The von Hippel-Lindau Tumor Suppressor Protein Mediates Ubiquitination of Activated Atypical Protein Kinase C*

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The von Hippel-Lindau tumor-suppressor protein (pVHL) forms a protein complex (VCB-Cul2) with elongin C, elongin B, Cul-2, and Rbx1, which functions as a ubiquitin-protein ligase (E3). The α-subunits of the hypoxia-inducible factors have been identified as targets for the VCB-Cul2 ubiquitin ligase. However, a variety of cellular defects caused by the depletion of pVHL cannot be explained solely by the ubiquitin-mediated degradation of hypoxia-inducible factor-α. We show here that a member of the atypical protein kinase C (PKC) group, PKCa, is ubiquitinated by the pVHL-containing E3 enzyme. An active PKCa mutant is ubiquitinated more extensively than wild-type PKCa in HEK293 cells, and the ubiquitination is further enhanced by the overexpression of pVHL. The activation of wild-type PKCa by serum stimulation of cells enhances the ubiquitination of the protein, supporting the notion that active PKCa is preferentially ubiquitinated by VCB-Cul2 ubiquitin ligase. Furthermore, we show that PKCa can be ubiquitinated in vitro in a cell-free ubiquitination assay using purified recombinant components including VCB-Cul2. Given the known function of aPKC in the regulation of cell polarity and cell growth, PKCa may be a target of pVHL in its function as a tumor suppressor.

The von Hippel-Lindau (VHL)1 tumor-suppressor gene is responsible for the inherited VHL cancer syndrome, and mutations of the VHL gene accompanied by loss of heterozygosity are also found in more than 50% of sporadic clear cell renal carcinomas and hemangioblastomas (1). The VHL protein (pVHL) has been shown to associate with elongin B, elongin C, Cullin-2 (Cul2), and Rbx1 to form the VCB-Cul2 complex. This complex is structurally analogous to SCF (Skp-1, Cullin-1, F-box protein) ubiquitin-protein ligase (2, 3) and has been shown to function as a ubiquitin ligase; pVHL is a substrate recognition subunit of the ligase complex as F-box proteins are in SCF (4). Ubiquitination is a multistep process that conjugates ubiquitin moieties to internal lysine residues of proteins. Successive conjugation of ubiquitin molecules generates polyubiquitin chains. Polyubiquitinated proteins are then degraded by the 26 S proteasome. Among the molecules involved in ubiquitin conjugation reactions, the E3 ubiquitin-protein ligases play a pivotal role in substrate recognition (5).

As a consequence of VHL gene inactivation, the expression level of vascular endothelial growth factor is increased and subsequent stimulation of angiogenesis is observed (1). Hypoxia-inducible factor-1 and -2 (HIF-1, -2) are key transcription factors for the induction of vascular endothelial growth factor gene expression. HIFs are composed of α and β subunits. The activity of HIFs are regulated by the oxygen-dependent degradation of the α subunit of HIF-1 and HIF-2 (HIF-α) via the ubiquitin-proteasome pathway (6–10). However, various phenomena observed in VHL-deficient cells, including growth factor-independent cell growth (11), loss of contact inhibition (12), and abnormal organization in cytoskeletal proteins (13), cannot be attributed solely to the accumulation of HIFs. Thus, it is supposed that there are other targets for the VCB-Cul2 ubiquitin ligase. Although fibronectin (14), Sp1 (15), and atypical PKC (aPKC) (16, 17) have been shown to associate with pVHL, it is not clear whether these proteins are substrates of VCB-Cul2 for ubiquitination. Moreover, the physiological significance of these interactions remains to be elucidated.

Atypical PKC comprises two members, PKCa (PKCζ) and PKCc. They are implied in signaling through lipid metabolites, including phosphatidylinositol 3-phosphates (PIP3) (18). A series of studies suggests that aPKC plays an important role in various cellular processes, including proliferation (19), survival (20), and establishment and maintenance of cell polarity (21–26). In mamalian epithelial cells, aPKC forms a complex with ASIP and mPAR-6, mammalian homologues of Caenorhabditis elegans PAR-3 and PAR-6, and localizes at tight junctions, a structural cue of cell polarity (25). Because modifications of cell polarity and cell-cell contact are fundamental steps in the tumorigenesis of epithelial cells, the interaction of pVHL and aPKC suggests the intriguing possibility that aPKC is a target of VCB-Cul2 for ubiquitination. Our previous finding that aPKC directly binds to the β-domain of pVHL, a target recognition site, further supports this possibility (17).

To test whether aPKC can be a substrate of VCB-Cul2 ubiquitin ligase, we monitored the ubiquitination of recombinant...
PKCa in vivo and in vitro. In experiments using transfected HEK293T cells, we show that pVHL mediates the ubiquitination of the activated form of PKCa through an interaction with the regulatory domain of PKCa. By using an in vitro reconstitution system for ubiquitination, we could clearly show that the ubiquitination of PKCa is mediated by pVHL-containing E3.

EXPERIMENTAL PROCEDURES

Plasmids—The expression plasmids were constructed on SRHisB under the SRα promoter. SRHisB-VHLWT (full 1–213), SRHisB-VHL122 (aa 1–122), and SRHisB-VHLΔ37–130 (deletion of an 87–130) encode the indicated human VHL cDNA downstream of the T7-His tag sequence to produce N-terminal tagged proteins. The expression plasmid for N-terminally HA-tagged ubiquitin was constructed on pcDNA3.1 (kindly provided by Dr. K. Tanaka, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). The expression plasmid for N-terminally Myc-tagged Cul2 was also constructed on pcDNA3.1. Expression plasmids SRDwPKCa (full-length PKCa), SRDARD (regulatory domain, aa 1–240, of PKCa), MLKD (kinase domain, aa 198–586, of PKCa), and MLA4RA (active mutant of PKCa, aa 48–586 with mutations of arginines 117, 118, 121, and 122 to substitutions) have been described previously (27).

Cell Culture—Human embryonic kidney cells, HEK293T, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Inc.) containing 10% (v/v) fetal bovine serum (FBS). For the activation of wild-type PKCa, cells were exposed to 100 nM MG132 for 6 h before harvest. After centrifugation, cells were treated with 10 mM fluoride, 2 mM vanadate, and 20 mM NaF. Western Blotting—After incubation on ice, 0.1% cysteine was added and the suspension was incubated on ice for an additional 10 min. After centrifugation at 15,000 g for 20 min, the supernatants were incubated with an antibody (28) and Protein G-Sepharose 4FF (Amersham Pharmacia BioTech) for 1 h at 4°C. After centrifugation, the pellets were washed four times with lysis buffer and twice with final wash buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 10 mM N-ethylmaleimide, 50 mM β-glycerophosphate, 10 μM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 2 mM vanadate, 20 mM NaF, and 10 μg/ml aprotonin. After a 20-min incubation on ice, 0.1% cysteine was added and the suspension was incubated on ice for an additional 10 min. After centrifugation at 15,000 × g for 20 min, the supernatants were incubated with an anti-PKCa antibody (28) and Protein G-Sepharose 4FF (Amersham Pharmacia Biotech) at 4°C for 2 h. The immunocomplexes were washed four times with lysis buffer and twice with final wash buffer containing 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.5% deoxycholate, 50 mM β-glycerophosphate, 10 μM leupeptin, 2 mM phenylmethylsulfon fluoride, 2 mM vanadate, and 20 mM NaF.

Purification of Recombinant Proteins and in Vitro Ubiquitination—UbcH5b, Ubc12, and Nedd8 were expressed and purified from Escherichia coli as described previously (4). Ifection of baculoviruses to Hi Five cells and purification were performed as described (4). Purified or immunoprecipitated PKCa was incubated together with E1, UbcH5b, ATP regeneration system (0.5 mM ATP, 10 mM creatine phosphate, and 5 μg of creatine phosphokinase), Nedd8 conjugation system (300 ng of APP-BP1-Uba3, 200 ng of Ubc12, and 1 μg of Nedd8), and the indicated amount of VCB-Cul2 complex (see Fig. 6 legend) in the presence or absence of 10 μg of ubiquitin at 37°C for 2 h. After stopping the reaction by adding 4× SDS sample buffer, the reaction mixtures were electrophoresed on to 6% SDS-PAGE and detected by anti-PKCa antibody (Transduction Laboratories).

Western Blotting—Cell extracts or immunocomplexes were separated by SDS-PAGE on 12% polyacrylamide gels and transferred electrophoretically to Immobilon-P polyvinylidene difluoride membranes (Millipore). The blotted membranes were soaked in phosphate-buffered saline containing 5% skim milk overnight at 4°C. The membranes were blocked with 5% nonfat dry milk in Tris-buffiled saline containing 0.05% Tween 20 (TBS) for 1 h, and then incubated with the indicated antibody in TBS containing 0.1% bovine serum albumin for 1 h at 37°C. After washing with TBS, the membranes were incubated with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (KPL) and then visualized using an ECL system (Amersham Pharmacia Biotech). Densitometric analysis was performed with Molecular Analyst (Bio-Rad).

The antibodies used were: anti-PKCa (28), anti-PKCα (Transduction Laboratories), anti-HA (Roche Molecular Biochemicals), anti-T7 Tag (Novagen), and anti-human VHL (Pharmingen).

RESULTS

The Activated Form of PKCa Is Preferentially Ubiquitinated in HEK293T Cells—The identification of the VCB-Cul2 complex as an ubiquitin-protein ligase prompted us to examine whether PKCa is ubiquitinated by the pVHL-containing complex. We transiently expressed PKCa in HEK293T cells together with T7 epitope-tagged VHL (T7-VHL) and HA-tagged ubiquitin (HA-Ub) and monitored the conjugation of HA-Ub to immunoprecipitated PKCa. As shown in Fig. 1A, faint smear bands of higher molecular mass were recognized by the anti-HA antibody in the immunoprecipitates from cells transfected with WT PKCa, indicating that WT PKCa is ubiquitinated (lane 3). We have observed previously that pVHL binds to the constitutively active PKCa mutant (PKCa4RA) more tightly than to wild-type PKCa (17). Therefore, we predicted that this mutant would be ubiquitinated more efficiently by the pVHL-dependent ubiquitination system. In fact, we were able to show relatively clear ubiquitination of PKCa4RA in HEK293T cells (Fig. 1A, lane 2).

PKCa interacts directly with pVHL (17), and the interaction depends on the N-terminal β-domain of pVHL, which is predicted to be a binding site for proteins targeted by VCB-Cul2 for ubiquitination (29). Taken together with the results described above, these observations suggest that pVHL recognizes PKCa for ubiquitination in vivo. To prove this point, we first tested the dose dependence of overexpressed pVHL (T7-VHL) on the ubiquitination of PKCa4RA by transfecting different amounts of T7-VHL expression vector (Fig. 1B). As determined by densitometric analysis, the ubiquitination of PKCa4RA was most prominent when 0.1 μg of T7 expression vector was used (Fig. 1B, lanes 4–6). It should be noted that the ubiquitination of PKCa4RA was also observed without the expression of T7-VHL; this may depend on endogenous VHL protein in 293T cells (Fig. 1B, lane 3). To confirm the contribution of VCB-Cul2 to the ubiquitination of PKCa, we employed a dominant-negative mutant of pVHL that is predicted to suppress the interaction of wild-type pVHL with PKCa4RA. For the evaluation of the dominant-negative effect on ubiquitination, we used a C-terminal deleted pVHL (T7-VHL122), which binds to PKCa but cannot form a VCB complex (17). As shown in Fig. 1C, the ubiquitination of PKCa4RA was greatly diminished by co-expression of T7-VHL122, both in the presence and absence of exogenous pVHL.

pVHL Is Involved in the Ubiquitination of Physiologically Activated PKCa—Although we suspected that activated PKCa is preferentially ubiquitinated by the pVHL-containing ligase, the efficient ubiquitination of mutant PKCa by VCB-Cul2 might be because of its abnormal protein structure. To detect the ubiquitination of physiologically activated PKCa, we monitored the ubiquitination of wild-type PKCa before and after the serum stimulation of cells, which induces the activation of PKCa in vivo (30). The amount of ubiquitinated PKCa increased as early as 5 min after the addition of serum and was sustained for at least 120 min (Fig. 2A). The ubiquitinated PKCa decreased to the basal level at 360 min after serum stimulation, very similar to the activation of serum-induced activation of pPKCa in HepG2 cells (30). These observations strongly support the notion that the active form of PKCa is preferentially ubiquitinated.

To test the contribution of VCB-Cul2 to the ubiquitination of activated PKCa, we monitored the effect of T7-VHL122, which acts as a dominant-negative mutant of pVHL, on the ubiquitination level. As shown in Fig. 2B, the ubiquitination of PKCa in serum-stimulated cells was greatly diminished by co-expres-
Degradation of the Ubiquitin-conjugated Active Form of PKCα Is Blocked by a Proteasome Inhibitor in Vivo—The most common physiological significance of ubiquitin conjugation is the marking of proteins to be degraded by the 26 S proteasome (31). To detect the ubiquitin-conjugated PKCα in the experi-

FIG. 1. VHL mediates ubiquitination of the active form PKCα. A, ubiquitination of the constitutively activated and nonactive forms of PKCα. PKCα4RA, or PKCα WT was transiently coexpressed with T7-VHL and HA-Ub in 293T cells. Immunoprecipitates obtained with an anti-PKCα antibody and whole-cell lysates were analyzed by immunoblotting using anti-HA, anti-PKCα, or anti-T7 antibodies. Without activation by serum stimulation, wild-type PKCα was faintly ubiquitinated (lane 3), but the constitutively active PKCα4RA was markedly ubiquitinated (lane 2). B, ubiquitination of constitutively active PKCα was enhanced by VHL expression. Increasing amounts (0.01, 0.05, or 0.1 μg of DNA) of SRHisB-VHLWT were cotransfected into 293T cells together with the PKCα4RA and HA-Ub expression vectors. Immunoprecipitates and cellular extracts were analyzed as in A. Endogenous VHL in 293T cells participates in the ubiquitination activity of VHL nontransfected cells (lane 3), but relative ubiquitination activity was increased in a dose-dependent manner by the expression of VHL (lanes 4–6). C, ubiquitination of the constitutively activated form of PKCα4RA by endogenous or exogenous VHL (lanes 1 and 3) is inhibited in a dominant-negative fashion by the co-expression of T7-VHL122, which lacks the ability to form a VCB complex (lanes 2 and 4). 293T cells were transfected with the indicated expression vectors. 0.1 μg of DNA of the T7-VHL WT expression vector and 15 μg of DNA of the T7-VHL122 expression vector were used. Transfected 293T cells were serum-stimulated as in A for 30 min and then immunoprecipitated and analyzed as in Fig. 1.
The regulatory domain of PKC is essential for ubiquitination of PKCα. We formerly reported that pVHL binds to the regulatory domain of aPKC (17), these results indicate that the formation of the VCB-Cul2 enzymatic complex is definitely required for the ubiquitination of PKCα. PKCα is ubiquitinated in vitro by VCB-Cul2—Results from in vivo studies strongly suggest that VCB-Cul2 ubiquitin ligase can recognize and ubiquitinate PKCα. However, direct evidence that the VCB-Cul2 complex acts as a ubiquitin ligase for PKCα is still missing. Therefore, an in vitro ubiquitination assay for PKCα was established using purified components. All the recombinant components for the ubiquitination of PKCα were either expressed in E. coli or baculovirally expressed in insect cells and purified as shown in Fig. 6A, followed by testing for ubiquitination of PKCα by VCB-Cul2 in vitro. As shown in Fig. 6B, a mobility shift of PKCα was observed in a ubiquitin and VCB-Cul2-dependent manner. That ubiquitin was conjugated to the mobility-shifted PKCα was demonstrated by Western blot analysis using an anti-ubiquitin antibody (data not shown). Because ubiquitination of baculovirally expressed PKCα might be because of the presence of malfolded proteins caused by the overexpression, we next examined whether en-

![Diagram](image_url)
pVHL-mediated Ubiquitination of PKCα

The activity of the atypical types of PKC isozymes, including PKCζ and -λ, is induced by an interaction with PIP₃, a lipid derivative produced by phosphoinositide 3-kinase (32), or by phosphorylation of a threonine residue catalyzed by PDK1 (33). The activated enzymes may be rapidly down-regulated to terminate unnecessary PKC activity in cells. Proteolysis is a common mechanism of down-regulation reported for both conventional and novel PKC isozymes (αPKC and nPKC). The ubiquitination and the degradation of cPKCα and nPKCα by its has been reported to be triggered by the induction of their activation by 12-O-tetradecanoylphorbol-13-acetate treatment of cells (34). However, the ubiquitin ligase which is responsible for the ubiquitination of c/nPKC has not been identified. As for αPKC, this is the first report dealing with the down-regulation of the activated enzyme and the identification of the VCB-Cul2 complex as an active αPKC-specific ubiquitin ligase.

**DISCUSSION**

In this report, we showed that pVHL mediates ubiquitination of PKCα in vitro and in vivo and that the activated form of PKCα is preferentially targeted for ubiquitination. We also showed that both the α-domain and β-domain of pVHL are required for the VCB complex to function as a ubiquitin ligase for PKCα. These results replicate the results of our previous binding study of pVHL and αPKC (17), which showed that αPKC directly associates with the β-domain of pVHL, and that this interaction occurs preferentially with the active form of αPKC. From these results, we propose that the VCB complex is responsible for the down-regulation of activated αPKC via the ubiquitin-proteolytic pathway, which terminates αPKC-mediated cellular signaling.

**Fig. 5.** Two functional domains of VHL are required for PKCα ubiquitination. SRDHisB-VHLWT, VHL122 (defect in the elongin binding region), or VHLΔ387–130 (defect in the αPKC binding region) were transfected with the PKCαWT, Myc-Cul2, and HA-Ub expression vectors into 293T cells, and the transfected cells were serum-stimulated for 30 min with 20% FBS for the activation of wild-type PKCα. Immunoprecipitates were analyzed by immunoblotting with the indicated antibody. Neither deletion mutant of VHL showed ubiquitination of physiologically activated PKCα.

**Fig. 6.** The purified VCB-Cul2 complex ubiquitinates PKCα in vitro. A, all the components used for the in vitro ubiquitination assay were sufficiently purified. The purity of all the proteins for the in vitro ubiquitination assay was assessed by Coomassie Brilliant Blue staining. 500 ng of E1 (lane 1), 500 ng of UbH5B (lane 2), 1 μg of PKCα (lane 4), 400 ng of Ubc12 (lane 6), and 400 ng of Nedd8 (lane 7) presented single bands, and 1.5 μg of the VCB-Cul2-Rbx1 complex (lane 3) presented five bands (for Cul2 (76 kDa), pVHL (30 kDa), elongin C (18 kDa), and Rbx1 (14 kDa)). 400 ng of APP-BP1-Uba3 complex (lane 5) exhibited two bands, for APP-BP1 (65 kDa) and Uba3 (50 kDa). All lanes demonstrated that there was no serious contamination of the proteins. B, the ubiquitination of PKCα is mediated by VCB-Cul2 E3 ubiquitin-protein ligase. Purified PKCα was incubated together with E1, UbH5B, the ATP regeneration system (0.5 mM ATP, 10 mM creatine phosphate, and 5 μg of creatine phosphokinase), the Nedd8 conjugation system (300 ng of APP-BP1-Uba3, 200 ng of Ubc12, and 1 μg of Nedd8), and the indicated amount of VCB-Cul2 complex in the presence (lanes 1–6) or absence (lanes 7–12) of 10 μg of ubiquitin at 37 °C for 2 h. The amount of VCB-Cul2 complex added to the assay mixture was 200 ng (lanes 2 and 8), 400 ng (lanes 3 and 9), 800 ng (lanes 4 and 10), 1600 ng (lanes 5 and 11), and 3200 ng (lanes 6 and 12). No VCB-Cul2 was added to lanes 1 and 7. After stopping the reaction by adding 4X SDS sample buffer, the reaction mixtures were electrophoresed on 6% SDS-PAGE gels and detected by an anti-PKCα antibody (Transduction Laboratories). C, immunoprecipitated endogenous PKCα is ubiquitinated by VCB-Cul2 in vitro. In vitro ubiquitination reactions for immunoprecipitated PKCα from NIH3T3 cells were performed as described under “Experimental Procedures” except for the following. Lane 1 lacks UbcH5b, lane 2 lacks VCB-Cul2-Rbx1, and lane 3 lacks ATP regeneration system (ATP). The reaction mixtures were analyzed as in B. Ubiquitination of PKCα was observed only in the presence of E2, ATP regeneration system, and VCB-Cul2 (lane 4).
Ubiquitination and degradation of proteins often depend on post-translational modifications. Target proteins for the SCF complex-type ubiquitin ligases, such as β-catenin, p27kip1, and IκB, are recognized by F-box proteins only when phosphorylated at defined amino acid residues (35, 36). This molecular mechanism allows phosphorylation-dependent quick degradation of proteins, which plays crucial roles in the regulation of the cell cycle, growth, and differentiation. Degradation of HIF-α, a target of VCB-Cul2 ubiquitin ligase, depends on oxygen, and the degradation is blocked under hypoxic conditions (6, 7, 9). Recent studies revealed that the hydroxylation of a proline residue located in the oxygen-dependent degradation domain is essential for the binding to pVHL (37, 38). Then how does pVHL recognize active PKCζ? Protein kinases of the PKC family consist of a regulatory domain and a kinase domain, and the regulatory domain contains a cysteine-rich lipid binding region and a pseudosubstrate region. In nonactivated PKC, the pseudosubstrate region is predicted to interact with the kinase domain, and this intramolecular interaction may inhibit the kinase activity. It is supposed that the binding of a certain second messenger, such as PIP2, to the regulatory domain may lead to the dissociation of the pseudosubstrate region from the kinase domain and to the interaction with substrate molecules. This process involves a conformational change of the aPKC molecule; during this change, a specific region of regulatory domain that faces the inside of the molecule in the inactive form might be exposed to the outer environment. We showed that the free regulatory domain binds to pVHL in the absence of the kinase domain (17) and is ubiquitinated in vivo (Fig. 4). Neither the interaction of pVHL nor the ubiquitination was observed with kinase domains from which the regulatory domain contains a cysteine-rich lipid binding region and a pseudosubstrate region. When we compared the steady-state level of aPKC between VHL-deficient renal cell carcinoma cells and their derivatives with an introduced ectopic VHL gene, no significant difference was observed (data not shown). This observation is in apparent contradiction to the prediction that pVHL plays an important role in the degradation of aPKC. However, because the inactive form of aPKC, a main component of the cellular aPKC protein, is expected to be resistant to the pVHL-driven degradation pathway, the steady-state protein level of aPKC is predicted to be independent of the protein level of pVHL. The activation may occur in a small fraction of the aPKC pool, and this restricted activation allows the activation of second and third fractions of aPKC soon after the activation and degradation of the first fraction. pVHL may involved in such quick and repeatable on/off control of the aPKC activity, and a disorder of this switching system may result in tumorigenesis of epithelial cells.

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