Identification of the von Hippel–Lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex

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Mutations of von Hippel–Lindau disease (VHL) tumor-suppressor gene product (pVHL) are found in patients with dominant inherited VHL syndrome and in the vast majority of sporadic clear cell renal carcinomas. The function of the pVHL protein has not been clarified. pVHL has been shown to form a complex with elongin B and elongin C (VBC) and with cullin (CUL)-2. In light of the structural analogy of VBC-CUL-2 to SKP1-CUL-1-F-box ubiquitin ligases, the ubiquitin ligase activity of VBC-CUL-2 was examined in this study. We show that VBC-CUL-2 exhibits ubiquitin ligase activity, and we identified UbcH5a, b, and c, but not CDC34, as the ubiquitin-conjugating enzymes of the VBC-CUL-2 ubiquitin ligase. The protein Rbx1/ROC1 enhances ligase activity of VBC-CUL-2 as it does in the SKP1-CUL-1-F-box protein ligase complex. We also found that pVHL associates with two proteins, p100 and p220, which migrate at a similar molecular weight as two major bands in the ubiquitination assay. Furthermore, naturally occurring pVHL missense mutations, including mutants capable of forming a complex with elongin B–elongin C-CUL-2, fail to associate with p100 and p220 and cannot exhibit the E3 ligase activity. These results suggest that pVHL might be the substrate recognition subunit of the VBC-CUL-2 E3 ligase. This is also, to our knowledge, the first example of a human tumor-suppressor protein being directly involved in the ubiquitin conjugation system which leads to the targeted degradation of substrate proteins.

Von Hippel–Lindau disease (VHL) is a dominant inherited syndrome characterized by the predisposition to develop various kinds of benign and malignant tumors, including clear cell renal carcinomas, pheochromocytomas, and hemangioblastomas of the central nervous system and retina (1, 2). VHL is caused by germline mutations of one allele of the VHL tumor-suppressor gene and inactivation or loss of the other wild-type (WT) allele in tumors (1, 2). Inactivation or deletion of both alleles of VHL was found in over 80% of sporadic clear cell renal carcinomas and cerebellar hemangioblastomas (3). The notable characteristic of VHL-negative neoplasms is their hypervascularity (4). It has been shown that hypoxia-inducible mRNAs, including vascular endothelial growth factor (VEGF) mRNA, are deregulated and constitutively expressed in cells lacking VHL tumor-supressor gene product (pVHL) (5–7). Reintroduction of WT pVHL into pVHL-negative cells restores normal regulation of hypoxia-inducible mRNAs (5–7). Evidence suggests that VHL may affect hypoxic regulation by several mechanisms. First, pVHL can destabilize VEGF and other hypoxically regulated mRNAs, suggesting that pVHL may regulate expression of an RNA-binding protein that regulates the fate of hypoxia-inducible mRNAs (5–8). Results of a recent study indicate that pVHL can, directly or indirectly, target hypoxia-inducible transcription factors, including hypoxia-inducible factor (HIF)-1 and HIF-2, for degradation by the proteasome (9).

pVHL, consisting of 213 amino acids, is expressed in all tissues (1, 2). Although the primary sequence of pVHL shares no homology to that of any known proteins, pVHL has been shown to associate with elongin B, elongin C (VBC), and cullin (CUL)-2 in a complex termed VBC-CUL-2 (10–12). Some of the naturally occurring cancer-causing mutations destabilize the interaction of pVHL with elongin B–elongin C (BC)-CUL-2 (13), suggesting the importance of the pVHL- BC-CUL-2 interaction for pVHL function. However, there are also other VHL mutational hotspots that do not disrupt the complex with BC-CUL-2 (1).

The ubiquitin-mediated protein degradation system has been shown to be involved in a wide variety of cellular functions, including cell-cycle progression and signal transduction. Ubiquitination is a multistep process that depends on the activities of several enzymes. First, ubiquitin is activated by ATP to form a high-energy thiol ester intermediate with the E1 ubiquitin-activating enzyme. Activated ubiquitin is then transferred to an E2 ubiquitin-conjugating enzyme from E1. In the presence of an E3 ubiquitin protein ligase, E2 transfers ubiquitin to the specific substrate. In some cases, ubiquitin is first transferred to E3, then to the substrate. Ubiquitin forms an isopeptide bond between its C-terminal glycine and an ε-amino group of a lysine residue of the substrate protein. Polyubiquitin chains are then formed by generating successive isopeptide bonds between the C-terminal glycine and lysine-48 of conjugated ubiquitin molecules. Polyubiquitinated proteins are degraded by the 26S proteasome (14, 15). Among the molecules involved in ubiquitin conjugation reactions, the E3 ubiquitin ligases play pivotal roles in substrate recognition. Thus far, four types of E3s, including E3α, Hect domain proteins, cyclosome- or anaphase-promoting complex, and SKP1-CUL-1-F-box (SCF), have been identified (16). Members of the cullin family of proteins have been shown to be involved in cell-cycle regulation in *Caenorhabditis elegans* and yeast (17). The best characterized cullin, yeast CDC53, an ortholog of mammalian CUL-1, is a subunit of SCF ubiquitin ligases, which target varieties of proteins mainly involved in cell-cycle regulation and gene expression (18, 19). CUL-1 functions as an E3 ligase that targets IkBα for ubiquitination together with SKP1 and the F-box protein β-TrCP (20, 21). In SCF

Abbreviations: VHL, von Hippel-Lindau disease; pVHL, VHL tumor-suppressor gene product; VBC, pVHL–elongin B and C; CUL, cullin; VEGF, vascular endothelial growth factor; SCF, SKP1-CUL-1-F-box protein; SOCS, suppressor of cytokine signaling; WT, wild type; HA, hemagglutinin; HSV, herpes simplex virus; HIF, hypoxia-inducible factor; BC, elongin B–elongin C.

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ubiquitin ligases, CUL-1 recruits CDC34, the E2, Rbx1/ROC1, and SKP1, a protein functioning as a bridge to any of several substrate recognition subunits called F-box proteins. In the case of VBC-CUL-2, CUL-2 binds to pVHL via the bridging complex, elongin BC (22). Notably, CUL-2 is a member of the cullin family, elongin C is a homolog of SKP1, and elongin B is a ubiquitin-like protein. Another common element in this complex is the Rbx1/ROC1 protein, which was originally identified as a pVHL-associated protein (23), a CUL-4A-associated protein (24), and an SCF-associated protein (25), which enhances the ubiquitin ligase activity of SCF ubiquitin ligases (23–26). In light of the strong structural analogy of VBC-CUL-2 to SCF ubiquitin ligases (27, 28), it has been proposed that VBC-CUL-2 may function as an E3 ubiquitin ligase (22, 23, 27, 29).

In the present study, we show that VBC-CUL-2 exhibits ubiquitin ligase activity with E2s of UbcH5 family and that pVHL mutants, including mutants capable of forming a complex with BC-CUL-2, cannot ubiquitinate pVHL-specific proteins. On the basis of our results, we propose that pVHL might be the substrate-binding subunit of a ubiquitin ligase complex, playing a role analogous to F-box proteins in SKP1-CUL-1-F-box protein (SCF) ligases. Mutations in pVHL either cause a disruption of the complex between pVHL and BC-CUL-2 or render pVHL unable to bind the specific proteins to be ubiquitinated. Our results suggest that the pathological consequence of VHL mutations may be caused by the lack of E3 ubiquitin ligase activity toward specific substrates for ubiquitination.

Materials and Methods

Cell Culture. A VHL-defective human renal cell carcinoma cell line, 786–0, transfected with hemagglutinin (HA)-tagged WT pVHL, truncated pVHL (amino acids 1–115), or vector control [ref. 30; kindly provided by W.G. Kaelin (Dana-Faber Cancer Institute, Boston, MA)], and HeLa cells transfected with vector control, Flag-tagged WT-pVHL, or the Y98N, R167Q mutants of pVHL, were cultured in DMEM supplemented with 10% fetal calf serum (GIBCO–BRL), 100 units/ml penicillin G, and 100 μg/ml streptomycin in a 5% CO_2 in air at 37°C. Cells were lysed in 0.2% Nonidet P-40/20 mM Tris·Cl, pH 7.5/150 mM NaCl/10% glycerol/2 mM DTT/1 mM PMSF. Insoluble fractions were removed by centrifugation at 14,000 $\times$ *g* for 20 min at 4°C.

Expression of Recombinant Proteins in *Escherichia coli*. N-terminally RGS(His)$_6$-tagged Ubc3, E2–25K, E2–20K cDNA cloned by reverse transcription–PCR from HeLa cells, UbcH5a, UbcH7 cDNA (provided by M. Scheffner, Deutsche Krebsforschungscentrum, Heidelberg, Germany), and UbcH5b, UbcH5c cDNA (provided by A. M. Weissman, National Cancer Institute, Bethesda, MD) were subcloned into the pT7–7 bacterial expression vector. BL21(DE3) transformed with the appropriate expression plasmids were cultured in 2$\times$ YT medium, 400 μM of isopropyl-D-thiogalactoside was added when A600 was 0.6, and after 2 hr of induction of protein expression, cells were harvested. E2 proteins were purified by Ni-nitrilotriacetic acid agarose affinity chromatography as described (31). pVHL, elongin B, and elongin C expressed in *E. coli* were purified, and VBC complex was generated as described (12). ^{32}P-labeled VBC was generated as follows. VBC was radio-labeled with protein kinase A in the presence of γ-^{32}P-ATP and purified over a Nick column (Amersham Pharmacia Biotech) to remove unincorporated γ-^{32}P-ATP. Glutathione *S*-transferase-ubiquitin was expressed in DH5α cells and affinity purified on glutathione-Sepharose (Amersham Pharmacia Biotech).

Expression of Recombinant Proteins in Insect Cells. N-terminally (His)$_6$-tagged mouse E1 cDNA (kindly provided by F. Yamao, National Institute of Genetics, Mishima, Japan) and N-terminally T7-tagged human Rbx1 cDNA cloned by reverse transcription–PCR from HeLa cells were subcloned into pVL1393 (Invitrogen). N-terminally HA-tagged CUL-2, C-terminally HPC4-tagged elongin B, C-terminally herpes simplex virus (HSV)-tagged elongin C, and C-terminally Flag-tagged WT and mutant pVHLs were subcloned into pBac-PAK8 (CLONTECH). Recombinant baculovirus was generated by using Bac-PAK6 baculovirus expression system (CLONTECH). Hi Five cells were cultured in Grace's insect medium supplemented with 10% fetal calf serum at 27°C and infected with appropriate recombinant virus. Sixty hours after infection, cells were harvested and lysed in 1% Triton X-100/50 mM Tris·Cl, pH 8.0/150 mM NaCl/10% glycerol/2 mM DTT/1 mM PMSF. Insoluble fractions were removed by centrifugation at 14,000 $\times$ *g* for 20 min at 4°C. Mouse E1 proteins were purified by Ni-nitrilotriacetic acid agarose affinity chromatography.

Immunoprecipitation. Lysates from WT-pVHL, truncated pVHL, or vector-transfected 786–0 cells were immunoprecipitated with HA11-conjugated agarose beads (Babco, Richmond, CA). Lysates from WT-pVHL, Y98N, R167Q, or vector-transfected HeLa cells and lysates from Hi Five cells were immunoprecipitated with Flag M2-conjugated agarose beads (IBI). After 2-hr rotation at 4°C, beads were washed with 1 ml of lysis buffer five times followed by two washings with 20 mM Tris·Cl, pH 7.5/2 mM DTT.

***In Vitro* Ubiquitin Conjugation Assay.** ^{32}P-labeled ubiquitin was generated as follows. Glutathione *S*-transferase-ubiquitin was radio-labeled on the glutathione-Sepharose beads by using protein kinase A in the presence of γ-^{32}P ATP. ^{32}P-labeled ubiquitin was then eluted with 20 mM glutathione and cleaved with thrombin at 25°C for 4 hr. Thrombin was inactivated at 75°C for 15 min, and precipitated proteins were removed by centrifugation. Eight microliters of packed and washed antibody-conjugated beads containing WT or mutant pVHL complexes were incubated with 20 mM Tris·Cl, pH 7.5/5 mM MgCl2/2 mM DTT/100 ng of E1/200 ng of E2/0.5 μg of ubiquitin aldehyde/300,000 cpm (0.5 μg) of ^{32}P-labeled ubiquitin in the presence of ATP and ATP regeneration system (0.5 mM ATP/10 mM creatine phosphate/10 μg of creatine phosphokinase) at 37°C for 30 min. The total volume of the reaction mixture was 20 μl. *In vitro* ubiquitination assays were performed in the presence of 5 μg of bovine ubiquitin (Sigma) for CUL-2 or fibronectin instead of ^{32}P-labeled ubiquitin and incubated at 37°C for 2 hr. Reactions were stopped by adding 4$\times$ SDS sample buffer. After being boiled for 10 min, samples were electrophoresed in SDS/PAGE gels, as shown in figure legends.

Western Blot. Immunoprecipitates from Hi Five cells with anti-Flag M2 beads or those from 786–0 cells with anti-HA beads were separated in SDS/PAGE gel as described in the figure legends and transferred to poly(vinylidene difluoride) membrane (Millipore). After being blocked in 5% skim milk–PBS containing 0.1% Tween 20, membranes were incubated with anti-HA (Babco), anti-HSV (Novagen), anti-Flag M2 (IBI) antibodies, anti-CUL-2 antisera, or antifibronectin antisera (Collaborative Research), followed by incubation with horseradish peroxidase-conjugated appropriate second antibody (Amersham Pharmacia Biotech). Membranes were visualized with the Supersignal Western blotting detection system (Pierce), according to the manufacturer's instructions.

Far Western Blot. Immunoprecipitates of WT-pVHL, Y98N, R167Q or vector-transfected HeLa cells with Flag M2-conjugated agarose beads were electrophoresed in 5% SDS/PAGE, followed by transfer to nitrocellulose membrane (Amersham Pharmacia Biotech). Proteins were renatured on the membrane as described previously (32). After being blocked

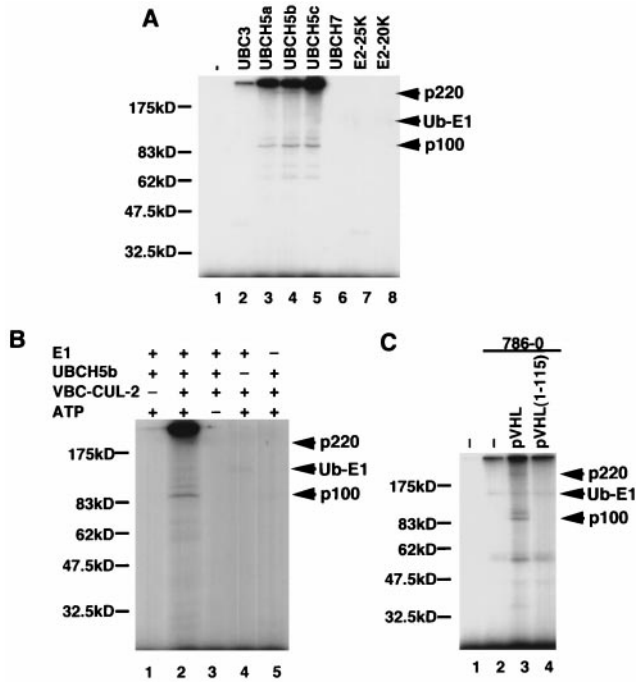

Fig. 1. VBC-CUL-2 exhibits the E3 activity together with E2s of UbcH5 family. (*A*) The VBC-CUL-2 complex immunoprecipitated from HA-tagged WT-pVHL expressing 786–0 cells was incubated with recombinant E1, ^{32}P-labeled ubiquitin, and ATP regeneration system at 37°C for 30 min in the presence of UBC3 (lane 2), UbcH5a (lane 3), UbcH5b (lane 4), UbcH5c (lane 5), UbcH7 (lane 6), E2–25K (lane 7), or E2–20K (lane 8), or in the absence of any E2 (lane 1). (*B*) *In vitro* ubiquitination reactions were performed as described in *Materials and Methods*, except for the followings: lane 1 lacks VBC-CUL-2, lane 3 lacks ATP regeneration system, lane 4 lacks UbcH5b, and lane 5 lacks E1. (*C*) Immunoprecipitates with anti-HA agarose beads from HA-tagged WT-pVHL expressing (lane 3), HA-tagged truncated pVHL (amino acids 1–115) expressing (lane 4), or parent 786–0 cells (lane 2) were subjected to the *in vitro* ubiquitination assay together with anti-HA beads alone (lane 1) in the presence of E1, UbcH5b, ^{32}P-labeled ubiquitin, and ATP regeneration system at 37°C for 30 min. Reactions were stopped by adding 4× sample buffer and electrophoresed in 10% SDS/PAGE, followed by autoradiography.

with 5% nonfat skim milk–PBS containing 0.05% Nonidet P-40, the membrane was incubated with ^{32}P-labeled VBC complex in the blocking solution, followed by autoradiography.

Results

Generation of Higher Molecular Mass Ubiquitin Conjugates in the Presence of VBC-CUL-2 Complex and UbcH5. The structural analogy of the VBC-CUL-2 complex with the SCF complex, which is a known E3 ligase, led us to examine whether the VBC-CUL-2 complex exhibits ubiquitin ligase activity. The VBC-CUL-2 complex was immunoprecipitated from 786–0 VHL-defective human renal carcinoma cells stably transfected with WT-pVHL and incubated with various purified E2 proteins together with purified E1, ^{32}P-labeled ubiquitin, and an ATP regeneration system (Fig. 1*A*). High molecular mass ubiquitin conjugates were clearly observed when the complex was incubated with UbcH5a, b, or c (Fig. 1*A*, lanes 3–5), but not with E2–25K, E2–20K, or UbcH7 (lanes 6–8). Smaller amounts of ubiquitin conjugates of 100 kDa (p100) and 220 kDa (p220) were also seen after incubation with UbcH5a, b, and c (lanes 3–5). High molecular mass ubiquitin conjugates were faintly observed with Ubc3 (CDC34); however, the 100-kDa and 220-kDa conjugates could not be seen with Ubc3 (lane 2). The activities of all the E2s were confirmed by both thiol-ester formation to ubiquitin and ubiquitin conjugation activity to endogenous HeLa proteins (data not shown). These observations suggest that the immunoprecipitate containing VBC-CUL-2 functions as an E3 ubiquitin ligase in the presence of UbcH5s. To confirm the E3 activity, we next examined the requirements for the components of ubiquitin conjugation reactions, which are E1, E2 (UbcH5b), and ATP (Fig. 1*B*). Higher molecular mass ubiquitin conjugates and ubiquitin conjugates of 100 kDa and 220 kDa were observed when the VBC-CUL-2 complex was incubated with all the components (lane 2) and not observed when any of the components were missing (lanes 3–5). In addition, no ubiquitinated signal was observed in the absence of VBC-CUL-2 (lane 1). We next examined the ubiquitin conjugate formation in the presence of immunoprecipitates with anti-HA agarose beads from WT-pVHL-expressing, truncated pVHL (amino acids 1–115)-expressing, or parent 786–0 cells (Fig. 1*C*). The levels of the higher molecular mass ubiquitin conjugates observed in the presence of the immunoprecipitates from mutant pVHL or parent 786–0 cell (lanes 2, 4) were significantly less compared with those observed with immunoprecipitates from WT-pVHL-expressing cells (lane 3). In addition, ubiquitin conjugates of 100 kDa and 220 kDa were observed only with the precipitates from WT-pVHL-expressing cells (lane 3). Collectively, these results strongly suggest that VBC-CUL-2 itself possesses the E3 activity.

Identification of p220 and p100 as VBC-Specific Binding Proteins. We then looked whether we could detect any pVHL-associated proteins of approximate molecular mass of 100 kDa and 220 kDa. However, we did not readily observe proteins with such molecular mass in pVHL-containing immunoprecipitates by silver staining, because there was high background in these areas. We therefore used the Far Western assay to detect pVHL-associated proteins. Immunoprecipitates from cells expressing WT or mutant pVHLs were transferred to a membrane after SDS/PAGE, and the membrane was probed with ^{32}P-labeled VBC complex. Two discrete bands with apparent molecular masses of ≈220 kDa (p220) and ≈100 kDa (p100) were detected in the immunoprecipitate from cells expressing WT-pVHL. The bands were not present after proteinase K treatment of the immunoprecipitates, suggesting they were protein and not nucleic acid (data not shown). When the blot was stained with Ponceau red, we could detect an 80-kDa and a 200-kDa band in the WT-pVHL immunoprecipitates (data not shown). The 80-kDa band was confirmed to be CUL-2, whereas the 200-kDa band was identified to be fibronectin by Western blot (data not shown). Fibronectin has been shown previously to bind to the VBC complex (32). However, neither fibronectin nor CUL-2 was ubiquitinated under our reaction conditions, and they did not react with the VBC probe in the Far Western experiment (data not shown). These results suggest that CUL-2 and fibronectin associate with pVHL under native immunoprecipitation conditions, but their VBC-binding domain does not refold properly on the blot to be detected by the recombinant VBC probe. Therefore, we conclude that the 100-kDa and 220-kDa bands are low abundant VBC-associated proteins that could be identical with the proteins conjugated with ubiquitin by VBC-CUL-2 described above, because they comigrate on an SDS/PAGE gel even considering the conjugation of ^{32}P-ubiquitin (≈9 kDa) (Fig. 1). The p100 and p220 proteins were not detected in immunoprecipitates from cells expressing VHL-R167Q (the most frequent tumor-associated VHL mutation) or VHL-Y98N (the second most frequent tumor-associated VHL mutation) or from control vector-transformed cells (Fig. 2). These observations demonstrate that these cancer-causing VHL mutations abolish binding of VBC to p100 and p220 proteins.

Rbx1/ROC1 Enhances the Ubiquitin Ligase Activity of the VBC-CUL-2 Complex. Rbx1/ROC1, which was previously identified as a pVHL-associated protein (23), enhances the ubiquitin ligase

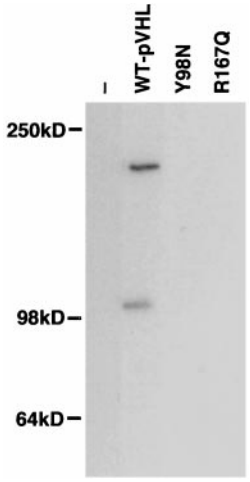

Fig. 2. Identification of P220 and P100 as WT-pVHL specific associated molecules. Immunoprecipitates of HeLa cells expressing Flag-tagged WT, Y98N, R167Q, or vector-transfected HeLa cells with Flag M2 beads were electrophoresed in 5% SDS/PAGE, followed by transfer to nitrocellulose membrane. After proteins were renatured on the membrane, the membrane was incubated with ^{32}P-labeled VBC complex, followed by autoradiography.

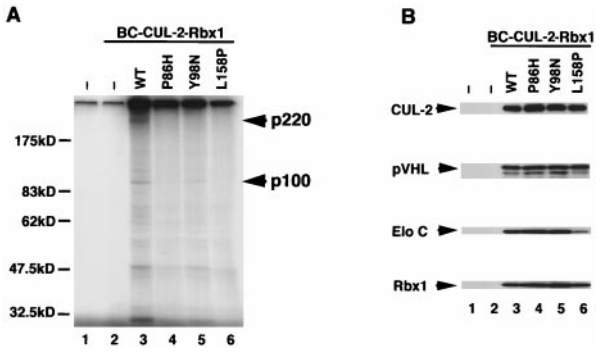

Fig. 4. VHL mutants found in patients failed to ubiquitinate p220 and p100. Hi Five cells infected with recombinant baculovirus encoding CUL-2, elongin B, elongin C, and Rbx1 (lane 2) together with WT-pVHL (lane 3), P86H (lane 4), Y98N (lane 5), or L158P (lane 6) encoding recombinant baculoviruses. Lysates of infected cells or noninfected cells (lane 1) were immunoprecipitated with anti-Flag M2 beads. (*A*) Immunoprecipitates were subjected to *in vitro* ubiquitination assay as described in Fig. 1*C*. Samples were electrophoresed in 9% SDS/PAGE, followed by autoradiography. (*B*) Immunoprecipitates were electrophoresed in 4–20% gradient SDS/PAGE. After being transferred, the membrane was probed with anti-HA, anti-Flag M2, anti-HSV, or anti-T7 antibodies, to detect CUL-2, pVHL, elongin C (Elo C), or Rbx1, respectively.

activity of SCF ubiquitin ligases (23–26). We therefore tested the effect of Rbx1/Roc1 on VBC-CUL-2-dependent ubiquitination. Insect cells were coinfected with baculoviruses encoding CUL-2, elongin B, elongin C, and pVHL in the presence or absence of Rbx1/ROC1-encoding virus, and the VBC-CUL-2 complex was immunoprecipitated. The amounts of pVHL, elongin C, and CUL-2 were almost identical in the immunoprecipitates (Fig. 3*B*). The immunoprecipitates were then used in *in vitro* ubiquitination reaction. Higher molecular mass ubiquitin conjugates were generated in the absence of Rbx1/ROC1. However, the amount of the conjugates increased by the presence of Rbx1/ROC1 in the VBC-CUL-2 complex (Fig. 3*A*), suggesting that Rbx1/ROC1 indeed enhances the ubiquitin ligase activity of VBC-CUL-2 as it does for SCF ligase complexes. This observation further substantiates the similarity between SCF and VBC-CUL-2. In addition to the higher molecular mass ubiquitin conjugates, the ubiquitin conjugates of p220 and p100 were observed when VBC-CUL-2 was subjected to the ubiquitination reactions, and the signal became stronger by the presence of Rbx1/ROC1. The observation of ubiquitin conjugates in the absence of Rbx1/ROC1 seems contradictory to the previous observation that deletion of Rbx1/ROC1 from SCF complex abolishes the ubiquitin ligase activity of the complex (23, 24, 26). We think the difference is likely because of the coprecipitation of endogenous Rbx1/ROC1 homolog from insect cells with VBC-CUL-2 complex, because Rbx1/ROC1 is highly conserved from yeast to human (23). These results suggest that possible homologs of p220 and p100, present in insect cells, might be ubiquitinated by VBC-CUL-2.

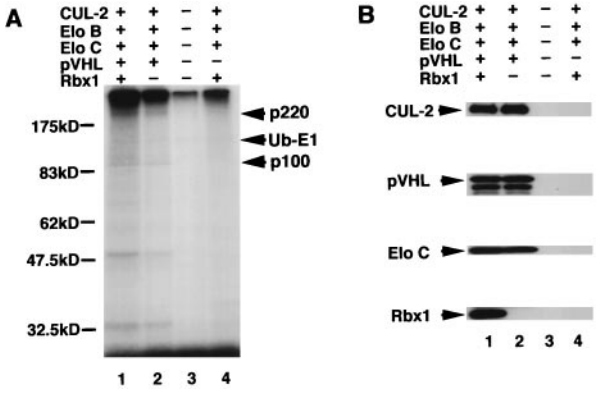

Fig. 3. Rbx1/ROC1 enhances the E3 activity of VBC-CUL-2. Hi Five cells infected with recombinant baculoviruses encoding CUL-2, elongin B (Elo B), elongin C (Elo C) together with either pVHL (lane 2), or Rbx1 (lane 4) or both (Lane1). Lysates of infected cells or noninfected cells (lane 3) were immunoprecipitated with anti-Flag M2 beads. (*A*) Immunoprecipitates were subjected to *in vitro* ubiquitination assay as described in Fig. 1*C*. Samples were electrophoresed in 10% SDS/PAGE, followed by autoradiography. (*B*) Immunoprecipitates were electrophoresed in 4–20% gradient SDS/PAGE. After being transferred, the membrane was probed with anti-HA, anti-Flag M2, anti-HSV, or anti-T7 antibodies, to detect CUL-2, pVHL, elongin C (Elo C), or Rbx1, respectively.

VHL Mutants Found in VHL Patients Result in a Disruption of Ligase Activity. Mutations found in VHL patients or sporadic clear cell renal carcinomas are thought to abrogate the function of pVHL (1, 2). Therefore, it was important to determine whether mutations found in VHL patients or sporadic clear cell renal carcinomas abolish the ubiquitin ligase activity. Insect cells were infected with WT or mutant pVHL (P86H, Y98N, and L158P) expressing recombinant baculovirus together with the viruses encoding elongin B, elongin C, CUL-2, and Rbx1/ROC1. Complexes immunoprecipitated from infected cells were subjected to *in vitro* ubiquitin conjugation reactions. The amounts of WT-pVHL or mutant pVHLs in the immunoprecipitates were nearly identical (Fig. 4*B*). Very little elongin C was immunoprecipitated with the L158P mutant, because this mutation resides in the elongin BC-binding motif and disrupts the VBC-CUL-2 complex like the R167Q hotspot mutation. The ubiquitin ligase activity of mutant VHL-BC-CUL-2 complexes was weak or absent as compared with that of WT VBC-CUL-2. Importantly, the ubiquitin conjugates of 100 kDa were not detected with immunoprecipitates containing L158P or P86H but were weakly detected with Y98N. Strikingly, the conjugates of 220 kDa were not detected at all in any of the pVHL mutants tested and could be detected only in the presence of the WT-pVHL-containing complex. These observations suggest that loss of ubiquitin ligase activity might be linked to the loss of function of pVHL mutants leading to tumorigenesis.

Discussion

Identification of VBC-CUL-2 as an E3 Ubiquitin Ligase Complex. The ubiquitin-mediated protein degradation system has been shown to be involved in many cellular functions. The selection of targets, which is mediated in large part by E3s, is critical for the correct functioning of this system. It has been suggested that hundreds of E3s may exist, whereas only four types of E3 ligases were identified (15). Among them, SCF ubiquitin ligases are of great interest because the presence of 60 F-box proteins in *C. elegans* suggests that SCF complexes form a large family of E3 ligases (34). Moreover, cullins are clearly members of a multigene family and have some homology to the C-terminal half of APC2, a subunit of anaphase-promoting complex/cyclosome. There exist seven cullins in *C. elegans* and at least six cullins in human (17). Cullins share several characteristics. First, cullins are known to be modified by an ubiquitin-like molecule, Nedd-8 (27, 35, 36). Second, cullins bind to Rbx1/ROC1, a ring-finger protein (23, 24). Because CUL-1 is a component of SCF ubiquitin ligases and Rbx1/ROC1 is an essential component of the SCF ubiquitin ligase, it has been suggested that other cullins are also components of E3 ubiquitin ligases (37). However, ubiquitin ligase activity related to cullins (except CUL-1) has not yet been reported. Our observation is, to our knowledge, the first example that cullins other than CUL-1 possess ubiquitin ligase activity together with its binding proteins, suggesting the possibility that each cullin may function as a component of E3 ubiquitin ligases.

Both elongin A and pVHL have been shown to bind to elongin BC via the so-called BC-box (38), although CUL-2 cannot bind to the elongin ABC complex (38). Suppressor of cytokine signaling (SOCS) proteins, originally identified as suppressors of cytokine signaling (39–41), have been shown to bind elongin BC because they contain a BC-box within their SOCS box motif (42, 43). A database search revealed that around 20 kinds of proteins containing ankyrin repeats, WD-40 repeats, or SPRY domain have been identified as SOCS proteins (43). The WD-40 repeats, ankyrin repeats, or SPRY domains are known to be involved in protein–protein interaction. Indeed, some F-box proteins such as β-TrCP or CDC4, which are known to be substrate recognition subunits of SCF, are known to have WD-40 repeats for their substrate-binding domain (19–21). Therefore, such complexes may represent other E3 ligase families containing cullins, with VBC-CUL-2 being the prototype. In a recent report, the structure of the VBC complex was solved and revealed that the interaction between pVHL and elongin C is very similar to that between F-box proteins and SKP1 in the SCF complex (28). The F-box shows structural similarity to the BC box in pVHL, and elongin C is a homolog of SKP1. These results suggest that the overall architecture of the VBC-CUL-2 complex and the SCF complex is conserved, suggesting that pVHL might function as a substrate recognition subunit like the F-box proteins do in SCF. The VBC-CUL-2-associated proteins p100 and p220 do not bind to the Y98N mutant, which does not affect binding to the BC complex (12). Thus, it is unlikely that p100 and p220 bind to elongin BC or other pVHL-associated proteins, because they are recognized by the VBC complex directly by Far Western blotting. We conclude that it is most likely that pVHL itself is responsible for binding to p100 and p220, which comigrate with the 100-kDa and 220-kDa ubiquitin conjugates. Therefore, it is possible that p100 and p220 are identical with the major ubiquitinated bands at 100 and 220 kDa and are substrates of the VBC-CUL-2 ligase activity. Another possibility is that p100 and p220 are VBC-associated proteins that may play different roles in the VBC-CUL-2 complex. We favor the first possibility and therefore tentatively consider pVHL to be the receptor subunit of the VBC-CUL-2 ubiquitin ligase that determines the substrate specificity of the ubiquitin ligase complex.

Mutation of pVHL and Ubiquitin Ligase Activity. It has been shown that the ubiquitin system is involved in tumorigenesis in some settings. The oncogenic human papilloma E6 protein degrades p53 in conjunction with E6-AP E3 ligase (45). Failure of the ubiquitin system to degrade β-catenin leads the cells to oncogenic transformation (46). However, it has not been reported that mutations of a receptor subunit of E3 ligase lead to tumorigenesis. In tumors of VHL patients or sporadic clear cell renal carcinomas, nonsense and missense mutations of pVHL are observed equally. Some missense mutations found in the BC box disrupt BC-CUL-2 binding, leading to the dissociation of the ligase complex. However, there are also other VHL missense mutations such as P86H, a mutational hotspot, which does not disrupt the complex with BC-CUL-2, suggesting that complex disruption is not the sole cause of loss of tumor-suppressor function of VHL (1). In the present study, we showed that VBC-CUL-2 exhibits ubiquitin ligase activity and that p220 and p100 are specific associated proteins of VBC-CUL-2 that migrate at a similar molecular mass to the major ubiquitinated bands in the ubiquitination assay. Although weak ubiquitination of the 100-kDa conjugate was observed in some mutant pVHLs, the 220-kDa conjugate was not observed in any pVHL mutants tested. We suspect that some naturally occurring mutations cannot abolish the ubiquitin ligase activity of VBC-CUL-2 completely. This may partly explain the phenotypical diversity of VHL syndrome. The Y98 mutation has been associated with a disease phenotype of pheochromocytoma without renal cell carcinoma, whereas the L158 or R167 mutations are associated with pheochromocytoma with renal cell carcinoma (1). Therefore, the expression of specific pVHL mutations might be linked to the specific alterations in ubiquitination of certain VBC-CUL-2 substrates.

In a recent report, the crystal structure of the VBC complex was solved and showed the existence of a potential binding site in pVHL that was exposed to the solvent, termed the β-domain. It was proposed that this region, encompassing exon 1 (amino acids 1–113), might be involved in binding to other proteins, whereas a more C-terminal region between amino acids 155–189, termed the α-domain, is responsible for binding to elongin BC (28). Most missense mutations that do not affect the binding to the BC-CUL-2 complex, resulting in a stable VBC-CUL-2 complex, are present in the β-domain. It was an open question which function of pVHL is disrupted by these mutations. The data in this study suggest that this region might be involved in the association with specific proteins for ubiquitination, and that point mutations in this region disrupt this interaction but do not affect the pVHL-elongin BC interaction.

One notable characteristic of pVHL-negative neoplasms is the accumulation of hypoxia-inducible mRNAs (5–8). Recently, the stability of HIF-1 and HIF-2, which enhance the transcription of hypoxia-inducible mRNAs, has been shown to be controlled by pVHL (9). It has been hypothesized that the stability of an RNA-binding protein, which can bind to 3′ untranslated region of hypoxia-inducible mRNAs, a region critical for the stability of the mRNAs, is controlled by pVHL (8). Thus, HIF-1, HIF-2, and an unknown RNA-binding protein might be the pVHL-specific substrates for ubiquitination. Therefore, the isolation of pVHL-specific substrates for ubiquitination, possibly p100 and p220 shown here, will be needed to further characterize the VHL tumor-suppressor pathway.

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