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Studying interactions of four proteins in the yeast two-hybrid system: Structural resemblance of the pVHL/elongin BC/hCUL-2 complex with the ubiquitin ligase complex SKP1/cullin/F-box protein

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ABSTRACT **The yeast two-hybrid system is a powerful technique that detects interactions between two proteins and has been useful in identifying new binding partners. However, the system fails to detect protein–protein interactions that require the presence of additional components of a multisubunit complex. Here we demonstrate that the vector YIpDCE1 can be used to express elongins B and C in yeast, and that these proteins form a stable complex that interacts with the von Hippel–Lindau tumor-suppressor gene product (pVHL). Only when pVHL and elongins B and C (VBC) are present does an interaction with the cullin family member, hCUL-2, occur, forming the heterotetrameric pVHL/elongin BC/hCUL-2 complex. This system was then used to map the binding region of hCUL-2 for the VBC complex. The first amino-terminal 108 aa of hCUL-2 are necessary for interaction with the VBC complex. The elongin BC dimer acts as a bridge between pVHL and hCUL-2 because pVHL and hCUL-2 can form distinct complexes with elongins B and C. These results reveal a striking structural resemblance of pVHL/elongin BC/hCUL-2 complex with the E3-like ubiquitin ligase complex SKP1/Cullin/F-box protein with respect to protein composition and sites of interactions. Thus, it seems possible that pVHL/elongin BC/hCUL-2 complex will possess ubiquitin ligase activity targeting specific proteins for degradation by the proteasome.**

von Hippel–Lindau disease is a hereditary cancer syndrome that predisposes affected individuals to a variety of benign and malignant tumors, including clear cell carcinomas (RCC) of the kidney, pheochromocytomas, and vascular tumors of the central nervous system and retina (1, 2). Germline mutations of one allele of the von Hippel–Lindau tumor-suppressor gene (VHL) and inactivation of the remaining allele in a given cell are the cause of von Hippel–Lindau disease (1, 2). Inactivation of both VHL alleles has been reported for the majority of sporadic RCCs and cerebellar hemangioblastomas consistent with Knudson's two-hit hypothesis (1, 2).

The human VHL gene encodes a 213-residue protein that is expressed in all tissues (1, 2). The VHL gene product (pVHL) associates in the cell with elongins B and C, two small proteins of 18 and 15 kDa, respectively (3–5). Elongin BC also associates with elongin A, a transcription elongation factor of RNA polymerase II, and stimulates its activity (6). We demonstrated that von Hippel–Lindau tumor-suppressor gene product (pVHL) can act as a transcription elongation inhibitor *in vitro* by virtue of its ability to compete with elongin A for the association with the two regulatory elongin subunits B and C (3). However, we found most of pVHL in a stable complex with elongins B and C, and interchange of pVHL with elongin A was not observed. The fact that pVHL is stably associated with elongin BC led us to speculate that this trimeric complex has a cellular function distinct from transcription elongation. The elongin BC binding site, which spans 13 amino acids, is loosely conserved between pVHL and elongin A (6). The trimeric VBC complex associates *in vivo* and *in vitro* with hCUL-2, forming a heterotetrameric complex pVHL/elongin BC/hCUL-2 (7, 8).

Many naturally occurring mutations in VHL have either been shown or are predicted to abrogate assembly with elongins B and C and hCUL-2, suggesting a functional role for these interactions (5, 9).

hCUL-2 is a member of a multigene family, the cullins, which have been implicated in the regulation of cell cycle in *Caenorhabditis elegans* and yeast (10). We showed recently that reintroduction of the wild-type VHL gene restores the ability of VHL-negative RCC cancer cells to exit the cell cycle and enter G_0/quiescence in low serum (11). These results implicate VHL as a tumor suppressor gene involved in the regulation of cell cycle exit consistent with its gatekeeper function in the kidney. The pVHL/elongin BC/hCUL-2 complex also plays a role in the down-regulation of the expression of vascular endothelial growth factor (VEGF), platelet-derived growth factor-β, and glucose transporter-1 (8, 12, 13). VHL-associated tumors are characterized by prominent neovascularization and high VEGF levels (1, 2). Cells lacking functional pVHL overexpress these hypoxia-inducible genes that are regulated at the level of transcription and mRNA stability (12–15). However, it is not understood how the pVHL/elongin BC/hCUL-2 complex functions to control these diverse activities.

The best characterized cullin, yeast CDC53, is a 94-kDa subunit of the conserved SKP1/Cullin/F-box protein (SCF) ubiquitin ligases (16, 17). Additional components of the yeast SCF ubiquitin ligases are SKP1 and alternative substrate-specific F-box proteins. Depending on the F-box protein used, SCF is known to ubiquitinate distinct proteins, targeting them for destruction by the 26S proteasome. SCF substrates include G_1-cyclins, cyclin-dependent kinase (CDK) inhibitors, and the CDK-inhibitory kinase SWE1 (18–23). hCUL1 associates with hSKP1 and the human F-box protein SKP2, forming an SCF ubiquitin ligase complex (24–26). The activities and functional domains of other cullin family members are not understood.

Abbreviations: β-gal, β-galactosidase; VHL, von Hippel–Lindau tumor-suppressor gene; RCC, clear cell carcinomas; pVHL, von Hippel–Lindau tumor-suppressor gene product; SCF, SKP1/Cullin/F-box protein.

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The scope of this study was to further characterize the protein interactions within the pVHL/elongin BC/hCUL-2 complex. The components and their interactions in this complex could then be compared with other SCF ubiquitin ligase complexes. To this end, a yeast two-hybrid system that allows the expression of four exogenous proteins (pVHL, hCUL-2, and elongins B and C) was developed. By using this system, it was shown that the presence of elongins B and C are required for pVHL/hCUL-2 binding, with elongin BC acting as a bridging complex between pVHL and hCUL-2. In addition, the binding site of hCUL-2 was localized to its amino-terminal 108 aa. These residues are conserved in *C. elegans* CUL-2 but not in other cullin family members. According to our results, the pVHL/elongin BC/hCUL-2 complex shows a striking resemblance to the SCF ubiquitin ligase complex with respect to protein composition and sites of association.

EXPERIMENTAL PROCEDURES

Yeast Strains. The yeast strain CG1945 was obtained from CLONTECH. Synthetic Defined plus Complete Supplement Mixture (SD+CSM) media were obtained from Bio101. Yeast were transformed with various constructs of the YIpDCE1 vector, which contains two expression cassettes (27), by using the lithium acetate method, and were grown on SD + CSM−Ade plates. Constructs integrated into CG1945 included linearized YIpDCE1, YIpDCE1 with elongin B in multiple cloning site 1, YIpDCE1 with elongin C in multiple cloning site 2, and YIpDCE1 with both elongins B and C. Colonies, which grew on SD + CSM−Ade plates, were white as a result of restored adenine synthesis. Colonies were checked by PCR for correct integration of the vector, as described (27). Those with correctly integrated vector were then tested for expression of elongins B and C by Western blot analysis by using commercial goat polyclonal antibodies (Santa Cruz Biotechnology; data not shown). Yeast strains CG1945, CG1945-B, CG1945-C, and CG1945-BC were transformed with various pACT2 and pAS2–1 plasmids described below and selected for growth on SD + CSM−Ade−His−Leu−Trp plates or SD + CSM−Ade−Leu−Trp plates followed by β-galactosidase (β-gal) assays directly on colony patches on filters or in liquid assay, as described below.

Vectors. HA-tagged hCUL-2 was amplified by PCR from plasmid pcDNA3-hCUL-2 (7) by using Pfu DNA polymerase (Stratagene) with primers containing *Eco*RI and *Bam*HI sites and subcloned into pAS2–1 (CLONTECH) or with primers containing *Bam*HI and *Xho*I sites and subcloned into pACT2 (CLONTECH). Carboxyl-terminal, amino-terminal, and internal deletion mutants of hCUL-2 were generated by one-step or two-step PCR. Alanine-scanning mutants of hCUL-2 were generated by two-step PCR. All hCUL-2 variants were cloned into the *Eco*RI and *Bam*HI sites of pAS2–1. VHL and mutants of VHL were amplified by PCR or by two-step PCR from plasmid pQE 30-VHL and subcloned into the *Bam*HI and *Eco*RI sites of pACT2 and pAS2–1. *C. elegans* CUL-2 was amplified by PCR from plasmid pBS SK-Ce-CUL-2 (10) by using primers with *Nde*I and *Bam*HI sites and subcloned into pAS2–1. hCUL-1 was amplified by PCR from plasmid pcDNA3-hCUL-1 (7) by using primers with *Nco*I and *Bam*HI sites and subcloned into pAS2–1. Elongin B was subcloned from pSX-elongin B (3) into the multicloning site 1 of YIpDCE1 at *Sal*I and *Bam*HI. Elongin C was amplified by PCR from pXS-elongin C (3) and subcloned into YIpDCE1's multicloning site 2 at *Kpn*I and *Xho*I. Elongins B and C cDNAs were amplified by PCR and subcloned into the *Nco*I and *Bam*HI sites of pAS2–1 and pACT2. Elongin A cDNA was amplified by PCR from pSVL-elongin A (3) and subcloned into the *Nco*I and *Bam*HI sites of pACT2 or released with *Nde*I and *Bam*HI and subcloned into pAS2–1. Constructs were sequenced by using an Applied Biosystems sequencer. Western blots were performed to assess the production of the various fusion proteins. pVHL-fusions were detected by using a rabbit polyclonal antibody against the whole protein, and hCUL-2-fusions were detected by using monoclonal HA.11 (Babco, Richmond, CA) against the hemagglutinin epitope.

***β*-Gal Assays.** Colony patch filter assays (5-bromo-4-chloro-3-indolyl β-D-galactoside as substrate) and liquid culture assays (*o*-nitrophenyl β-D-galactoside) were performed in triplicate as described in the CLONTECH product protocol for the Matchmaker yeast two-hybrid system.

RESULTS

A Modified Yeast Two-Hybrid System for Testing Interactions of Four Proteins. Previous reports have suggested that pVHL interacts only with hCUL-2 if it is able to complex with elongin BC (7, 8). One way of measuring the interaction of two proteins is to use the yeast two-hybrid system. pVHL/elongin BC/hCUL-2 is a heterotetrameric protein complex composed of pVHL, elongins B and C, and hCUL-2. We developed yeast strains that constitutively expressed one gene (elongin B or elongin C) or two genes simultaneously (elongins B and C) to measure the interaction of pVHL with hCUL-2 and to map potential binding sites (Fig. 1). We used a previously developed vector YIpDCE1 that allows for the constitutive expression of two genes simultaneously (27). To test whether the elongin BC complex was necessary for interaction of pVHL with hCUL-2, hCUL-2 was subcloned into the binding domain plasmid pAS2–1 and VHL into the activation domain plasmid pACT-2. Yeast was cotransformed with plasmids pAS2–1-hCUL-2 and pACT2-VHL. Interactions of pVHL and hCUL-2 were measured as a function of growth on SD + CSM−His plates and *lacZ* reporter gene activation. Expression of the four proteins and mutant derivatives was confirmed by Western blot analysis (data not shown).

In yeast with integrated YIpDCE1 or YIpDCE1 containing elongin B or C alone, no interaction between pVHL and hCUL-2 was detected (Fig. 2). However, when elongins B and C were expressed simultaneously in yeast, an interaction between hCUL-2 and pVHL occurred. The strength of the interaction was comparable to the association of SV40 T-antigen and p53 as expressed from control plasmids (Fig. 2 and data not shown). hCUL-1, a homologue of hCUL-2 that is known not to interact with VBC *in vitro* (7), was also cloned into pAS2–1 and cotransformed with pACT2-VHL into the different yeast strains. No interaction occurred even in the presence of elongins B and C expression (Fig. 2). These data indicate that the interaction of pVHL with hCUL-2 requires both elongins B and C, which are known to form a complex in the cell (6, 9).

The Binding Site for hCUL-2 Depends on Exon 3 of VHL. To identify the site of the hCUL-2 interaction on pVHL, we used three different deletion mutants of VHL for interaction with hCUL-2 in the presence of elongins B and C. Deletion of exon 1 removes half of the VHL protein (amino acids 1–113) but still allows for binding to hCUL-2 (Fig. 2). A naturally occurring mutant found in sporadic RCCs that deletes exon 2 from VHL (amino acids 114–154) was tested. It was able to interact with hCUL-2 when transformed into yeast expressing elongins B and C (2). However, the naturally occurring VHL deletion mutant of exon 3 (amino acids 157–213) was no longer able to interact with hCUL-2 as previously shown *in vitro* and *in vivo* (7, 8). These results demonstrate that the binding site of hCUL-2 to pVHL depends on exon 3 where the elongins B and C interaction region lies (amino acids 157–177). An earlier report showed that the binding site for elongins B and C and hCUL-2 is identical and lies within the residues 157–177 of pVHL (8). Exons 1 and 2 of pVHL (residues 1–154) are frequently mutated in VHL and sporadic kidney tumors, yet mutations in this region do not affect elongin BC/hCUL-2

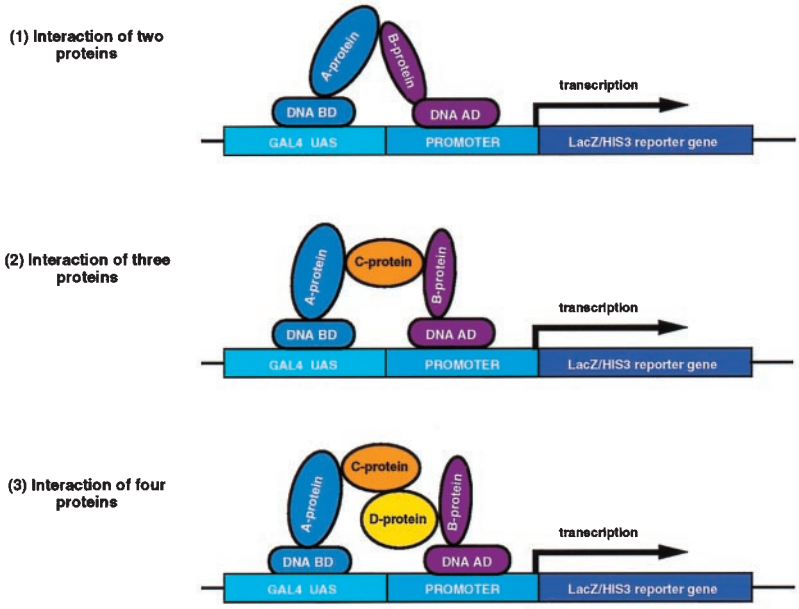

FIG. 1. Schematic representation of the modified yeast two-hybrid system. Depiction of various yeast two-hybrid systems testing for the interaction of two proteins (*Top*) three proteins (*Middle*) and four proteins (*Bottom*) resulting in the activation of transcription of the *LacZ* and HIS3 reporter genes.

binding. These mutations must affect pVHL function in other ways.

Identification of the hCUL-2 Region That Mediates the Interaction with pVHL. The modified two-hybrid system was used to identify the region of hCUL-2 that mediates its interaction with the pVHL/elongin BC complex. To this end, various deletion mutants of hCUL-2 were generated (Fig. 3). Carboxyl- terminal deletion mutants of hCUL-2 removing up to 587 (of 745) amino acids continued to show interactions with pVHL comparable to full length hCUL-2 in the presence of elongins B and C. This result localized the binding region somewhere within the first 158 amino acids. Larger carboxyl-terminal deletion mutants were not stable when expressed in yeast as detected by Western blot analysis and therefore could not be used in this assay (data not shown). Deleting as few as four amino acids from the amino terminus disrupted the ability of hCUL-2 to bind to pVHL (Fig. 3).

We then tested internal hCUL-2 deletion mutants within the first 158 residues. In addition, short (seven residues) alanine scans in that region were tested for interaction with pVHL (Fig. 3). The deletions of hCUL-2 showed that the entire first 108 residues are required for interaction with pVHL in the presence of elongins B and C. The alanine scan mutants, beginning at residue 20 and continuing to residue 57, disrupted the hCUL-2/pVHL interaction in each case. No simple short amino acid sequence in hCUL-2 could be found sufficient to interact with pVHL, and replacement of short amino acid stretches with alanines always disrupted the interaction. Failure to make small changes in hCUL-2 that still interact with pVHL in the presence of elongins B and C suggests that the entire amino-terminal 108 residues of hCUL-2 are required. The amino terminus of hCUL-2 is highly conserved in the CUL-2 homologue of *C. elegans* (38% identity) but not in any other cullins that are known to date. We therefore tested whether *C. elegans* CUL-2 interacts with human pVHL/elongins BC, and indeed it associated as tightly as hCUL-2 (Table 1).

Identification of Elongin BC, hCUL-2/BC, and pVHL/BC Complexes. The above results suggest a model where pVHL forms a complex with elongins B and C, and this complex interacts with the amino-terminal 108 residues of hCUL-2. Because the region of pVHL required for binding of elongins B and C and hCUL-2 is indistinguishable, it is possible that the elongin BC complex serves as a bridge between pVHL and hCUL-2. If this were the case, then it should be possible to show that elongins B and C form separate complexes with either pVHL or hCUL-2. First, we observed that elongins B and C form a complex in yeast as reported for mammalian tissues and cell lines (ref. 6; Table 2, top section). Both pVHL and hCUL-2 form separate complexes with the elongin BC complex but not with elongin B or C alone (Table 2, top, middle, and bottom sections). This is in disagreement with *in vitro* data showing purified recombinant pVHL and elongin C can bind (9, 28). Apparently in our *in vivo* yeast system, neither pVHL nor hCUL-2 is able to bind to elongin C alone but does so only in the presence of elongin B (Table 2, top, middle, and bottom sections).

Furthermore, we detected pVHL binding to elongin C in the presence of elongin B and pVHL binding to elongin B in the presence of elongin C (Table 2, middle and bottom sections). It was shown earlier that pVHL binds to elongin C directly, and elongin C binds with a separate domain to elongin B, suggesting that elongin B does not directly bind to pVHL (28). Note that in the hCUL-2/elongin BC interaction assays, only one configuration scores positive (Table 2, middle and bottom sections). This is when hCUL-2 is in the AD-plasmid, elongin C is in the BD-plasmid, and elongin B is expressed from the integrated plasmid. In addition, elongin A showed association with elongins B and C in one configuration with elongin B expressed from the integrated plasmid. These apparent discrepancies may reflect subtle differences between the structure of the two-hybrid fusion partners. Accordingly, elongin B or C alone cannot serve as bridge between pVHL and hCUL-2. The elongin BC complex is required for the pVHL/hCUL-2 association and is independent of whether hCUL-2 was fused to the activation domain or the DNA binding domain of GAL4 (Table 1). These data suggest that pVHL is binding via residues 157–177 to elongin BC, which in turn interact with the amino terminus of hCUL-2 (Fig. 4).

We also tested whether elongin A can bind to hCUL-2 via the elongin BC complex (Table 1). However, binding of elongin A to hCUL-2 was not detected, though we could measure elongin A binding to C and BC (Table 2, top and middle sections). This result is in agreement with data from

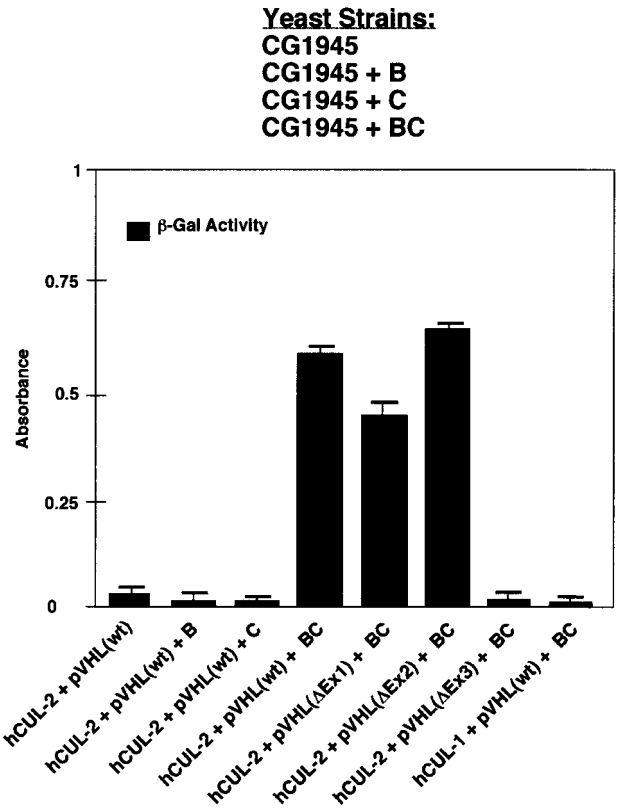

FIG. 2. Wild-type pVHL interacts with hCUL-2 only in the presence of elongins B and C in yeast. hCUL-1 and hCUL-2 cDNAs were expressed in pAS2–1 and VHL cDNAs in pACT2. Plasmids were transformed into different CG1945 yeast strains expressing control sequence, elongin B, elongin C, or elongin BC together. β-Gal activity was measured in permeabilized yeast by using *o*-nitrophenyl-β-D-galactoside as the substrate. Absorbance values are relative to β-gal activity in yeast expressing the control plasmids pVA3–1 and pTD1–1 (p53 fragment and SV40T antigen). Expression of proteins was verified by Western blot analysis (data not shown).

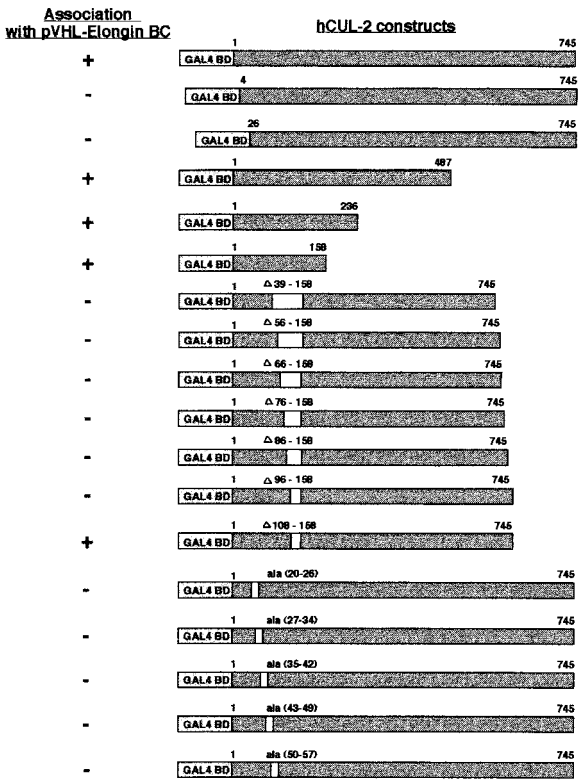

FIG. 3. Mapping the pVHL binding region on hCUL-2. Various pAS2–1-hCUL-2 constructs were cotransformed with pACT1-VHL into CG1945-BC yeast, and growth on SD + CSM−Ade−Leu−Trp−His and β-gal activity were measured. Amino acid residues are indicated on top of every hCUL-2 construct, as well as residues deleted or replaced by alanines. Expression of proteins was verified by Western blot analysis (data not shown).

another group, which could never detect an elongin A/hCUL-2 interaction in cells (8). These results suggest that elongin A binding to elongin BC is not sufficient to associate to hCUL-2, but a specific conformation of the BC complex is required for this interaction to occur, which is given in the case of the VBC complex.

DISCUSSION

Our data demonstrate the usefulness of the modified yeast two-hybrid system for studying multimeric protein complexes. Previously, others have reported using a three-component system that involved the addition of a third protein to facilitate or inhibit interaction between binding domain fusion and activation domain fusion proteins (29–31). The four-component system described here may be useful in studying protein interactions that occur only when all of the subunits of a complex are present, as is the case for pVHL and hCUL-2. There is evidence that other cullins function in large complexes, and these molecules might be conveniently studied in this yeast multimeric-hybrid system (24–26). The simple nutritional requirements and short doubling time of yeast make the yeast tetrahybrid system efficient to rapidly characterize protein structure/function interactions and to screen for new interacting proteins.

The mapping of the binding region of hCUL-2 for the VBC complex may be useful for generating a dominant-negative or unregulated hCUL-2 construct in mammalian cells to investigate whether binding to pVHL is important for tumor suppression. In addition, the amino terminus of other members of the conserved cullin family members may be important in binding to regulatory molecules, as is the case with pVHL and hCUL-2. Indeed, CDC53, the yeast cullin of the SCF ligase complex, binds with its amino-terminal 200 residues to SKP1, with SKP1 being an elongin C homologue (8, 22). SKP1 then binds to different F-box proteins that are the substrate recognition subunits for the SCF ubiquitin ligase (Fig. 4). In our case, elongins B and C bind to pVHL via its elongin BC box, which then allows hCUL-2 to bind. Therefore, by analogy, pVHL might function as substrate recognition subunit of the pVHL/elongin BC/hCUL-2 complex. Elongin A, another F-box protein, associates with elongin BC but does not bind to

Table 1. Association of cullins with pVHL/elongin BC

Strain	AD-plasmid	BD-plasmid	His	β-Gal
CG1945(BC)	(VHL)	(CUL-2)	+	+
CG1945(BC)	(CUL-2)	(VHL)	+	+
CG1945(BC)	(VHL)	(VHL)	−	−
CG1945(BC)	(CUL-2)	(CUL-2)	−	−
CG1945(BC)	(VHL)	(CeCUL-2)	+	+
CG1945(BC)	(CUL-2)	(A)	−	−
CG1945(BC)	(A)	(CUL-2)	−	−
CG1945(BC)	(VHL)	(CUL-1)	−	−

Characterization of associations of various cullins with pVHL in the presence of elongins B and C in yeast. Various pAS2-1 and pACT2 constructs were cotransformed into CG1945-BC yeast and growth on SD+CSM−Ade−Leu−Trp−His and β-gal activity were measured. Expression of proteins was verified by Western blot analysis (data not shown).

Table 2. Association of hCUL-2, pVHL, and elongin BC

Strain	AD-plasmid	BD-plasmid	His	β-Gal
CG1945	(VHL)	(B)	−	−
CG1945	(VHL)	(C)	−	−
CG1945	(VHL)	(CUL-2)	−	−
CG1945	(VHL)	(VHL)	−	−
CG1945	(CUL-2)	(B)	−	−
CG1945	(CUL-2)	(C)	−	−
CG1945	(CUL-2)	(CUL-2)	−	−
CG1945	(CUL-2)	(VHL)	−	−
CG1945	(B)	(B)	−	−
CG1945	(B)	(C)	+	+
CG1945	(B)	(CUL-2)	−	−
CG1945	(B)	(VHL)	−	−
CG1945	(C)	(B)	+	+
CG1945	(C)	(C)	−	−
CG1945	(C)	(VHL)	−	−
CG1945	(C)	(CUL-2)	−	−
CG1945	(A)	(B)	−	−
CG1945	(A)	(C)	+	+
CG1945	(B)	(A)	−	−
CG1945	(C)	(A)	+	+
CG1945(B)	(VHL)	(C)	+	+
CG1945(B)	(VHL)	(CUL-2)	−	−
CG1945(B)	(VHL)	(VHL)	−	−
CG1945(B)	(CUL-2)	(C)	+	+
CG1945(B)	(CUL-2)	(CUL-2)	−	−
CG1945(B)	(CUL-2)	(VHL)	−	−
CG1945(B)	(C)	(CUL-2)	−	−
CG1945(B)	(C)	(VHL)	+	+
CG1945(B)	(A)	(C)	+	+
CG1945(B)	(C)	(A)	+	+
CG1945(B)	(A)	(CUL-2)	−	−
CG1945(B)	(CUL-2)	(A)	−	−
CG1945(C)	(VHL)	(B)	+	+
CG1945(C)	(VHL)	(CUL-2)	−	−
CG1945(C)	(VHL)	(VHL)	−	−
CG1945(C)	(CUL-2)	(B)	−	−
CG1945(C)	(CUL-2)	(CUL-2)	−	−
CG1945(C)	(CUL-2)	(VHL)	−	−
CG1945(C)	(B)	(CUL-2)	−	−
CG1945(C)	(B)	(VHL)	+	+
CG1945(C)	(A)	(B)	−	−
CG1945(C)	(B)	(A)	−	−
CG1945(C)	(A)	(CUL-2)	−	−
CG1945(C)	(CUL-2)	(A)	−	−

Characterization of associations between hCUL-2, pVHL, and elongins B and C in yeast. Various pAS2-1 and pACT2 constructs were cotransformed into CG1945 (top section), CG1945-B (middle section), and CG1945-C (bottom section) yeast, and growth on SD + CSM−Ade−Leu−Trp−His and β-gal activity were measured. Expression of proteins was verified by Western blot analysis (data not shown).

hCUL-2, suggesting that elongin A uses the elongin BC subunits for a different purpose.

In *C. elegans*, one can find seven cullins, 10 SKP1 homologues, and 60 F-box proteins (17). The relatively equal number of genes suggests that each cullin may bind to its own SKP1 homologue. The binding specificity may occur by the cullin's amino terminus because this region is the least conserved region among the cullins (17, 22). The SKP1 homologues would bind to different substrate recognition subunits, resulting in many combinatorial possibilities for SCF and other cullin-containing ubiquitin ligases (17). The interaction of *C. elegans* CUL-2 with human pVHL/elongin BC suggests that the pVHL/elongin BC/hCUL-2 pathway is conserved in nematodes.

Our study has revealed a striking resemblance between SCF and pVHL/elongin BC/hCUL-2, in that both contain a cullin and SKP1-like protein. Both bind to the SKP1-like molecules with the conserved amino terminus of the respective cullin (Fig. 4). The SKP1-like protein serves as a bridging protein to bind to a substrate recognition subunit in the case of SCF and to pVHL in the case of the pVHL/elongin BC/hCUL-2 complex. SKP1 binds via the F-box to the different substrate binding proteins, and elongin BC bind to pVHL via the elongin BC box (8, 18). We are now screening for other possible substrate recognition subunits of pVHL/elongin BC/hCUL-2 complex using hCUL-2 together with elongin BC as a bait in the modified yeast two-hybrid system.

Another similarity is that both cullins contain a UBC homology domain at their carboxyl terminus (Fig. 4). In the case of CDC53 and hCUL-1, it is the interaction domain for the ubiquitin-conjugating enzyme CDC34 (22, 24, 25). The ubiquitin ligase APC (anaphase-promoting complex) is a multiprotein complex with a subunit (APC2) that contains a region highly homologous to the UBC domain of cullins. This region was suggested to bind to the ubiquitin-conjugating enzyme UBC4 in APC (32, 33). It is therefore likely that either CDC34 or another pVHL/elongin BC/hCUL-2 complex specific ubiquitin conjugating enzyme is associated with hCUL-2 through the UBC domain in the carboxyl terminus of hCUL-2. Given this striking similarity of SCF and the pVHL/elongin BC/hCUL-2 complex, it is possible that the pVHL/elongin BC/hCUL-2 complex exhibits ubiquitin ligase activity together with either CDC34 or another yet-to-be-identified UBC. Possible substrates for the pVHL/elongin BC/hCUL-2 complex

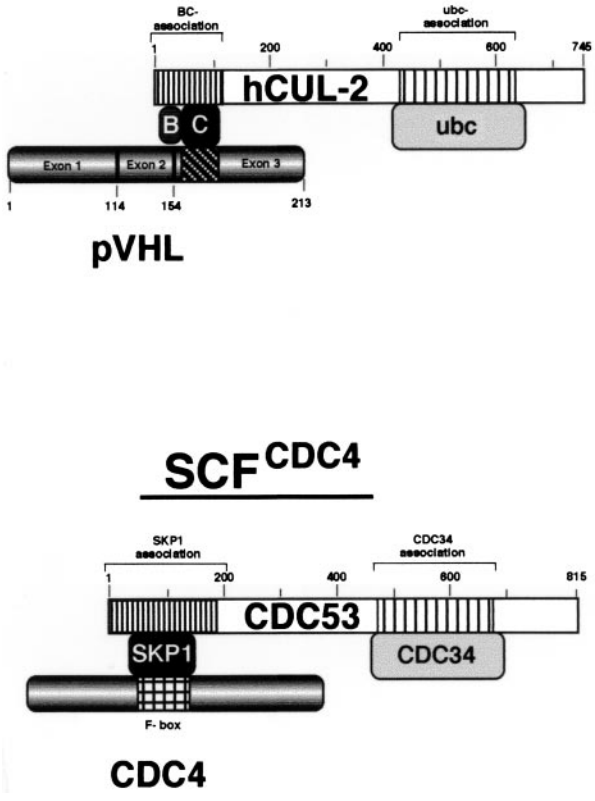

FIG. 4. Similarity of the pVHL/elongin BC/hCUL-2 complex and the SCF^{CDC4} complex. The pVHL/elongin BC/hCUL-2 complex (*Upper*) consists of hCUL-2, elongins B and C, and pVHL. The SCF^{CDC4} complex consists of CDC53 or CUL-A, SKP1, and the F-box protein CDC4, as well as the ubiquitin-conjugating enzyme CDC34. hCUL-2 contains a region at its carboxyl terminus that is highly conserved in all cullins (17). This region in CDC53 mediates the association with the ubiquitin-conjugating enzyme CDC34. The SKP1 protein is a homologue of elongin C. hCUL-2 is a homolog of CDC53.

targeted degradation include mRNA-binding proteins that regulate the stability of hypoxia-inducible factors like vascular endothelial growth factor, platelet-derived growth factor-β, and glucose transporter-1, or the hypoxia-inducible transcription factor HIF-1 α, which is regulated by ubiquitin-dependent degradation (34).

One interesting finding of these studies is the fact that pVHL binds only with the elongin BC region (157–177) to hCUL-2. In sporadic RCCs as well as in VHL patients, the VHL gene may contain one of numerous missense mutations in exon 1 or 2, and yet these mutations are not predicted to affect elongin BC/hCUL-2 binding (1). There is the possibility that missense mutations are rapidly degraded *in vivo*. Another possibility is that pVHL mutations in exon 1 and 2 affect other vital functions, such as binding to substrates, which are then conjugated to ubiquitin and targeted for degradation. If this is the case, the consequence of mutations in exon 3 or exon 1 and 2 would be the same. In the former, the pVHL/elongin BC/hCUL-2 complex would disassemble; in the latter, possible substrates would not be recognized by pVHL/elongin BC/hCUL-2 complex. In either case, the substrates would no longer be targeted for degradation.

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