10 μl per IP). The beads were washed five times with 1 ml of lysis buffer and the final bead pellets were resuspended in 40 μl of 2X sample buffer, boiled and resolved by SDS–PAGE and then proteins detected by Western blotting. Immunoreactivity was analyzed by chemiluminescence using ECL (Amersham).

2.5. Immunofluorescence

COS7 were transfected with YFP–VHL and myc-PKCζII in coverslips. After 24 h of transfection the coverslips were washed once with PBS, fixed for 10 min with 2% PFA in PBS and then washed three times with PBS. The cells were then permeabilised and blocked with 0.1% Triton X100, 1% bovine serum albumin (BSA) in PBS. After 20 min of permeabilisation the coverslips were incubated with a primary antibody mouse anti-myc (1/250 in PBS 1% BSA) for 1 h, washed three times with PBS and then incubated for 45 min with Cy3-conjugated secondary antibodies (1/500). Finally, coverslips were washed three times with PBS and once with distilled water before mounting in slides with

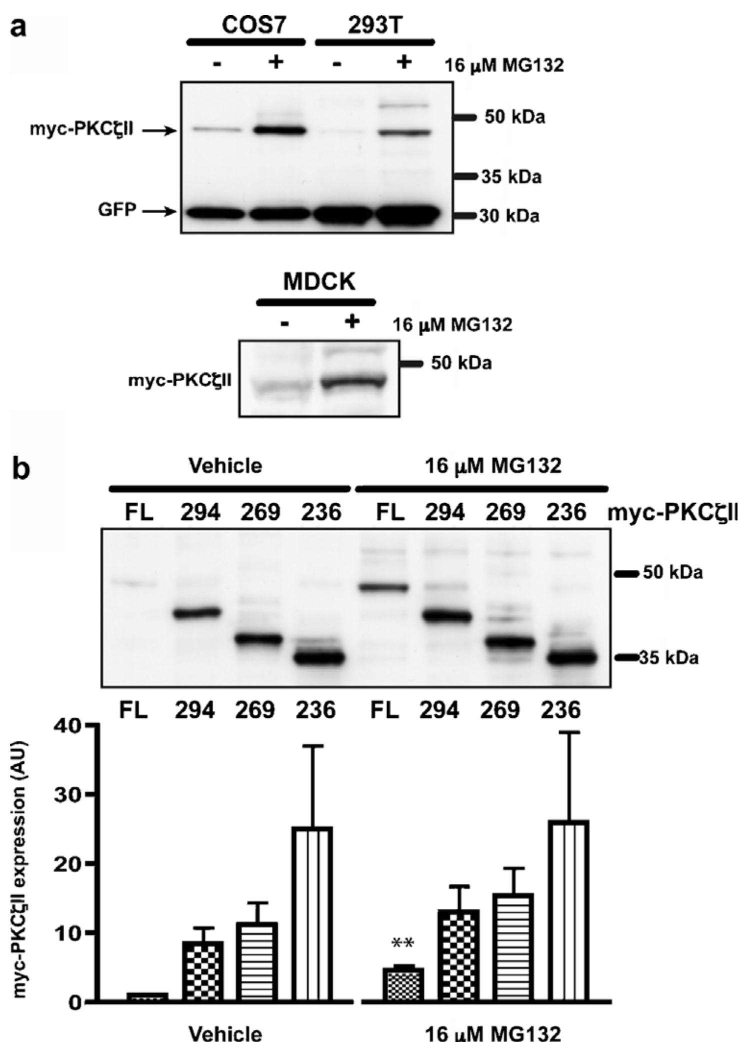

Fig. 1. PKCζII is constitutively degraded by the proteasome. (a) COS7 and 293T were co-transfected with pcDNA3 myc-PKCζII full length (FL) and pEGFP empty vector and MDCK cells were transfected with pcDNA3 myc-PKCζII FL alone. One day after transfection the cells were treated or not with 16 μM MG132 for 6 h. Cell lysates were subjected to Western blot analysis using anti-myc and anti-GFP antibodies. (b) 293T cells were transfected with pcDNA3 myc-PKCζII FL and C-terminus truncated fragments (Δ294, Δ269 and Δ236). The day after transfection the cells were treated or not with 16 μM MG132 for 6 h. Cell lysates were subjected to Western blot analysis using anti-myc antibody. Densitometric analysis of western blots representing myc-PKCζII FL or the truncation mutants (Δ294, Δ269 and Δ236) protein levels are aligned with the experiment shown. This data derives from three independent experiments and shows means and S.D.; ** $P < 0.005$.

Mowiol. Mounted slides were examined using a confocal laser scanning microscope (LSM 510, Carl Zeis Inc.) equipped with Krypton/Argon lasers and with 63×/1.4 Plan-APOCHROMAT oil-immersion objective. Double-labeling images (1024 × 1024 pixels) were analyzed in sequential scanning mode by exciting YFP at 488 nm and Cy3 at 543 nm. Each image corresponds to an equatorial section of the cell.

3. Results and discussion

3.1. PKCζII is constitutively degraded by the proteasome

Previous studies establishing PKCζII as a regulatory device for polarisation indicated that the protein is subject to rapid turnover [7]. To test the possible role of the proteasome, different cell lines (COS7, 293T and MDCK) were transfected with a myc-PKCζII construct and cultures treated with or without the proteasome inhibitor MG132 (Fig. 1a). Interestingly, the treatment of COS7, 293T or MDCK cell lines with MG132 significantly increases the expression of myc-PKCζII. This was not a pleiotropic effect since co-transfection with a control vector expressing GFP was unaffected by MG132 (Fig. 1 upper panel). This result suggests that PKCζII is constitutively degraded by the ubiquitin-proteasome system.

PKCζII deletion mutants were tested to investigate what was required for the engagement of this proteasome sensitive pathway. As illustrated in Fig. 1b and quantified in the lower panel, PKCζII itself is stabilised by MG132 in 293T cells. The 294 mutant displayed modest but not significant stabilisation, while further deletions (269, 236) were intrinsically expressed more efficiently and were also insensitive to MG132. Hence effective engagement with this degradative machinery requires the C-terminal region of PKCζII spanning amino acids 269–376.

3.2. pVHL mediates PKCζII degradation

Typically proteins degraded through the proteasome are targeted by E3 ligases. The related translation product PKCζ has been shown to interact with the E3 ubiquitin ligase pVHL [12]. This has been reported to be involved specifically in the degradation of the T410 phosphorylated aPKC [14], a kinase domain phosphorylation site absent from PKCζII. To assess whether pVHL is involved in PKCζII degradation, WT and mutant forms of pVHL were co-expressed with myc-PKCζII in 293T cells and expression monitored. WTpVHL had no overt effect on PKCζII expression; similarly, the Y98H mutant has no influence. However the C162W and R167W pVHL mutants both stabilised PKCζII to the levels seen in the presence of MG132 (Fig. 2a and quantified in Fig. 2b). These two mutants are not able to bind the Cul2 complex (for review see [15]) and hence have dominant effects on pVHL targeted proteins, since they can interact but fail to induce ubiquitination.

To establish the specificity and function of the pVHL mutant proteins, the endogenous levels of Hif1α were assessed along with a myc-14.3.3β control. As predicted from prior studies, the over-expressed Y98H, C162W and R167W mutants all protect Hif1α leading to its accumulation when compared to the expression of WTpVHL. The myc-14.3.3β control was not influenced by any of the mutant or WT pVHL proteins (Fig. 2c).

In order to compare the effects of pVHL and those of MG132, WT or the R167W mutant of pVHL were co-expressed with PKCζII deletion mutants (Fig. 2d). The R167W mutant increased PKCζII expression 4.5 ± 1.6-fold (compared to 4.6 ± 1.1-fold for MG132; see above). Furthermore, essentially the same pattern of stabilisation is observed for the R167WpVHL mutant with PKCζII deletions. The WTpVHL had no effect. Thus the dominant effect of R167WpVHL phenocopies treatment with MG132, indicating that pVHL is key to engaging PKCζII on the proteasomal and degradative pathway.

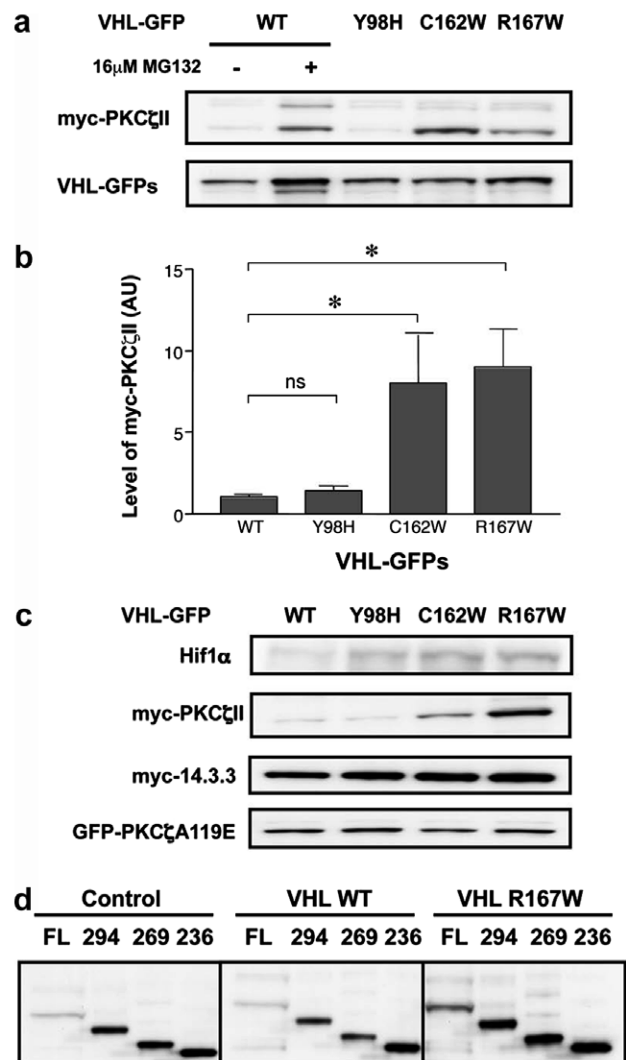

Fig. 2. pVHL mediates PKCζII degradation. (a) 293T cells were co-transfected with pcDNA3 myc-PKCζII FL and pVHL–EGFP constructs (wild type (WT) and mutants Y98H, C162W and R167W). Cell lysates were subjected to Western blot analysis using anti-myc and anti-GFP antibodies. (b) Densitometric analysis of western blots representing myc-PKCζII FL protein levels. Each bar represents three independent experiments. Ns – not significant, $^*P > 0.05$; $P < 0.05$ (Student's *t*-test). (c) 293T cells were co-transfected with pVHL–EGFP constructs and Hif1α or myc-PKCζII FL or myc-14.3.3 or GFP–PKCζA119/E. Cell lysates were subjected to Western blot analysis using anti-Hif1α or anti-myc or anti-GFP antibodies. (d) 293T cells were transfected with pcDNA3 myc-PKCζII FL or C-terminal truncation fragments (294, 269 and 236) with or without pVHL–GFP WT or R167W as indicated. Cell lysates were subjected to Western blot analysis using anti-myc antibody.

3.3. The PB1 domain of PKCζII is required for interaction with pVHL

Although deletion of the C-terminal 93 amino acids of PKCζII is sufficient to stabilise the protein, it is not clear

whether this represents sequences required for E3 ligase interaction or for the subsequent ubiquitination and degradation of PKCζII. This was established by investigating directly the interaction of pVHL and PKCζII. In initial studies the unusual localisation of the YFP-pVHL fusion protein was employed to determine interaction of pVHL and PKCζII in intact cells [13]. On expression of myc-PKCζII the protein typically accumulates in the nucleus as described previously (Fig. 3a; [7]). However on co-expression with YFP-pVHL, PKCζII is recruited to the cytosolic YFP-pVHL positive structures. This is consistent with an interaction of these two proteins in cells. The same co-localisation was observed for the deletion mutants as illustrated for the 294 and 236 mutants (Fig. 3a, lower panels). A direct biochemical assessment of interaction was carried out through co-immunoprecipitation, employing myc-PKCζII and the C-terminal deletion series described above (Fig. 3b). It was established that the WT protein and all three deletion mutants can be recovered in PKCζII immunocomplexes (Fig. 3c).

Further deletion defined the PB1 domain as that interacting with pVHL (Fig. 3d). This domain has been shown to interact with Par6 [16], which serves here as a control for the PB1 domain interaction. Notably, when pVHL is present, the recovery of Par6c in the PKCζII immuno-complex is substantially reduced indicative of mutual exclusivity of these two PB1 domain interacting partners. It has not been possible to formally establish the absence of pVHL in a Par6C–PKCζII complex

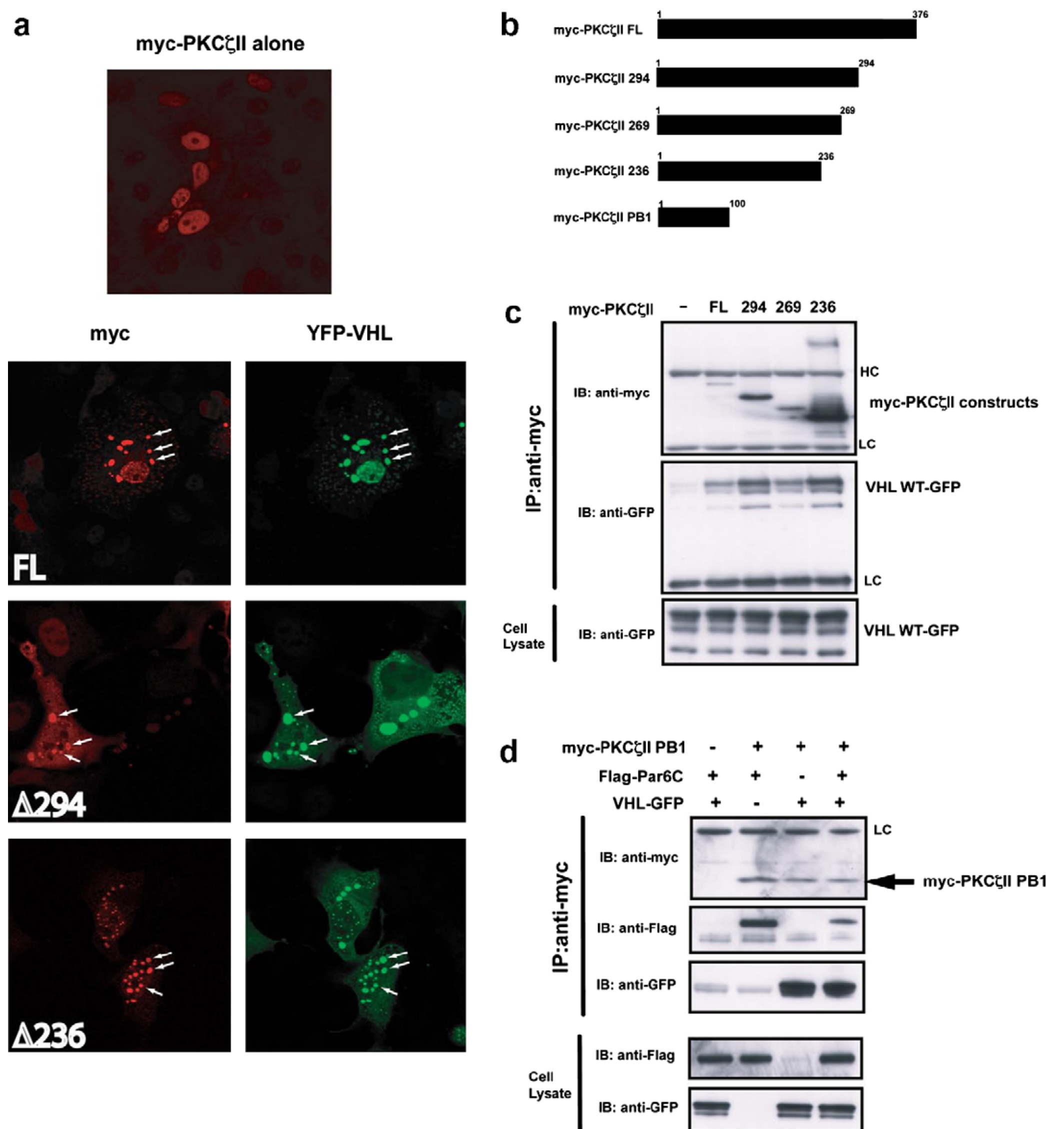

Fig. 3. The PB1 domain of PKCζII is required for interaction with pVHL. (a) COS7 cells were transfected with pcDNA3 myc-PKCζII FL, the myc-PKCζII Δ294 or Δ236 mutants with or without pYFP–VHL WT as indicated. Fixed cells were subjected to immunofluorescence labeling with anti-myc antibody. Localisation of myc-PKCζII protein and mutants is in red and that of YFP-pVHL protein is in green. Colocalisation of the proteins is illustrated by the arrows in each panel. (b) Schematic representations of myc-PKCζII constructs. (c) COS7 cells were transfected with pcDNA3 myc-PKCζII FL or C-terminal truncation fragments (294, 269 and 236) and VHL–GFP. Cell lysates were subjected to immunoprecipitation using an anti-myc antibody and immunoprecipitated materials were analysed by western blot with anti-myc and anti-GFP antibodies. (d) COS7 cells were transfected with myc-PKCζII PB1 and with Flag-Par6C or VHL–GFP. Cell lysates were subjected to immunoprecipitation using an anti-myc antibody and immunoprecipitated materials were analysed by Western blot with anti-myc, anti-Flag and anti-GFP antibodies.

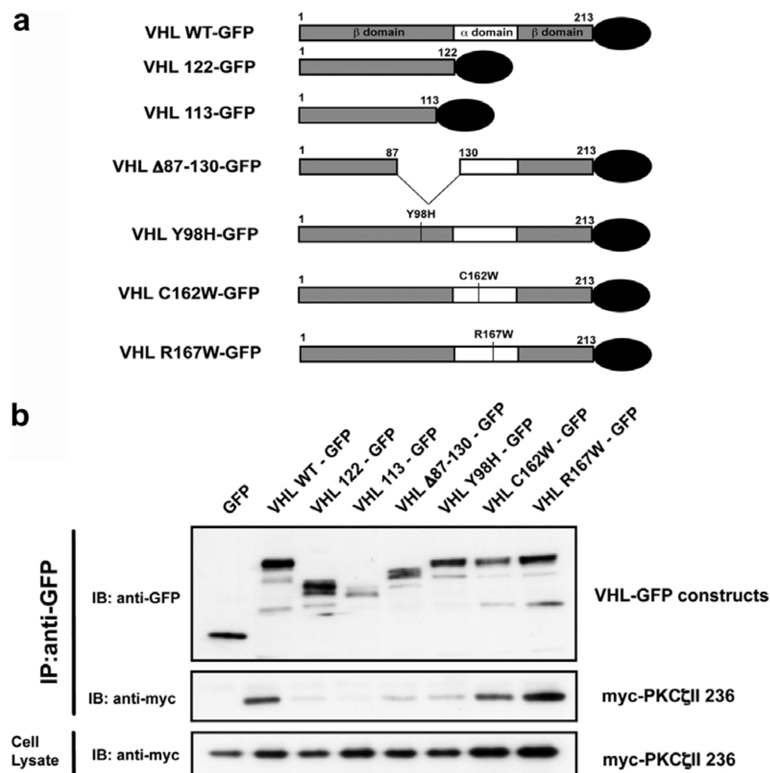

Fig. 4. The N-terminal β-domain of pVHL is required for PKCζII interaction. (a) Schematic representation of pVHL constructs. (b) COS7 cells were transfected with myc-PKCζII 236 and VHL–GFP constructs. Cell lysates were subjected to immunoprecipitation with an anti-GFP antibody and immunoprecipitated materials were analysed by western blot with anti-myc and anti-GFP antibodies.

because Par6C was found to interact with pVHL independently of PKCζII (data not shown).

3.4. *The N-terminal β-domain of pVHL is required for PKCζII interaction*

To define the site(s) of interaction of PKCζII on pVHL, a series of deletions and mutations were analysed, specifically those associated with pVHL subclass mutations (Fig. 4a). Co-immunoprecipitations of these were carried out using the myc-PKCζII 236 construct, which is readily expressed in COS7 cells. Myc-PKCζII 236 was found to interact with WT pVHL as well as the two mutants of the pVHL α domain (C162W and R167W), which stabilised myc-PKCζII as shown above. This result suggests that the stabilisation of PKCζII observed with the co-expression of these two mutants of pVHL in 293T cells is indeed mediated by their dominant-negative effects over endogenous pVHL, consequent to their inability to interact with the VCB–Cul2 complex. Interestingly the mutant Y98H pVHL, which does not stabilise PKCζII, interacts only weakly with myc-PKCζII 236. A similar result is observed with the deletion construct Δ87–130 of pVHL and the truncation mutant of pVHL, 122 (Fig. 4b). Finally, pVHL truncated to 113 does not interact with PKCζII suggesting that the domain of interaction of PKCζII in pVHL is located between residues 87 and 122 of pVHL as previously reported for PKCζ [12].

3.5. *Concluding remarks*

The evidence presented here demonstrates that PKCζII is degraded in part via a proteasome depedent pathway. It is further shown that this pathway involves the E3 ligase pVHL with which PKCζII interacts in cells and in co-immunoprecipitations. The domains involved in the kinase-ligase interaction have been mapped to the PKCζII PB1 domain and the previously defined pVHL effector binding domain [12]. Interestingly, the PB1 domain of PKCζII does not display proteasome sensitivity in cells, a property that in fact maps to the C-terminus of PKCζII. Hence the recruitment and the ability of pVHL to determine PKCζII turnover are situated at distinct ends of the protein.

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