Identification of Elongin C and Skp1 Sequences That Determine Cullin Selection*

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The multiprotein von Hippel-Lindau (VHL) tumor suppressor and Skp1-Cul1-F-box protein (SCF) complexes belong to families of structurally related E3 ubiquitin ligases. In the VHL ubiquitin ligase, the VHL protein serves as the substrate recognition subunit, which is linked by the adaptor protein Elongin C to a heterodimeric Cul2/Rbx1 module that activates ubiquitylation of target proteins by the E2 ubiquitin-conjugating enzyme Ubc5. In SCF ubiquitin ligases, F-box proteins serve as substrate recognition subunits, which are linked by the Elongin C-like adaptor protein Skp1 to a Cul1/Rbx1 module that activates ubiquitylation of target proteins, in most cases by the E2 Cdc34. In this report, we investigate the functions of the Elongin C and Skp1 proteins in reconstitution of VHL and SCF ubiquitin ligases. We identify Elongin C and Skp1 structural elements responsible for selective interaction with their cognate Cullin/Rbx1 modules. In addition, using altered specificity Elongin C and F-box protein mutants, we investigate models for the mechanism underlying E2 selection by VHL and SCF ubiquitin ligases. Our findings provide evidence that E2 selection by VHL and SCF ubiquitin ligases is determined not solely by the Cullin/Rbx1 module, the target protein, or the integrity of the substrate recognition subunit but by yet to be elucidated features of these macromolecular complexes.

The von Hippel-Lindau (VHL) tumor suppressor complex is the founding member of the family of Elongin BC-containing E3 ubiquitin ligases, which are composed of a substrate recognition subunit, Elongins B and C, a member of the Cullin family of proteins (either Cul2 or Cul5), and the RING finger protein Rbx1 (also known as ROC1 or Hr1t) (1–4). The VHL protein is one of a large family of proteins that bind Elongins B and C through a conserved BC box motif, which is a 10-amino-acid degenerate motif of sequence (A,P,S,T)LXXXX(CXX)(A,-L,V) (5–9). In the context of the VHL ubiquitin ligase, the VHL protein serves as the substrate recognition subunit and is linked by the adaptor protein Elongin C to a heterodimeric Cul2/Rbx1 module that functions as a potent activator of ubiquitylation of target proteins by an E2 ubiquitin-conjugating enzyme. Elongin B, a ubiquitin-like protein, associates with the complex through interactions with Elongin C and appears to stabilize the binding of Elongin C to VHL.

The large family of SCF (Skp1-Cul1-F-box protein) E3 ubiquitin ligases share striking structural similarities with the VHL ubiquitin ligase. SCF ubiquitin ligases are composed of one of many F-box proteins, the Elongin C-like protein Skp1, Cullin family member Cul1 (called Cdc53 in the Saccharomyces cerevisiae), and Rbx1 (4, 21–30). In the context of SCF ubiquitin ligases, F-box proteins serve as substrate recognition subunits and are linked by the adaptor protein Skp1 to a Cul1/Rbx1 module that activates an E2 to ubiquitylate target proteins.

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The abbreviations used are: VHL, von Hippel-Lindau; VBC, the VHL-Elongin BC complex; GST, glutathione S-transferase; HPLC, high pressure liquid chromatography; SCF, Skp1-Cul1/Cdc53-F-box; Ub, ubiquitin; h, human; E1, Ub-activating enzyme; HIF, hypoxia-inducible factor; HA, hemagglutinin; PDB, Protein Data Bank.

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The large family of SCF ubiquitin ligases is largely unknown. In the context of SCF ubiquitin ligases, the E2 ubiquitin-conjugating enzyme Cdc34 (30–34). In contrast, at least in vitro, VHL ubiquitin ligase-dependent ubiquitylation of HIFα depends on members of the Ub5 family of E2s (2, 3).

Our laboratory is currently engaged in biochemical studies to define the functions of subunits of VHL and SCF ubiquitin ligases. In this report, we investigate the functions of the Elong-
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gin C and Skp1 adaptor proteins in reconstitution of active VHL and SCF ubiquitin ligases. We identify Elongin C and Skp1 structural elements responsible for selective interaction with their cognate Cullin/Rbx1 modules. In addition, by exploiting altered specificity Elongin C-Skp1 and Cdc4-VHL chimeric proteins, we provide insights into the determinants of E2 selectivity of VHL and SCF ubiquitin ligases.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Anti-VHL monoclonal antibody (Ig32) was purchased from BD Pharmingen. Anti-Myc (9E10) and anti-HA (12CA5) monoclonal antibodies were obtained from Roche Applied Science. Anti-HSV monocular antibody was from Novagen. Anti-FLAG monoclonal antibody (M2) was purchased from Sigma. Anti-Cul2 and anti-Elongin C monoclonal antibodies were obtained from BD Transduction Laboratories. Anti-Cdc53 and mouse Rbx1 containing N-terminal His6 and Myc tags were purified by the same procedure.

**Expression of Recombinant Proteins in SF21 Insect Cells**—Wild-type human Elongin C and Elongin C mutants containing N-terminal epitope tags recognized by the HPC4 monoclonal antibody were subcloned into pbPak8. Recombinant baculoviruses were generated with the BacPak baculovirus expression system (Clontech). Baculoviruses encoding human Cul1 and Cul2 containing N-terminal HA epitope tags were described previously (7). Baculoviruses encoding human Cul2, human VHL, human VHL containing an N-terminal His6 tag, human Elongin B and mouse Rbx1 containing N-terminal Myc epitope tags, and human HIF1α containing N-terminal His6 and HPC4 tags were described previously (3). Baculoviruses encoding S. cerevisiae Cdc53 and mouse Rbx1 containing N-terminal His6 and Myc tags were described previously (4).

**Preparation of Hydroxylated HIF1α—**SF21 cells were infected with baculoviruses encoding human HIF1α containing N-terminal His6 and HPC4 tags. HIF1α was purified from SF21 cell lysates by Ni2+-agarose chromatography as described previously (3). Approximately 5 μg of purified HIF1α was incubated at room temperature for 2 h with 200 μl of TNT wheat germ extract translation system (Promega) programmed with a pcDNA3 expression vector encoding the EGLN1 prolyl hydroxylase (20) and containing 100 μM FeCl3, 2 mM sodium ascorbate, and 5 mM α-ketoglutarate. Prolyl hydroxylated HIF1α was then purified from the reaction mixture by Ni2+-agarose chromatography (3).

**Assays of HIF1α and Sic1 Ubiquitylation in Vitro**—Human Ubc5a (Human hemocyanin, His6 tagged), human HPC4 (猴), and S. cerevisiae Cdc4 (YcdC44), all containing N-terminal His6 tags, S. cerevisiae Uba1 (yUba1) containing N-terminal Myc and His tags, and mammalian GST-ubiquitin were prepared as described (3). Reconstituted VBC-Cullin/Rbx1 complexes or aliquots of TSK DEAE-NPR HPLC-purified VBC-Cullin/Rbx1 or VHL-Cullin/Rbx1 complexes were incubated with 50 ng of yUba1, 500 ng of GST-Ubc5a, 20 ng of prolyl hydroxylated Sic1, and varying amounts of the E2 ubiquitin-conjugating enzymes hUbc5a, hUbc3, or yCdc34 as indicated in the text and figures in 10 μl of ubiquitylation reaction buffer containing 40 mM Hepes-NaOH (pH 7.9), 60 mM potassium acetate, 2 mM dithiothreitol, and 1.5 mM ATP. Reaction mixtures were incubated for 1 h at 25 °C, fractionated by SDS-polyacrylamide gel electrophoresis, and analyzed by Western blotting with anti-HIF1α antibodies.

**RESULTS**

As illustrated in Fig. 1, the Elongin C and Skp1 proteins share significant amino acid sequence similarity and belong to a protein superfamily with shared three-dimensional structure. Elongin C and Skp1 function as adaptors that recruit heterodimeric Cul2/Rbx1 and Cul1/Rbx1 modules, respectively, into VHL and SCF ubiquitin ligation complexes. In the case of the VHL ubiquitin ligase, Elongin C recruits a Cul2/Rbx1 module to the complex by interacting specifically with a BC box in the VHL protein (Ref. 40 and references therein) and with an N-terminal Cul2 region (41). In the case of SCF ubiquitin ligation complexes, Skp1 functions by an apparently similar mechanism as Elongin C to recruit a Cul1/Rbx1 module to the complex as Elongin C to recruit a Cul1/Rbx1 module to the complex.
plex by interacting specifically with an F-box in F-box proteins and with an N-terminal Cul1 region (42, 43).

To begin to study the relationship between the roles of Elongin C and Skp1 in reconstitution of their respective ubiquitin ligases, we sought to identify Elongin C sequences required for its interaction with Cul2 and for its recruitment of the Cul2/Rbx1 module into the VHL complex. To accomplish this, we constructed baculoviruses encoding a systematic series of Elongin C deletion mutants and analyzed the abilities of these Elongin C mutants to support the assembly of VBC and of the complete VBC-Cul2/Rbx1 ubiquitin ligase in Sf21 insect cells. Sf21 cells were coinfected with baculoviruses encoding VHL, Elongin B, Cul2, Rbx1, and wild type or mutant Elongin C with an N-terminal epitope tag recognized by the HPC4 monoclonal antibody. Elongin C-containing complexes were immunoprecipitated with HPC4 monoclonal antibodies and analyzed by Western blotting for the presence of individual subunits of the VHL complex. As shown in Fig. 2A, recruitment of Cul2 to the VHL complex was strongly dependent on both Elongin B and C. Elongin C was quite sensitive to deletions. Only three of the mutants could assemble similarly to wild type Elongin C into the VBC complex; two of these, Δ41–50 and Δ51–60, were defective in recruitment of Cul2 into the VHL ubiquitin ligase complex (Fig. 2B). These results suggested that Elongin C sequences between residues 41 and 60 are not crucial for VBC assembly but are important for Elongin C interaction with Cul2 and recruitment of Cul2 into the VHL ubiquitin ligase complex. Notably, some residues within the corresponding region of Skp1 appear to contribute to formation of the Skp1-Cul1 interface (43).

To investigate further the role of Elongin C sequences 41–60 in recruitment of Cul2 into the VHL ubiquitin ligase, we carried out domain swaps between Elongin C and Skp1 in an effort to identify an Elongin C-Skp1 chimeric protein capable of recruiting a Cul1/Rbx1 module into the VHL complex. In one mutant, designated M2, Elongin C residues 40–47 were replaced with Skp1 residues 25–32. These stretches of sequence in Elongin C and Skp1 fall largely within conserved helix 2 (Fig. 1) (40, 44). A second Elongin C-Skp1 chimeric protein, designated M1, was created by replacing Elongin C residues 47–57 with Skp1 residues 32–42. Elongin C residues 47–57 form a loop (loop 3) between helix 2 and strand 3 in human Elongin C; loop 3 was poorly resolved in the Elongin C x-ray structures (40, 45). The corresponding sequences from Skp1 fall within a protease-sensitive region that is also proposed to form a loop (loop 3) between helix 2 and strand 3 in human Skp1 (32). These stretches of sequence were used to replace Elongin C sequences in C M1 and C M2 mutants are shown in magenta. The green font with red shading indicates the residues that are predicted to be within 3 Å of a Cullin molecule and are likely to make direct contacts. Yellow shading indicates conserved bulky hydrophobic residues (Ile, Leu, Val, Met, Phe, Tyr, and Trp), blue type indicates conserved residues with small side chains (Ala, Gly, and Ser), and green shading indicates other conserved residues (including a group of positively charge side chains Lys, Arg, and His and acidic/amine side chains Asp, Glu, Asn, and Gln). In the secondary structure lines, s stands for a strand, and h stands for a helix.
complexes containing wild type Elongin C. In contrast, only complexes containing the Elongin C M1 mutant, in which the Elongin C flexible loop residues were replaced with the corresponding residues from Skp1, were capable of assembling into VHL complexes with Cul1/Rbx1. Taken together, these results argue that Elongin C sequences between amino acids 47 and 57 and Skp1 sequences between amino acids 32 and 42 are important for determining the specificity with which these adaptor proteins interact with their respective Cullins and for recruitment of Cullin/Rbx1 modules into their respective E3 ubiquitin ligase complexes.

Evidence from previous studies indicated that HIF1α ubiquitylation by the wild type VHL complex is carried out specifically by the E2 ubiquitin-conjugating enzyme Ubc5 (2, 3), whereas ubiquitylation of SCF target proteins is in most cases carried out by the E2 ubiquitin-conjugating enzymes hUbc5a, hCdc34, and hUbC5, expressed in and purified from E. coli. For these reasons, we considered the possibilities that (i) the Cullin protein might play a significant role in determining E2 selectivity and (ii) HIF1α ubiquitylation by the VHL ubiquitin-ligase complex containing the altered specificity Elongin C M1 mutant and the Cul1/Rbx1 module might depend on Cdc34 rather than Ubc5. To address these possibilities and to confirm the functionality of the altered specificity Elongin C M1 mutant, we tested the ability of the VHL ubiquitin ligase containing either wild type Elongin C and Cul2/Rbx1 or the Elongin C M1 mutant and Cul1/Rbx1 to activate HIF1α ubiquitylation in reactions carried out with the E2 ubiquitin-conjugating enzymes human Ubc5a (hUbc5a); human Cdc34 (hCdc34), or yeast Cdc34 (yCdc34).

The recombinant E2 ubiquitin-conjugating enzymes hUbc5a, hCdc34, and yCdc34 used in these experiments were purified to
that hUbc5a could also support SCFCdc4-dependent ubiquitylation of phosphorylated Sic1 (Fig. 4, C

lated HIF1 complex. Confirming the validity of this approach, hydroxy-
combinant heterotrimeric VBC with recombinant Cullin/Rbx1 by coexpression of VHL, Elongins B and C, Rbx1, reproducibly and in good yield prepare recombinant VHL complex containing the altered specificity Elongin C M1 mutated Elongin C were preincubated for 60 min at 4 °C in the presence of Cul1/Rbx1 or Cul2/Rbx1. The resulting mixtures were assayed for their abilities to support ubiquitylation of prolyl-hydroxylated HIF1α in the presence of the indicated amounts of yCdc34, hCdc34, or hUbc5a. Reaction products were analyzed by Western blotting with anti-HIF1α antibody and visualized using an Odyssey infrared imaging system (LI-COR Biosciences).

HIF1α ubiquitylation can be activated by a Cul1/Rbx1 module. In addition, they support the idea that the Cullin/Rbx1 module is not solely responsible for determining the E2 selectivity of Cullin-containing ubiquitin ligases, although the E2 is believed to dock on the Cullin/Rbx1 module at the C terminus of Cullin-containing ubiquitin ligases, although the E2 is believed to dock on the Cullin/Rbx1 module at the C terminus of Cullin containing residues 341−779, which includes the Cdc4 WD repeat domain but lacks the Cdc4 F-box (Fig. 7A). The Cdc4-VHL chimera binds stably to the Elongin BC complex through the VHL BC box but does not interact with Skp1 since it lacks an intact F-box (46).

The SCFCdc4 ubiquitin ligase targets phosphorylated Sic1 for ubiquitylation by the E2 ubiquitin-conjugating enzyme Cdc34 (21−23, 47). Cdc4 binds phosphorylated Sic1 via its WD repeat domain and recruits it for ubiquitylation by Cdc34. To determine whether the Cdc4-VHL chimeric protein can assemble into an E3 ubiquitin ligase containing Elongins B and C and a Cul2/Rbx1 module, Sf21 insect cells

![Fig. 5. Reconstitution of VHL ubiquitin ligase with isolated VBC and Cul2/Rbx1 complexes. VBC-Cul2/Rbx1, VBC, and Cullin/Rbx1 complexes were prepared as described under "Experimental Procedures." EGLN1-treated HIF1α was assayed as described under "Experimental Procedures" in ubiquitylation reactions containing ~800 ng of hUbc5a in the absence (lane 1) or presence (lane 2) of purified VBC-Cul2/Rbx1 complex or a mixture of purified VBC and Cul2/Rbx1 that had been preincubated for 60 min at 4 °C (lane 3). Reaction mixtures were subjected to 8% SDS-polyacrylamide gel electrophoresis.](Image)

![Fig. 6. Cul1-dependent ubiquitylation of HIF1α by hUbc5a. A, VBC complexes containing either wild type (WT) or M1 mutant Elongin C were preincubated for 60 min at 4 °C alone or in the presence of Cul1/Rbx1 or Cul2/Rbx1. The resulting mixtures were assayed for their abilities to support ubiquitylation of prolyl-hydroxylated HIF1α in the presence of 0.5 μg of hUbc5a or 5.4 μg of hCdc34. Reaction products were analyzed by Western blotting with anti-HIF1α antibody and visualized on film. B, VBC complexes containing either wild type or M1 mutant Elongin C were preincubated for 60 min at 4 °C in the presence of Cul1/Rbx1 or Cul2/Rbx1. The resulting mixtures were assayed for their abilities to support ubiquitylation of prolyl-hydroxylated HIF1α in the presence of the indicated amounts of yCdc34, hCdc34, or hUbc5a. Reaction products were analyzed by Western blotting with anti-HIF1α antibody and visualized using an Odyssey infrared imaging system (LI-COR Biosciences).](Image)
were coinfected with baculoviruses encoding the Cdc4-VHL chimera containing an N-terminal His tag, Elongins B and C, Cul2, and Rbx1. The Cdc4-VHL complex was purified from cell lysates by Ni$^{2+}$-agarose chromatography followed by TSK DEAE 5-PW HPLC. As shown in the Coomassie-stained SDS-polyacrylamide gel of Fig. 7B, the Cdc4-VHL chimera could be purified as part of a multiprotein complex with roughly stoichiometric amounts of Elongins B and C, Cul2, and Rbx1. As shown in Fig. 7C, Sic1 ubiquitylation by the Cdc4-VHL ubiquitin ligase complex was strongly dependent on the E2 ubiquitin-conjugating enzyme UbC5 rather than Cdc34. Sic1 ubiquitylation was dependent on the Cdc4 portion of the Cdc4-VHL chimeric protein since the purified VHL complex did not support Sic1 ubiquitylation in the presence of either UbC5 (Fig. 7D) or Cdc34 (data not shown).

**DISCUSSION**

In this report, we investigate the functions of the Elongin C and Skp1 proteins in reconstitution of VHL and SCF ubiquitin ligases. We identify Elongin C and Skp1 structural elements responsible for selective interaction with their cognate Cullin/Rbx1 modules. In addition, using altered specificity Elongin C and F-box protein mutants, we investigate models for the mechanism underlying E2 selection by VHL and SCF ubiquitin ligases.

Our findings suggest that the −11 amino acid loop 3 between helix 2 and strand 3 in the conserved three-dimensional structure of Elongin C and Skp1 (Fig. 1) plays an important role in Cullin selection by the VBC complex. Wild type Elongin C preferentially binds to Cul2. However, when loop 3 is replaced by its counterpart from Cul1-binding Skp1, the chimeric Elongin C M1 mutant can bind both Cul1 and Cul2, and it requires Cul1 for efficient HIF1α ubiquitylation.

Little is known about loop 3 structure and function. In the available structures of Elongin C (Protein Data Bank (PDB) accession numbers 1VCB and 1LM8), this region is disordered (40, 45), and in Skp1 (PDB accession number 1LDK), it appeared to interfere with crystallization and had to be deleted (43, 44). In an effort to gain better understanding of the function of loop 3, we rebuilt both the wild type loop and its replacement in Elongin C M1, using the corresponding sequences from either Elongin C or Skp1 with the isolated C chain of 1LDK as the template. The resulting model was docked onto Cul1 (the A chain of 1LDK) by global energy minimization or was homology-modeled onto the Skp1 structure (the C chain of 1LDK). The results obtained using both approaches are very similar and suggest that loop 3 might not be involved in a direct interaction with the Cullin protein (Fig. 8 and data not shown). In contrast with numerous direct, mainly hydrophobic interactions between helices that are conserved between Skp1 and Elongin C and between Cul1 and Cul2, respectively, all atoms in the predicted loop 3 structure in Elongin C M1 are separated by at...
least 6 Å from any atoms in Cul1, and this distance is even larger for the predicted loop 3 structure in wild type Elongin C. What might be the molecular mechanism by which loop 3 affects the specificity with which Elongin C interacts with Cul1?

There are several possibilities that could not be addressed by our approximate structural model. First, loop 3 might adopt a folded or bent conformation in the process of Elongin C-Cul1 interaction, which perhaps could produce additional contact(s) between Cul1 and the Elongin C M1 mutant but not between Cul1 and wild type Elongin C. Second, the ~6Å distance between Elongin C M1 loop 3 and Cul1 could be bridged by a water molecule, thus producing additional Elongin C M1-specific interactions. Finally, we note that our model predicts that loop 3 of Elongin C M1 would contribute an extra hydrogen bond to interact with a lysine residue in the middle of strand 1 (data not shown), perhaps inducing a change in the packing of the structural core of the Elongin C M1 mutant. Experimental structure-function studies are needed to determine whether any of these possibilities contribute to the specificity of protein-protein interactions that we observed; what is clear is that the mechanism of loop 3-dependent Cullin selection is subtle.

By exploiting the altered specificity Elongin C M1 mutant, we have investigated the importance of the Cul1/Rbx1 module in E2 selection by the VHL ubiquitin ligase complex. Our observation that hUbC5a and not Cdc5 is the preferred E2 for ubiquitylation of prolyl-hydroxylated HIF1α directed by a Cul1-containing VHL ubiquitin ligase suggests that the target protein is not solely hydrogen bond to interact with a lysine residue in the middle of strand 1 (data not shown), perhaps inducing a change in the packing of the structural core of the Elongin C M1 mutant. Experimental structure-function studies are needed to determine whether any of these possibilities contribute to the specificity of protein-protein interactions that we observed; what is clear is that the mechanism of loop 3-dependent Cullin selection is subtle.

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