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A Molecular Basis for Stabilization of the von Hippel-Lindau (VHL) Tumor Suppressor Protein by Components of the VHL Ubiquitin Ligase*

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The multiprotein von Hippel-Lindau (VHL) tumor suppressor (CBC^{VHL}, Cul2-Elongin BC-VHL) and SCF (Skp1-Cul1/Cdc53-F-box protein) complexes are members of structurally related families of E3 ubiquitin ligases that use a heterodimeric module composed of a member of the Cullin protein family and the RING finger protein Rbx1 (ROC1/Hrt1) to activate ubiquitylation of target proteins by the E2 ubiquitin-conjugating enzymes Ubc5 and Cdc34. VHL and F-box proteins function as the substrate recruitment subunits of CBC^{VHL} and SCF complexes, respectively. In cells, many F-box proteins are short lived and are proposed to be ubiquitylated by an autocatalytic mechanism and destroyed by the proteasome following assembly into SCF complexes. In contrast, the VHL protein is stabilized by interaction with the Elongin B and C subunits of CBC^{VHL} in cells. In this report, we have presented direct biochemical evidence that unlike the F-box protein Cdc4, which is ubiquitylated *in vitro* by Cdc34 in the context of the SCF, the VHL protein is protected from Ubc5-catalyzed ubiquitylation following assembly into the CBC^{VHL} complex. CBC^{VHL} is continuously required for negative regulation of hypoxia-inducible transcription factors in normoxic cells and of SCF complexes, many of which function only transiently during the cell cycle or in response to cellular signals. Our findings provide a molecular basis for the different modes of cellular regulation of VHL and F-box proteins and are consistent with the known roles of CBC^{VHL}.

Ubiquitylation and the subsequent proteasomal degradation of regulatory proteins control a large number of cellular processes, including cell cycle progression, transcription, and signal transduction. Ubiquitin-dependent protein degradation is an elaborate, multistage process that begins with enzymatic tagging of target proteins with a polyubiquitin chain and culminates with ubiquitin-dependent degradation of tagged proteins by the 26 S proteasome (1–4). In the first stage, the C terminus of ubiquitin is covalently bound through a thioester bond to the active site cysteine residue of an E1 ubiquitin-activating enzyme. Ubiquitin is then transferred from the E1 to an active site cysteine residue in one of a number of E2 ubiquitin-conjugating enzymes. Finally, in a reaction mediated by an E3 ubiquitin ligase, ubiquitin is conjugated directly via isopeptide bonds to ε-amino groups of lysines in the target protein and then to lysines in their ubiquityl moieties to complete synthesis of the polyubiquitin tag.

The E3 components of the ubiquitin pathway are responsible for recognizing and recruiting target proteins for polyubiquitylation. The E3 fall into two functional classes (3, 5). One class includes the homologous to E6-AP carboxyl terminus (HECT) domain proteins, which have an active site cysteine residue that receives ubiquitin from an E2 ubiquitin-conjugating enzyme and transfers it to target proteins. The other class includes the E3 that appear to activate ubiquitylation of target proteins at least in part by binding to both E2 ubiquitin-conjugating enzymes and target proteins and bringing them into close proximity. Among members of this class of E3s are the structurally related multiprotein SCF (Skp1-Cul1/Cdc53-F-box protein) and von Hippel-Lindau (VHL)[1] tumor suppressor (CBC^{VHL} or Cul2-Elongin BC-VHL) complexes, which use heterodimeric modules composed of a member of the Cullin protein family and the RING finger protein Rbx1 (also referred to as ROC1 or Hrt1) to activate ubiquitylation of target proteins by the E2 ubiquitin-conjugating enzymes Cdc34 and Ubc5 (6, 7).

SCF ubiquitin ligases include a member of the F-box family of proteins, which serve to recognize and recruit target proteins. F-box proteins are linked to a Cul1(Cdc53)/Rbx1 module by the Skp1 adaptor protein that binds to a degenerate, ~40-amino acid sequence motif called the F-box, which is present in F-box proteins (8, 9). As a component of the CBC^{VHL} ubiquitin ligase, the VHL tumor suppressor protein functions analogously to F-box proteins in the SCF complex to recruit target proteins for ubiquitylation (10–14). The VHL protein is linked to a Cul2/Rbx1 module by the ubiquitin-like Elongin B and Skp1-like Elongin C adaptor proteins. Elongins B and C form a stable subcomplex that binds to a short BC-box motif present in the VHL protein (15–17).

F-box proteins, including mammalian Skp2 and *Saccharomyces cerevisiae* Cdc4, Grr1, and Met30, have recently been shown to be short lived proteins that are rapidly turned over with ubiquitin-dependent degradation by the proteasome when assembled into SCF complexes in cells (18–21). Based on these

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[1] The abbreviations used are: VHL, von Hippel-Lindau; GST, glutathione *S*-transferase; DTT, dithiothreitol; WT, wild type; HPLC, high pressure liquid chromatography; CBC, Cul2-Elongin BC; SCF, Skp1-Cul1/Cdc53-F-box.

findings, it has been proposed that these F-box proteins are ubiquitylated by an autocatalytic mechanism in the context of the SCF and, further, that rapid turnover of F-box proteins provides a mechanism for rapid assembly and disassembly of distinct SCF complexes required for timely responses of the cell to different cell cycle or environmental cues.

In contrast to these F-box proteins, the VHL tumor suppressor protein is long lived in cells. In addition, whereas F-box proteins are stabilized in cells by mutations that prevent them from binding Skp1 and assembling into SCF complexes, VHL is destabilized by mutations that interfere with its ability to bind Elongins C and B and assemble into CBC^{VHL} (22). Notably, the hypoxia-inducible transcription factors HIF1α and HIF2α were recently shown to be targets of the CBC^{VHL} ubiquitin ligase (10–14). HIF1α and HIF2α are continuously synthesized in cells grown under normoxic conditions but are rapidly ubiquitylated by the CBC^{VHL} complex and degraded by the proteasome. Under hypoxic conditions (23–25) or in cells lacking a functional *VHL* gene (10, 26–28), HIF1α and HIF2α are stabilized and accumulate and activate expression of their target genes. Based on these findings, the longevity of the VHL protein in cells is likely to ensure that it can maintain close and continuous surveillance of hypoxia-inducible transcription factors, which may be activated at any time during the life of a cell.

In this report, we have investigated the molecular basis of the different modes of regulation of VHL and F-box proteins. We have demonstrated that unlike the F-box protein Cdc4, which is ubiquitylated *in vitro* by Cdc34 in the context of the SCF, the VHL protein is protected from Ubc5-catalyzed ubiquitylation in the context of CBC^{VHL} even though the RING finger protein Rbx1 is capable of directly targeting VHL for ubiquitylation when it is not assembled into the CBC^{VHL} complex. Our findings provide a plausible model to explain the differential stabilities of VHL and F-box proteins, and they suggest that the longevity of the VHL tumor suppressor protein is regulated at least in part by the geometry of the CBC^{VHL} complex through the spatial organization of its surface lysine residues.

EXPERIMENTAL PROCEDURES

Materials—Anti-VHL monoclonal antibody (Ig32) was purchased from BD PharMingen. Anti-Cul2 and anti-Elongin C monoclonal antibodies were obtained from Transduction Laboratories. Anti-Myc monoclonal antibody (9E10) was from Roche Molecular Biochemicals. Anti-protein C monoclonal antibody (HPC4) was a generous gift from C. T. Esmon (Oklahoma Medical Research Foundation). Anti-T7 monoclonal antibody was purchased from Invitrogen. Anti-HSV monoclonal antibody was obtained from Novagen. Anti-Elongin B rabbit polyclonal antibody has been described (29).

Expression of Recombinant Proteins in Escherichia coli—Human Ubc5a, human Ubc3 containing an N-terminal His_6 tag, *S. cerevisiae* Uba1 containing an N-terminal Myc tag and a C-terminal His_6, *S. cerevisiae* Cdc34 containing an N-terminal His_6, and mouse ubiquitin-K48R containing an N-terminal GST tag ($GST{\cdot}Ub^{K48R}$) were described previously (13, 30, 31). Proteins were expressed in *E. coli* strain BL21 (DE3) and purified by Ni^{2+}-agarose or glutathione-Sepharose affinity chromatography. After dialysis against 40 mM Hepes-NaOH (pH 7.9), 60 mM potassium acetate, 2 mM DTT, 1 mM $MgCl_2$, 0.5 mM EDTA (pH 7.9), and 10% (v/v) glycerol, proteins were stored at −80 °C.

Expression of Recombinant Proteins in Sf21 Insect Cells—Wild type human VHL and VHL mutants [K159R/K167R/K196R]VHL, [L158P]VHL, and [C162F]VHL; wild type human VHL and VHL mutant [L158P]VHL containing an N-terminal His_6 tag ($His{\cdot}VHL^{WT}$ and $His{\cdot}VHL^{L158P}$); human Cul2; human Elongin B; human Elongin B containing an N-terminal His_6 tag and a C-terminal HPC4 tag ($His{\cdot}HPC4^{EloB}$); mouse wild type Rbx1 and Rbx1 mutant [C94S]Rbx1 containing an N-terminal Myc tag ($Myc{\cdot}Rbx1^{WT}$ and $Myc{\cdot}Rbx1^{C94S}$); *S. cerevisiae* Rbx1 containing N-terminal His_6 and Myc tags (His-Myc·scRbx1); *S. cerevisiae* Cdc4 containing N-terminal His_6 and HSV tags (His-HSV·Cdc4); $Cdc4^{VHL}$ chimeric protein containing N-terminal His_6 and FLAG tags; and human Elongin C were subcloned into pBacPAK8. Recombinant baculoviruses were generated with the BacPAK baculovirus expression system (CLONTECH). The baculoviruses encoding mouse Rbx1 containing N-terminal His_6 and Myc tags, (His-Myc·Rbx1) (32), *S. cerevisiae* Cdc53 (33), and *S. cerevisiae* Skp1 containing three N-terminal FLAG tags (FLAG-Skp1) (34), have been described previously. Sf21 cells were cultured in Sf-900 II serum-free medium with 5% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 27 °C. Sf21 cells were coinfected with the recombinant baculoviruses indicated in the figures. Sixty hours after infection, cells were collected and lysed in ice-cold buffer containing 40 mM Hepes-NaOH (pH 7.9), 150 mM NaCl, 1 mM DTT, 0.5% (v/v) Triton X-100, 10% (v/v) glycerol, 5 μg/ml leupeptin, 5 μg/ml antipain, 5 μg/ml pepstatin A, and 5 μg/ml aprotinin. In some experiments, cells were resuspended in ice-cold buffer containing 40 mM Hepes-NaOH (pH 7.9), 150 mM NaCl, 20 mM imidazole (pH 7.9), 5 μg/ml leupeptin, 5 μg/ml antipain, 5 μg/ml pepstatin A, and 5 μg/ml aprotinin and lysed by French press (1 inch piston, 16,000 psi cell pressure; American Instrument Company).

Purification of Recombinant CBC and SCF Complexes from Sf21 Cell Lysates—Sf21 cells were coinfected with the baculoviruses indicated in the figure legends. Cells were harvested and lysed using a French press as described above. Following centrifugation at 10,000 × *g* for 20 min at 4 °C, the resulting supernatant was mixed with 1 ml of Ni^{2+}-agarose pre-equilibrated in buffer containing 40 mM Hepes-NaOH (pH 7.9), 150 mM NaCl, and 20 mM imidazole (pH 7.9). After 2 h, the Ni^{2+}-agarose was washed three times with the same buffer and packed into a 0.8-cm diameter column. The column was eluted stepwise with buffer containing 40 mM Hepes-NaOH (pH 7.9), 50 mM NaCl, 300 mM imidazole (pH 7.9), and 10% (v/v) glycerol. Peak fractions containing the recombinant proteins were diluted with 40 mM Tris-HCl (pH 7.9), 10% (v/v) glycerol, 1 mM DTT, and 0.5 mM EDTA and brought to a conductivity equivalent to that of the same buffer containing 40 mM KCl. Following centrifugation at 10,000 × *g* for 20 min at 4 °C, the resulting supernatant was applied to a TSK DEAE-NPR HPLC column (4.6 × 35 mM; Toso-Haas) pre-equilibrated in buffer containing 40 mM Tris-HCl (pH 7.9), 40 mM KCl, 10% (v/v) glycerol, 1 mM DTT, and 0.5 mM EDTA. The column was eluted at 0.2 ml/min with a 5-ml linear gradient from 40 to 550 mM KCl, and 0.2-ml fractions were collected.

Immunoprecipitations and Western Blotting—Sf21 and 293T cells were harvested and lysed as described above. Cell lysates were incubated for 2 h at 4 °C with protein A-Sepharose and the antibodies indicated in the figure legends. Protein A-Sepharose was washed three times in buffer containing 40 mM Hepes-NaOH (pH 7.9), 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA (pH 7.9), and 0.5% (v/v) Triton X-100. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to Hybond P membranes (Amersham Biosciences), and visualized by Western blotting with Supersignal West Pico chemiluminescent reagent (Pierce).

In Vitro Ubiquitylation Assay—To assay immunoprecipitated CBC^{VHL} and SCF^{Cdc4} complexes for their abilities to ubiquitylate the VHL protein, Sf21 cells coinfected with the baculoviruses indicated in the figures were lysed with ice-cold buffer containing 40 mM Hepes-NaOH (pH 7.9), 150 mM NaCl, 1 mM DTT, 0.5% (v/v) Triton X-100, 10% (v/v) glycerol, 5 μg/ml leupeptin, 5 μg/ml antipain, 5 μg/ml pepstatin A, and 5 μg/ml aprotinin. After centrifugation at 10,000 × *g* for 20 min at 4 °C, the supernatants were immunoprecipitated with 2 μg of anti-VHL (Ig32), anti-FLAG, or anti-HPC4 antibodies and 10 μl of protein A-Sepharose. The beads were mixed with ~50 ng of Uba1, ~3 μg of $GST{\cdot}Ub^{K48R}$, and either ~100 ng of hUbc5a or ~200 ng of ScCdc34 in a 20-μl reaction containing 40 mM Hepes-NaOH (pH 7.9), 60 mM potassium acetate, 2 mM DTT, 5 mM $MgCl_2$, 0.5 mM EDTA (pH 7.9), 10% (v/v) glycerol, and 1.5 mM ATP. Reaction mixtures were incubated for 1 h at 26 °C.

To assay the abilities of purified recombinant CBC and SCF complexes to ubiquitylate VHL, $Cdc4^{VHL}$, and Cdc4 proteins, aliquots of TSK DEAE-NPR HPLC column fractions indicated in the figures were mixed with ~50 ng of Uba1, ~3 μg of $GST{\cdot}Ub^{K48R}$, and either ~100 ng of hUbc5a or ~200 ng of ScCdc34 in a 10-μl reaction containing 40 mM Hepes-NaOH (pH 7.9), 60 mM potassium acetate, 2 mM DTT, 5 mM $MgCl_2$, 0.5 mM EDTA (pH 7.9), 10% (v/v) glycerol, and 1.5 mM ATP. Reaction mixtures were incubated for 1 h at 26 °C.

RESULTS AND DISCUSSION

The VHL Protein Is Protected from Ubiquitylation in the Context of the CBC^{VHL} Complex—The observation that VHL is more stable in cells when it is associated with Elongins B and

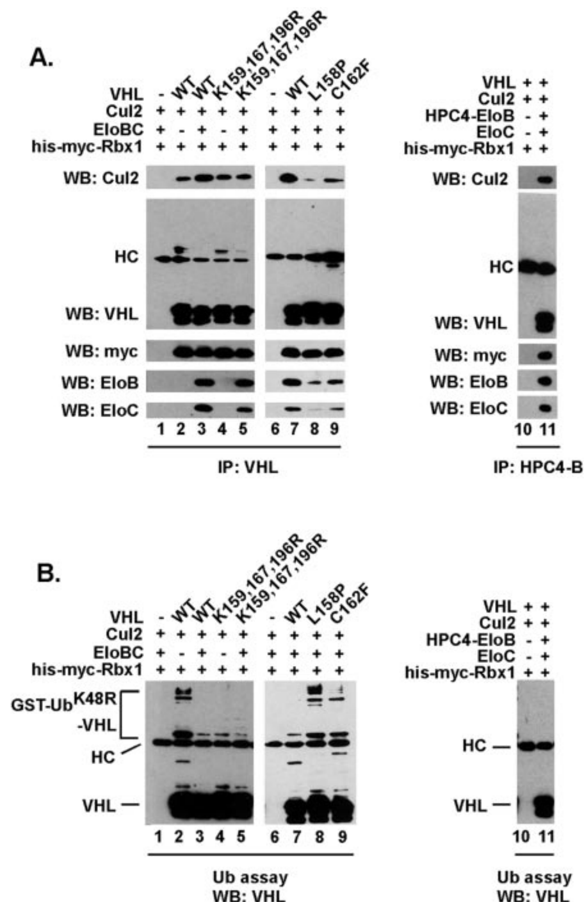

FIG. 1. **The VHL protein is protected from ubiquitylation in the context of the CBC^{VHL} complex.** *A*, Sf21 cells were infected with baculoviruses encoding the indicated proteins. Anti-VHL (Ig32, *lanes 1–9*) or anti-HPC4 (*lanes 10* and *11*) immunoprecipitates were subjected to 8 or 13% SDS-PAGE followed by Western blotting as described under "Experimental Procedures." The antibodies used to probe immunoblots are indicated to the *left* of the gel images. *B*, complexes immunoprecipitated with anti-VHL (Ig32, *lanes 1–9*) or antibodies against the HPC4 epitope tag on Elongin B (*lanes 10* and *11*) were assayed for their abilities to activate VHL ubiquitylation as described under "Experimental Procedures" (*lower panels*). *HC*, immunoglobulin heavy chain; *IP*, immunoprecipitate; *WB*, Western blot; *Ub* assay, ubiquitylation assay.

C (22) raised the possibility that the VHL protein might be protected from ubiquitylation and subsequent degradation by assembly into the CBC^{VHL} complex. To address this possibility directly, Sf21 insect cells were coinfected with various combinations of baculoviruses encoding Cul2, Rbx1, Elongins B and C, and either wild type VHL, VHL mutant [K159R,K171R,K196R]VHL, which has no lysines and therefore cannot be ubiquitylated, or VHL mutants [L158P]VHL and [C162F]VHL, which exhibit substantially reduced affinities for Elongins B and C. We note that although Elongin C is capable of interacting with VHL in the absence of Elongin B, Elongins B and C were always coexpressed together in these experiments for the following reasons. (i) The binding of Elongin C to VHL is stabilized by Elongin B both *in vitro* and in cells (16, 35). (ii) Elongin C is stabilized and expressed to considerably higher levels in insect cells that also express Elongin B (data not shown). (iii) The detectable Elongin C is always found in association with Elongin B during biochemical purification of Elongin C-containing complexes (Ref. 36 and data not shown).

VHL-containing complexes were immunoprecipitated with an anti-VHL monoclonal antibody and tested for their susceptibility to VHL ubiquitylation. In these experiments, immunopurified complexes (Fig. 1*A*, *lanes 1–9*) were incubated with an E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme Ubc5a, ubiquitin (GST·Ub^{K48R}), and ATP. Reaction products were analzyed by Western blotting with anti-VHL antibody. As shown in Fig. 1*B*, maximal formation of the more slowly migrating VHL-GST·Ub^{K48R} conjugates was observed in reactions performed with VHL complexes immunoprecipitated from insect cells expressing wild type VHL, Cul2, and Rbx1 (*lane 2*). Formation of these more slowly migrating species was strongly inhibited by the presence of Elongins B and C (*lanes 3* and *7*) and was not observed in VHL immunoprecipitates from insect cells expressing the [K159R,K171R,K196R]VHL mutant (*lanes 4* and *5*). Further supporting the notion that binding of Elongins B and C to VHL blocks its ubiquitylation, VHL mutants [L158P]VHL and [C162F]VHL are more efficiently ubiquitylated than wild type VHL, even when present in VHL immunoprecipitates from cells expressing high levels of Elongins B and C (*lanes 8* and *9*). To confirm and extend these findings, wild type VHL, Cul2, Rbx1, Elongin C, and an epitope-tagged Elongin B (HPC4·EloB) were coexpressed in insect cells and coimmunopurified with anti-HPC4 monoclonal antibodies. This procedure ensured that all of the immunoprecipitated VHL protein was associated with Elongins B and C. As shown in Fig. 1*A*, *lanes 10* and *11*, anti-HPC4 immunoprecipitates contained all five subunits of the CBC^{VHL} complex. Notably, the VHL protein contained in these Elongin B-containing complexes was resistant to ubiquitylation (Fig. 1*B*, *lanes 10* and *11*).

Rbx1 Directs Ubiquitylation of VHL in the Absence of Elongins B and C—The results of the experiments described above argue that VHL ubiquitylation can be negatively regulated by its binding to Elongins B and C and/or by its assembly into complete CBC^{VHL} complexes containing Cul2, Elongins B and C, and Rbx1. However, they provide no information about the mechanism of VHL ubiquitylation outside the context of CBC^{VHL}. In the course of investigating interactions among subunits of the CBC^{VHL} complex, we have observed that Rbx1 binds to VHL in the absence of exogenously expressed Cul2 and Elongins B and C (32), raising the possibility that Rbx1 might be capable of directly activating VHL ubiquitylation. To address this possibility, His$_6$-tagged wild type VHL or His$_6$-tagged [L158P]VHL were expressed in Sf21 insect cells alone, with Rbx1, or in combination with Rbx1, Cul2, and Elongins B and C. VHL or VHL-containing complexes were purified from cell lysates by consecutive Ni^{2+}-agarose chromatography and TSK DEAE-NPR HPLC. As shown in the Coomassie Blue-stained SDS-polyacrylamide gel in Fig. 2*A*, wild type VHL could be purified as an approximately stoichiometric component of the complete CBC^{VHL} complex. In addition, both wild type VHL and the [L158P]VHL mutant could be purified with approximately stoichiometric amounts of Rbx1 in the absence of exogenously expressed Cul2 and Elongins B and C.

Purified VHL and VHL-containing complexes were incubated with E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme Ubc5a, GST·Ub^{K48R}, and ATP. Consistent with the results in Fig. 1, we observed no detectable ubiquitylation of VHL in the context of the purified CBC^{VHL} complex. Purified VHL and [L158P]VHL formed VHL-Ub^{K48R} conjugates with electrophoretic mobilities corresponding to those expected for addition of a single GST·Ub^{K48R}. In contrast, high molecular mass VHL-GST·Ub^{K48R} conjugates were formed when VHL and [L158P]VHL were present as components of VHL^{Rbx1} complexes (Fig. 2, *B* and *C*).

To investigate further the requirement for Rbx1 in the formation of high molecular mass VHL-GST·Ub^{K48R} conjugates, we compared VHL ubiquitylation in the presence of wild type Rbx1 and the Rbx1 mutant [C94S], which does not support activation of either Cdc34-dependent ubiquitylation of Cln2 by

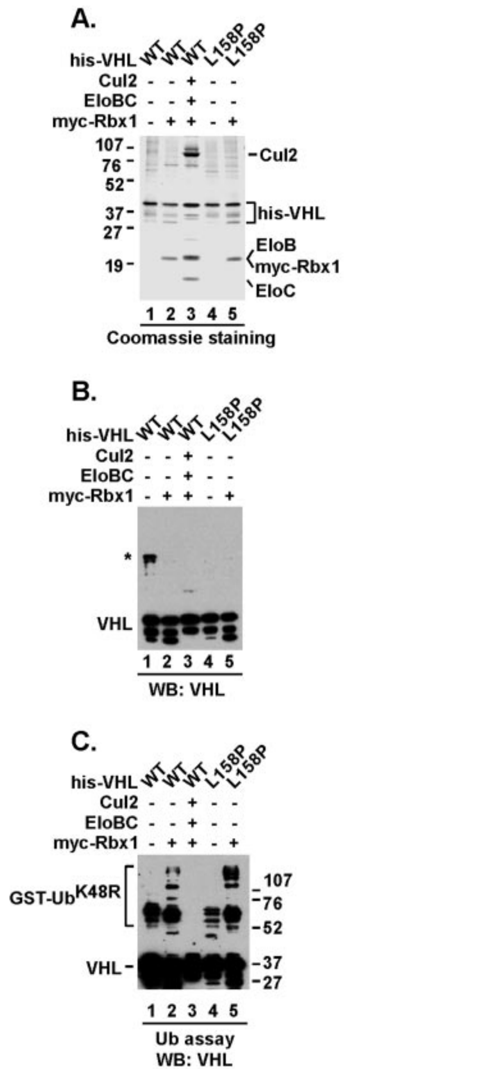

FIG. 2. **Rbx1-dependent ubiquitylation of chromatographically purified VHL.** *A*, the indicated recombinant proteins were expressed in Sf21 cells and purified by Ni^{2+}-agarose and TSK-DEAE HPLC as described under "Experimental Procedures." Purified VHL and VHL-containing complexes were subjected to 13% SDS-PAGE, and proteins were visualized by Coomassie Blue staining. *B*, the purified VHL and VHL-containing complexes shown in *panel A* were subjected to 13% SDS-PAGE and analyzed by Western blotting using the Ig32 anti-VHL monoclonal antibody. *C*, purified VHL and VHL-containing complexes shown in *panel A* were assayed as described under "Experimental Procedures" for their abilities to activate VHL ubiquitylation in the presence of ~100 ng of hUbc5a. Reaction products were subjected to 13% SDS-PAGE and analyzed by Western blotting using Ig32. The *asterisk* indicates the position of an unknown polypeptide recognized by Ig32.

SCF^{Grr1} complexes or Ubc12-dependent Rub1 modification of Cullin proteins (31). In these experiments, His_6-tagged VHL was coexpressed in insect cells with either Rbx1 or [C94S]Rbx1. The resulting complexes were purified from cell lysates by consecutive Ni^{2+}-agarose chromatography and TSK DEAE-NPR HPLC. As shown in Fig. 3*A*, approximately stoichiometric amounts of both wild type and mutant Rbx1 copurified with the VHL protein. In addition, high molecular mass VHL-GST·Ub^{K48R} conjugates were formed only in the presence of wild type Rbx1 (Fig. 3, *B* and *C*), indicating that VHL ubiquitylation depends on the presence of functional Rbx1.

The F-box Protein Cdc4 Can Be Ubiquitylated in the Context of the SCF^{Cdc4} Complex—The *S. cerevisiae* F-box protein Cdc4 is rapidly ubiquitylated and degraded by the proteasome in cells (18, 19). Based on the observations that (i) Cdc4 turnover

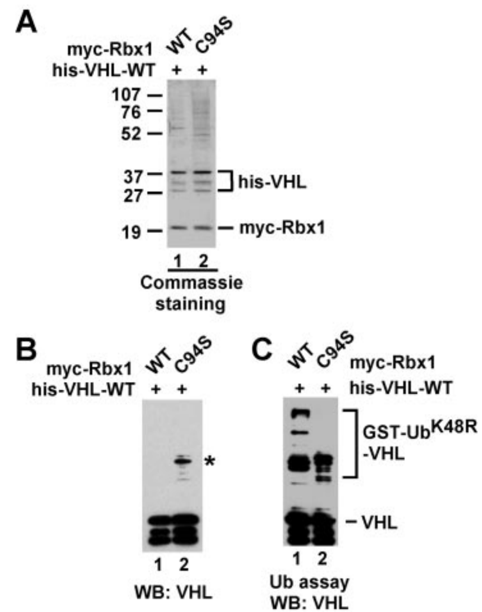

FIG. 3. **An Rbx1 RING finger mutant is impaired in its ability to support VHL ubiquitylation.** *A*, the indicated recombinant proteins were expressed in Sf21 cells and purified by Ni^{2+}-agarose and TSK-DEAE HPLC as described under "Experimental Procedures." Purified VHL^{Rbx1} complexes were subjected to 13% SDS-PAGE, and proteins were visualized by Coomassie Blue staining. *B*, the purified VHL^{Rbx1} complexes shown in *panel A* were subjected to 13% SDS-PAGE and analyzed by Western blotting using the Ig32 anti-VHL monoclonal antibody. *C*, purified VHL^{Rbx1} complexes shown in *panel A* were assayed as described under "Experimental Procedures" for their abilities to activate VHL ubiquitylation in the presence of ~100 ng of hUbc5a. Reaction products were subjected to 13% SDS-PAGE and analyzed by Western blotting using Ig32. The *asterisk* indicates the position of an unknown polypeptide recognized by Ig32.

depends upon its ability to interact with the SCF components Skp1 and Cdc53, and (ii) Cdc4 is stabilized when cells containing a temperature-sensitive Skp1 mutant are grown at the non-permissive temperature, it has been proposed that Cdc4 can be ubiquitylated in the context of an SCF^{Cdc4} complex (18, 19). However, direct evidence in support of this model has not been reported. To test this possibility, we asked whether a recombinant SCF^{Cdc4} complex, expressed in and purified from Sf21 insect cells, is capable of promoting Cdc4 ubiquitylation. N-terminal His_6- and HSV-tagged Cdc4 (His-HSV·Cdc4), *S. cerevisiae* Cdc53, N-terminal FLAG-tagged *S. cerevisiae* Skp1 (FLAG-Skp1), and N-terminal His_6- and Myc-tagged *S. cerevisiae* Rbx1 (His-Myc·ScRbx1) were coexpressed in Sf21 insect cells and purified from cell lysates by consecutive Ni^{2+}-agarose chromatography and TSK DEAE-NPR HPLC. The purified SCF^{Cdc4} complexes are shown in Fig. 4*A*, *left panel*. As shown in Fig. 4*A*, *right panel*, high molecular mass Cdc4-GST·Ub^{K48R} conjugates appeared in a time-dependent manner following incubation of the purified SCF^{Cdc4} complexes with E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme Cdc34, GST·Ub^{K48R}, and ATP.

Although these results strongly suggested that Cdc4 can be ubiquitylated in the context of the SCF^{Cdc4} complex, it was possible that the ubiquitylated Cdc4 in these reactions was not actually a component of SCF complexes but rather represented contaminating free Cdc4 in the purified SCF^{Cdc4} fraction. To prepare SCF^{Cdc4} complexes free of contaminating Cdc4, SCF subunits were coexpressed in insect cells and immunoprecipitated from cell lysates with antibodies recognizing the FLAG epitope on Skp1 (Fig. 4*B*, *left panel*). Immunoprecipitated complexes were then incubated with E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme Cdc34, GST·Ub^{K48R},

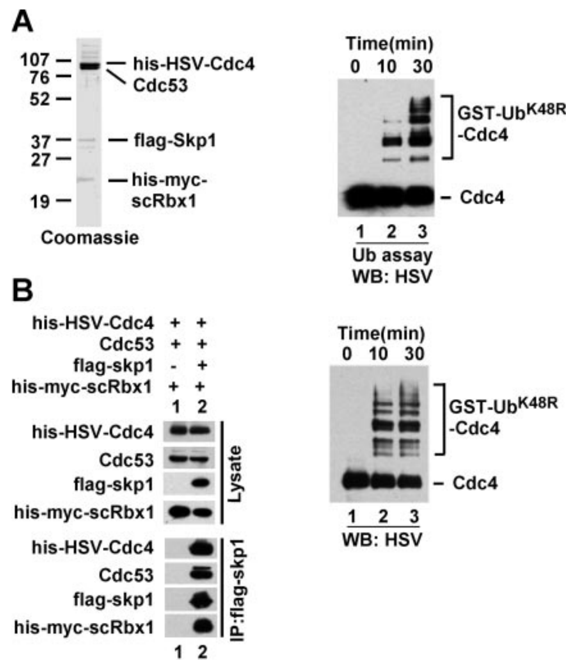

FIG. 4. **The F-box protein Cdc4 can be ubiquitylated *in vitro* in the context of the SCF^{Cdc4} complex.** *A*, the indicated recombinant proteins were expressed in Sf21 cells and purified by Ni^{2+}-agarose and TSK-DEAE HPLC as described under "Experimental Procedures." Purified SCF^{Cdc4} complexes were subjected to 13% SDS-PAGE, and proteins were visualized by Coomassie Blue staining (*left panel*). The purified complexes were assayed as described under "Experimental Procedures" for the ability to activate Cdc4 ubiquitylation activity in the presence of ~200 ng of scCdc34. Reaction mixtures were incubated for the times indicated. Reaction products were subjected to 8% SDS-PAGE, and proteins were visualized by Western blotting using anti-HSV antibody (*right panel*). *B*, total cell lysates and anti-FLAG immunoprecipitates from lysates of Sf21 cells infected with indicated baculoviruses were subjected to 8 or 13% SDS-PAGE. Proteins were visualized by Western blotting using antibodies directed against the proteins indicated in the *left panel*. The immunoprecipitates were assayed as described under "Experimental Procedures" for their abilities to activate Cdc4 ubiquitylation in the presence of ~ 200 ng of scCdc34. Reaction mixtures were incubated for the times indicated. Reaction products were subjected to 8% SDS-PAGE, and proteins were visualized by Western blotting using anti-HSV antibody.

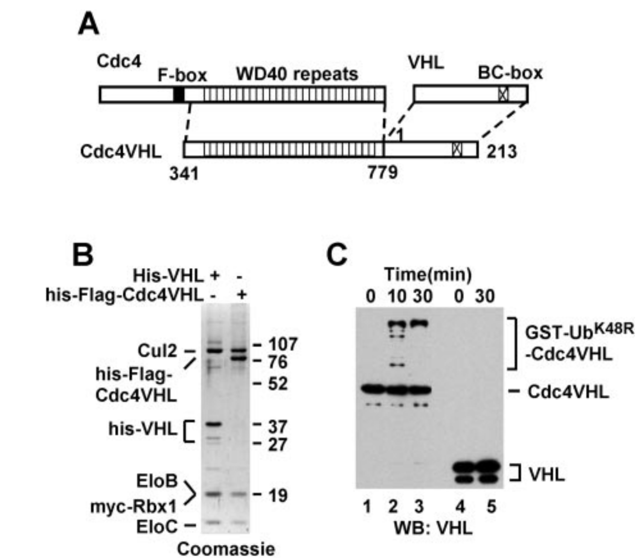

FIG. 5. **A Cdc4^{VHL} chimera is ubiquitylated in the context of CBC-Cdc4^{VHL}.** *A*, the Cdc4^{VHL} chimera contains residues 341–779 of Cdc4 fused to the complete VHL open reading frame. The Cdc4 F-box is indicated by the *black box*, and the VHL BC-box is indicated by the *hatched box*. *B*, the indicated recombinant proteins were expressed in Sf21 cells and purified as described under "Experimental Procedures." CBC complexes were subjected to 13% SDS-PAGE, and proteins were visualized by Coomassie Blue staining. *C*, CBC-Cdc4^{VHL} (*lanes 1–3*) or CBC^{VHL} (*lanes 4* and *5*) complexes were assayed for their abilities to activate ubiquitylation of Cdc4^{VHL} or VHL in the presence of ~100 ng of hUbc5a. Reaction mixtures were incubated for the indicated times. Reaction products were subjected to 11% SDS-PAGE, and proteins were visualized by Western blotting with the Ig32 anti-VHL monoclonal antibody.

and ATP. As shown in Fig. 4*B* (*right panel*), similar to the results obtained with chromatographically purified SCF^{Cdc4} complexes, high molecular mass Cdc4-GST·Ub^{K48R} conjugates were also formed in the presence of immunoprecipitated complexes. Thus, unlike the VHL protein in the CBC^{VHL} complex, the Cdc4 protein can be ubiquitylated in the context of the SCF^{Cdc4} complex.

To account for the observation that Cdc4, a target recruitment subunit of the SCF complex, can be ubiquitylated in the context of SCF^{Cdc4} (whereas VHL, a CBC target recruitment subunit, is protected from ubiquitylation in the context of CBC^{VHL}), we considered the possibility that CBC complexes might be inherently incapable of supporting ubiquitylation of associated target recruitment subunits. To address this hypothesis, we constructed an N-terminal His_6-tagged Cdc4^{VHL} chimera that contained the entire VHL open reading frame, including the BC-box and the Cdc4 WD-40 repeat domain but lacked the Cdc4 F-box (Fig. 5*A*). The Cdc4^{VHL} chimera was coexpressed in Sf21 insect cells with Cul2, Rbx1, and Elongins B and C; the resulting CBC-Cdc4^{VHL} complex was purified from cell lysates by consecutive Ni^{2+}-agarose chromatography and TSK DEAE-NPR HPLC. The purified CBC-Cdc4^{VHL} complex is shown in Fig. 5*B*. As shown in Fig. 5*C*, high molecular mass Cdc4^{VHL}-Ub^{K48R} conjugates were formed in a time-dependent manner following incubation of the purified CBC-Cdc4^{VHL} complex with E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme Ubc5a, GST·Ub^{K48R}, and ATP, indicating that the Cdc4^{VHL} chimera can be ubiquitylated in the context of the CBC-Cdc4^{VHL} complex. Thus, the resistance of VHL to ubiquitylation in the context of the CBC^{VHL} complex does not reflect an inherent inability of CBC complexes to ubiquitylate their target recruitment subunits, and therefore resistance to ubiquitylation must be a property of the VHL protein itself.

Summary and Perspective—In this report we have investigated the molecular basis of the different modes of regulation of the VHL tumor suppressor protein as the target recruitment subunit of the CBC^{VHL} ubiquitin ligase and of F-box proteins as target recruitment subunits of the structurally related SCF ubiquitin ligases. Our findings provide a plausible model to explain the different stabilities of the VHL and of F-box proteins in cells. Unlike the F-box proteins Cdc4, Grr1, and Met30, which can be ubiquitylated in the context of the SCF and degraded by the proteasome, the VHL protein is resistant to ubiquitylation in the context of the CBC^{VHL} complex, thus accounting at least in part for its long half-life in cells and consistent with its continuous requirement in negative regulation of the levels of hypoxia-inducible transcription factors throughout all phases of the cell cycle. Our observation that the Cdc4^{VHL} chimera can be ubiquitylated in the context of the CBC-Cdc4^{VHL} complex suggests that the resistance of VHL to ubiquitylation in the CBC^{VHL} complex is not an intrinsic property of the CBC complex conferred by one or more of its Cul2, Rbx1, and Elongin B and C subunits but instead is a property of the VHL protein regulated by the geometry of the CBC^{VHL} complex. The human VHL protein contains three lysine residues that are found in its C-terminal α-domain (37) at positions 159, 171, and 196 and that are potential ubiquitylation sites. The lysine at position 159, which is located within the Elongin

BC binding site and is hydrogen-bonded to Asn-108 of Elongin C, would be predicted to be protected from ubiquitylation by interaction of VHL with Elongins B and C. The lysines at positions 171 and 196 lie on exposed surfaces of VHL within the VHL-Elongin BC complex. Whether Cul2 and Rbx1 physically block access of the E2 to Lys-171 and -196 or whether some other aspect(s) of the geometry of the complex prevents their ubiquitylation awaits structural studies of the intact CBC^{VHL} complex. Interestingly, in recent experiments we have observed that, although the E2 ubiquitin-conjugating enzymes Ubc5 and Cdc34 can both be activated to synthesize polyubiquitin chains conjugated to Cul2 in the context of the CBC complex, the two enzymes transfer ubiquityl moieties to lysines at different sites on Cul2.[2] Thus, the overall geometry of lysines on the surface of target proteins is likely to contribute to regulation of their ubiquitylation by CBC and SCF ubiquitin ligases. Experiments are underway to decipher the rules governing the choice of surface lysines by different E2 enzymes and should shed light on this mode of regulation.

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