Identification of Elongin C Sequences Required for Interaction with the von Hippel-Lindau Tumor Suppressor Protein*

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Elongin C is a 112-amino acid protein that is found in mammalian cells as a positive regulatory subunit of the heterotrimeric RNA polymerase II elongation factor Elongin (SIII) and as a component of a multiprotein complex containing the von Hippel-Lindau (VHL) tumor suppressor protein. As a subunit of the Elongin complex, Elongin C interacts directly with the transcriptionally active Elongin A subunit and potently induces its elongation activity; in addition, Elongin C interacts with the ubiquitin-like Elongin B subunit, which regulates the interaction of Elongin C with Elongin A. As a component of the VHL complex, Elongin C interacts directly with both Elongin B and the VHL protein. Binding of the VHL protein to Elongin C was found to prevent Elongin C from interacting with and activating Elongin A in vitro, leading to the proposal that one function of the VHL protein may be to regulate RNA polymerase II elongation by negatively regulating the Elongin complex. In this report, we identify Elongin C sequences required for its interaction with the VHL protein. We previously demonstrated that the ability of Elongin C to bind and activate Elongin A is sensitive to mutations in the C-terminal half of Elongin C, as well as to mutations in an N-terminal Elongin C region needed for formation of the Elongin BC complex. Here we show that interaction of Elongin C with the VHL tumor suppressor protein depends strongly on sequences in the C terminus of Elongin C but is independent of the N-terminal Elongin C region required for binding to Elongin B and for binding and activation of Elongin A. Taken together, our results are consistent with the proposal that the VHL protein negatively regulates Elongin C activation of the Elongin complex by sterically blocking the interaction of C-terminal Elongin C sequences with Elongin A. In addition, our finding that only a subset of Elongin C sequences required for its interaction with Elongin A are critical for binding to VHL may offer the opportunity to develop reagents that selectively interfere with Elongin and VHL function.

The von Hippel-Lindau (VHL)1 tumor suppressor gene on chromosome 3p25.5 is mutated in the majority of sporadic clear-cell renal carcinomas and in VHL disease, an autosomal dominant familial cancer syndrome that predisposes affected individuals to a variety of tumors including renal carcinomas, cerebellar hemangioblastomas and hemangiomas, retinal angiomas, and pheochromocytomas (1). Molecular cloning of the VHL gene revealed that it encodes an ~30-kDa protein with no extensive homology to known proteins. A search for cellular proteins that interact with the VHL protein led to the discovery that it binds tightly and specifically to the C subunit of Elongin (SIII) (2, 3), a heterotrimeric transcription factor that activates elongation by RNA polymerase II by suppressing transient pausing by polymerase at many sites within transcription units (4, 5). Elongin is composed of a transcriptionally active A subunit of ~770 amino acids and two small positive regulatory B and C subunits of 118 and 112 amino acids, respectively (6–8). Elongin C, a direct activator of Elongin A, can bind Elongin A and induce its transcriptional activity in the absence of Elongin B. Elongin C also binds Elongin B, a ubiquitin-like protein that neither binds to nor activates Elongin A directly, but that facilitates interaction of Elongin C with Elongin A (6, 9). Binding of the VHL protein and Elongin A to either Elongin C or an Elongin BC complex is mutually exclusive, and binding of the VHL protein to the Elongin BC complex has been shown to block its ability to activate Elongin transcriptional activity in vitro (2).

The interaction of both the VHL protein and Elongin A with either Elongin C or an Elongin BC complex is mediated at least in part by a 12-amino acid sequence motif, TLXXXXV(XXLR), which is the only region of similarity between the VHL and Elongin A proteins (3, 6, 10). Supporting the idea that the VHL-Elongin BC interaction is important for VHL tumor suppressor activity, a significant fraction of all VHL mutants found in VHL kindreds and in sporadic clear-cell renal carcinomas fall within this conserved region required for Elongin BC binding (1), and a number of these VHL mutants have been tested and found to be impaired in their abilities to bind Elongin B and C (2, 3).

As part of our effort to understand the functional relationships between the VHL and Elongin proteins, we have carried out a systematic structure-function analysis of Elongin C to compare the regions of the protein that are important for binding and activation of Elongin A and for interaction with the VHL protein. In a previous study, we demonstrated that the ability of Elongin C to bind and activate Elongin A is sensitive to mutations in the C-terminal half of Elongin C as well as to mutations in an N-terminal Elongin C region needed for formation of the Elongin BC complex (9). In this report, we dem-
FIG. 1. Elongin C deletion mutants analyzed in this study. Elongin C mutations that most strongly affect the ability of Elongin C to bind to Elongin B (B binding), to assemble into Elongin ABC complexes (ABC formation), and to activate Elongin A (A activation) (9) are summarized at the top of the figure; mutations in the region indicated by the open box strongly affect activation of Elongin A by isolated Elongin BC complexes but do not significantly affect the activity of isolated Elongin ABC complexes. The structures of Elongin C deletion mutants investigated for their abilities to interact with the VHL protein are shown at the bottom of the figure.

onstrate that the C-terminal half of Elongin C is also important for binding to the VHL protein but that the N-terminal Elongin C region important for binding to Elongin B and for physical and functional interactions with Elongin A is dispensable for VHL binding.

**EXPERIMENTAL PROCEDURES**

**Construction and Expression of Elongin C Mutants**—N-terminal and internal Elongin C deletion mutants were constructed as described (9) using the Muta-Gen M13 in vitro mutagenesis kit (Bio-Rad) by oligonucleotide-directed mutagenesis (11) of M13mpET-elongin C (7, 12). Alanine replacement mutants were constructed using the same procedure with mutagenic oligonucleotides containing 12 nucleotides from the parental rat Elongin C cDNA on either side of the alanine substitution. Histidine-tagged Elongin B, Elongin C, and Elongin C mutants were expressed in E. coli using a pQE-60 vector (Qiagen) (13, 14). A 1-liter culture of FLAG epitope (DYKDDDDK) tag was expressed in VHL protein with an N-terminal 6-histidine tag and a C-terminal DNA sequencing using the DNA sequencing using the fluoridemass. The column was washed with 10 ml of 5.7 M guanidine hydrochloride, 20 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, and 10% (v/v) glycerol. Following dialysis, the mixtures were centrifuged for 15 min. The resulting supernatants were each applied to a TSK DEAE-NPR HPLC column (35 × 4.6 mm) (Tosohas) pre-equilibrated in a buffer (40 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 1% (v/v) glycerol) containing 40 mM KCl and fractionated using a SMART microchromatography system (Pharmacia Biotech Inc.) at 8 °C. The column was eluted at 0.3 ml/min with a 3-mL linear gradient from 40 to 500 mM KCl in the same buffer. A portion of each column fraction was analyzed by 10% Tris-Tricine SDS-PAGE gel electrophoresis (15), and the proteins were visualized by silver staining.

**Immunoprecipitation Assay of VC Complex Formation**—3 μg of FLAG-tagged VHL protein was mixed with 1–4 μg of either wild type or mutant Elongin C and diluted 5-fold with 40 mM Hepes-NaOH (pH 7.9), 100 mM KCl, 2 mM dithiothreitol, 50 μM ZnSO4, 0.1 mM EDTA, and 10% (v/v) glycerol. After incubation for 60,000 × g for 15 min. The resulting supernatants were each applied to a TSK DEAE-NPR HPLC column (35 × 4.6 mm) (Tosohas) pre-equilibrated in a buffer (40 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 1% (v/v) glycerol) containing 40 mM KCl and fractionated using a SMART microchromatography system (Pharmacia Biotech Inc.) at 8 °C. The column was eluted at 0.3 ml/min with a 3-mL linear gradient from 40 to 500 mM KCl in the same buffer. A portion of each column fraction was analyzed by 10% Tris-Tricine SDS-PAGE gel electrophoresis (15), and the proteins were visualized by silver staining.
amide gels were stained overnight with 0.1% (w/v) Coomassie Blue R250 in 50% (v/v) methanol and 10% (v/v) acetic acid and destained with 20% (v/v) methanol, 10% (v/v) acetic acid, and 5% (v/v) glycerol. Destained gels were dried between two pieces of porous cellophane membrane (Bio-Rad). Dried gels were scanned, and the relative intensities (mass ratios, C/VHL) of the protein bands were determined using the "Gel Plotting Macros" program (NIH Image 1.60).

RESULTS AND DISCUSSION

We previously demonstrated that transcriptionally active Elongin ABC and Elongin AC complexes, as well as stable VHL-Elongin BC (VBC) and VHL-Elongin C (VC) complexes, can be reconstituted by renaturation of bacterially expressed Elongin and VHL proteins purified from guanidine hydrochloride-solubilized inclusion bodies (2, 3, 7–9). To identify Elongin C sequences required for interaction with the VHL protein, a systematic series of N-terminal, C-terminal, and internal Elongin C deletion mutants were constructed (Fig. 1), expressed in E. coli, purified from inclusion bodies, and assayed for abilities to form chromatographically isolable VBC complexes. In these experiments, individual Elongin C mutants were refolded together with the wild type Elongin B and VHL proteins and subjected to TSK DEAE-NPR HPLC. Consistent with our previous results (2), the wild type VBC complex elutes from TSK DEAE-NPR as a discrete species with chromatographic properties distinct from those of both Elongin B, which flows through TSK DEAE-NPR at low ionic strength, and the wild type VHL and Elongin C proteins, which bind more tightly than the VBC complex to this resin and elute over relatively broad ionic strength ranges (Fig. 2A). Thus, coelution of the VHL, Elongin B, and Elongin C proteins from TSK DEAE-NPR is diagnostic of an interaction between the three proteins.

We previously observed that the Elongin C regions indicated at the top of Fig. 1 are important for its interactions with Elongin A and B. As shown in Fig. 2B, the N-terminal Elongin C deletion mutant C(15–112), as well as the internal Elongin C deletion mutants C(D31–40), C(D41–50), C(D51–60), and C(D81–90), which all bind Elongin B (9), were capable of forming isolable VBC complexes. Only the C-terminal Elongin C deletion mutant C(1–97) and the internal Elongin C deletion mutant C(D71–80), which both bind Elongin B (9), were clearly unable to form isolable VBC complexes. Ambiguous results were obtained in binding assays performed with the internal Elongin C deletion mutant C(D61–70), since Elongin BC complexes containing this mutant exhibit aberrant chromatographic behavior and elute from TSK DEAE-NPR at approximately the same ionic strength as the VHL and Elongin C proteins (9). Because the free VHL and Elongin C proteins are eluted from TSK DEAE-NPR at approximately the same ionic strength, ambiguous results were also obtained in binding assays performed with Elongin C mutants C(23–112), C(29–112), C(57–112), and C(D21–30), which do not bind Elongin B in this assay (9).

To measure formation of VC complexes and to overcome the
limitations inherent in the TSK DEAE-NPR binding assay, we developed an immunoprecipitation assay to assess the ability of VHL to bind to wild type Elongin C and Elongin C mutants. The VHL protein used in these studies has a C-terminal FLAG epitope tag (14) and can therefore be precipitated with the M2 anti-FLAG monoclonal antibody. Wild type and mutant Elongin C proteins were folded in the presence or absence of the VHL protein and mixed with M2 antibody and protein A-Sepharose. After three washes with 0.5 M NaCl and 1% Triton X-100, immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3A, lane 3, when immunoprecipitations were carried out in the presence of the VHL protein, Elongin C was found in the immunoprecipitate. Neither wild type Elongin C nor any of the Elongin C mutants were precipitated when immunoprecipitations were carried out in the absence of the VHL protein (Fig. 3A, lanes 2 and 4–19), arguing that the immunoprecipitation binding assay is a valid measure of interactions between the VHL protein and Elongin C mutants.

The results of immunoprecipitation binding assays are shown in Fig. 3, B and C. The amount of each Elongin C mutant immunoprecipitated (normalized to immunoprecipitated VHL) was quantitated by densitometry of the Coomassie-stained gels shown in the upper portion of each panel. These results resolved most of the ambiguities of the TSK DEAE-NPR binding assays. First, N-terminal Elongin C sequences, important for binding to Elongin B and for binding and activation of Elongin A, are dispensable for binding to the VHL protein. As shown in Fig. 3, lanes 6–20, the N-terminal Elongin C deletion mutants C(1–97) and C(57–112), as well as the internal Elongin C deletion mutant C(Δ21–30), were all efficiently co-immunoprecipitated with the VHL protein. A reduced amount of the N-terminal Elongin C deletion mutant C(23–112) was co-immunoprecipitated with the VHL protein; however, this appears to be due to a low yield of this mutant following renaturation from guanidine hydrochloride-solubilized inclusion bodies (Fig. 2 and data not shown). In addition, a superstoichiometric amount of both N-terminal Elongin C deletion mutants C(19–112), which both bind Elongin B and binds and activates Elongin A, and C(57–112), which binds neither Elongin B nor Elongin A, was co-immunoprecipitated with the VHL protein, possibly due to the tendency of Elongin C to form multimers (data not shown).

Second, internal Elongin C deletion mutant C(Δ71–80) was the only mutant that exhibited no detectable interaction with the VHL protein in the immunoprecipitation binding assay, although binding of C-terminal Elongin C deletion mutant C(1–97) was substantially reduced. The internal Elongin C deletion mutants C(Δ31–40), C(Δ41–50), C(Δ51–60), C(Δ61–70), and C(Δ81–90) all exhibited significant binding to the VHL protein. Elongin C mutant C(Δ91–100), which exhibited little or no binding to the VHL protein in the TSK DEAE-NPR binding assay, also bound to the VHL protein in the immunoprecipitation binding assay, suggesting that the immunoprecipitation binding assay may be somewhat less stringent than the TSK DEAE-NPR binding assay.

To characterize further the Elongin C sequences that lie between residues 70 and 80 and that appear to be most critical for binding to the VHL protein, we constructed and analyzed a set of clustered Elongin C alanine-scanning mutants with mutations between residues 70 and 83. Results of these experiments underscored the importance of this Elongin C region for interaction with the VHL protein. As shown in Fig. 4, all of the clustered Elongin C alanine-scanning mutants were severely impaired in their abilities to bind the VHL protein, although Elongin C mutants C(Ala70–72) and C(Ala73–75) had some residual binding activity. Elongin C point mutant C(V73A) exhibited normal VHL binding, whereas Elongin C point mutants C(Y76A), C(Y79A), and C(Y83A) exhibited reduced VHL binding.

A TBLASTN search of the GenBank non-redundant data base using rat Elongin C as the query sequence identified two Caenorhabditis elegans and one Saccharomyces cerevisiae gene-encoding putative Elongin C homologs (Fig. 5). Comparison of the predicted amino acid sequences of mammalian Elongin C and the potential C. elegans and S. cerevisiae Elongin C homologs revealed that the three regions of greatest similarity were: an N-terminal region (amino acids 18–34), which includes the N-terminal Elongin C sequences that we showed previously (9) are critical for binding to Elongin B and for binding to and activation of Elongin A; a central region (amino acids 67–85), which overlaps Elongin C regions critical for activation of Elongin A (amino acids 61–70) and for binding to the VHL protein (residues 71–80); and a C-terminal region (residues 92–111), which, when mutated, affects the ability of Elongin C to bind to and activate Elongin A and to bind to the VHL protein. Thus, the Elongin C regions most highly conserved between the mammalian, C. elegans, and S. cerevisiae
proteins correspond to Elongin C regions identified in our assays as most critical for binding to Elongin B, binding and activation of Elongin A, and binding to the VHL protein.

Because mutations in the C-terminal Elongin C conserved region affect the ability of Elongin C both to bind the VHL protein and to bind and activate Elongin A, we wished to investigate this Elongin C region in more detail and to compare the sequences important for interaction with the VHL protein and with Elongin A. We previously characterized a set of clustered C-terminal Elongin C alanine-scanning mutants to activate A and to bind the VHL protein. Residues mutated to alanine are shown on the left. B, Elongin B; C, Elongin C; L, load; FT, flow-through.

In summary, in this report we have identified Elongin C

FIG. 5. Alignment rat Elongin C with potential C. elegans and S. cerevisiae homologs. The best alignment of rat Elongin C with potential C. elegans (Ce) homologs on cosmids F54F7 (GenBank™ accession no. Z67755), cosmids W03H1 (GenBank™ accession no. U41509) and F31A9 (GenBank™ accession no. U58738), and S. cerevisiae (Sc; GenBank™ accession no. U44030) was determined with the MACAW program (16) using the BLOSUM 80 score table (17). Positions indicated by white letters on dark gray background have mean scores that fall within the top 71–90% of scores in the score table; positions indicated by black letters on light gray background have mean scores that fall within the top 51–70% of scores in the score table.

FIG. 6. Assay for formation of VBC complexes containing C-terminal Elongin C alanine-scanning mutants. A, C-terminal Elongin C alanine-scanning mutants were assayed for their abilities to form VBC complexes by TSK DEAE-NPR HPLC as described under “Experimental Procedures.” B, comparison of the abilities of C-terminal Elongin C alanine-scanning mutants to activate A and to bind the VHL protein. Residues mutated to alanine are shown on the left. B, Elongin B; C, Elongin C; L, load; FT, flow-through.

sequences required for interaction with the VHL tumor suppressor protein. In a previous study, we showed that activation of Elongin A by Elongin C requires sequences throughout the entire Elongin C molecule, including a short N-terminal region required for binding to Elongin B as well as Elongin C C-terminal sequences, which are dispensable for binding to Elongin B. Here we demonstrate that interaction of Elongin C with the VHL tumor suppressor protein depends strongly on sequences in the C terminus of Elongin C but not on the N-terminal Elongin C region required for Elongin B binding and for binding and activation of Elongin A. These results are consistent with the proposal that the VHL protein negatively regulates Elongin C activation of the Elongin complex by sterically blocking interaction of C-terminal Elongin C sequences with Elongin A. In addition, our finding that only a subset of Elongin C sequences required for interaction with Elongin A are critical for binding to VHL may offer an opportunity to develop reagents that selectively interfere with Elongin and VHL function.

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REFERENCES

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