The Elongin B Ubiquitin Homology Domain
IDENTIFICATION OF ELONGIN B SEQUENCES IMPORTANT FOR INTERACTION WITH ELONGIN C

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Mammalian Elongin B is a 118-amino acid protein composed of an 84-amino acid amino-terminal ubiquitin-like domain and a 34-amino acid carboxyl-terminal tail. Elongin B is found in cells as a subunit of the heterodimeric Elongin BC complex, which was originally identified as a positive regulator of RNA polymerase II elongation factor Elongin A and subsequently as a component of the multiprotein von Hippel-Lindau tumor suppressor and suppressor of cytokine signaling complexes. As part of our effort to understand how the Elongin BC complex regulates the activity of Elongin A, we are characterizing Elongin B functional domains. In this report, we show that the Elongin B ubiquitin-like domain is necessary and sufficient for interaction with Elongin C and for positive regulation of Elongin A transcriptional activity. In addition, by site-directed mutagenesis of the Elongin B ubiquitin-like domain, we identify a short Elongin B region that is important for its interaction with Elongin C. Finally, we observe that both the ubiquitin-like domain and carboxyl-terminal tail are conserved in Drosophila melanogaster and Caenorhabditis elegans. Elongin B homologs that efficiently substitute for mammalian Elongin B in reconstitution of the transcriptionally active Elongin ABC complex, suggesting that the carboxyl-terminal tail performs an additional function not detected in our assays.

Elongin B is a subunit of the heterodimeric Elongin BC complex, which was originally identified as a positive regulator of RNA polymerase II elongation factor Elongin A (1, 2). Subsequently the Elongin BC complex was identified as a component of both the multiprotein von Hippel-Lindau tumor suppressor complex (3, 4), where it appears to function as a positive regulator of RNA polymerase II elongation factor Elongin A (1, 2). Subsequently the Elongin BC complex was identified as a component of both the multiprotein von Hippel-Lindau tumor suppressor complex (3, 4), where it appears to function as a positive regulator of RNA polymerase II elongation factor Elongin A (1, 2).

As part of our effort to understand the structure of the Elongin BC complex and its mechanism of action in regulation of Elongin A transcriptional activity, we are carrying out a systematic structure-function analysis of each of the Elongin subunits. In this report, we demonstrate that the Elongin B ubiquitin-like domain is necessary and sufficient for interaction of Elongin B with Elongin C and for regulation of Elongin A activity. In addition, by site-directed mutagenesis of the Elongin B ubiquitin-like domain, we identify a short Elongin B region that is important for its interaction with Elongin C and that is evolutionarily conserved in Elongin B homologs from D. melanogaster and C. elegans.

EXPERIMENTAL PROCEDURES

Materials—Unlabeled ultrapure ribonucleoside 5'-triphosphates were purchased from Pharmacia Biotech Inc. [α-32P]CTP (>650 Ci/mmol) was obtained from Amersham Corp. Ni2+-nitroliotriacetic acid-agarose (Ni2+-agarose)1 was from Invitrogen. Placental ribonuclease

1 The abbreviations used are: Ni2+-agarose, Ni2+-nitroliotriacetic acid-agarose; RNasin, placental ribonuclease inhibitor; AdML, adenosivirus 2 major late; EST, expressed sequence tag; HPLC, high pressure liquid chromatography; HSV, herpes simplex virus; TF, transcription factor.
inhibitor (RNasin) and acetylated bovine serum albumin were from Promega. Guanidine hydrochloride (sequanal grade) was purchased from Pierce. Phenylmethylsulfonyl fluoride and polyvinyl alcohol (average molecular weight 30,000–70,000) were obtained from Sigma. Phenylmethylsulfonyl fluoride was dissolved in dimethyl sulfoxide to 1 M and stored at −20 °C. DNA was dissolved in water (0.5% [v/v]) and centrifuged or filtered through a 0.2-μm filter prior to use.

**DNA Template for Transcription—**pDN-AdML plasmid DNA (20) was isolated from Escherichia coli using the Triton-lysozyme method (21). Plasmid DNA was beaded twice in 0.5Ci in CsCl-ethidium bromide density gradients, precipitated with ethanol, and dissolved in TE buffer (20 mM Tris.HCl, pH 8.0, and 1 mM EDTA). A restriction fragment prepared by digestion of pDN-AdML DNA with EcoRI and NdeI was used as template in transcription reactions. The fragment was purified from 1.0% low melting temperature agarose gels using GELase (Epicentre Technologies) according to the manufacturer’s instructions. After phenol-chloroform extraction and ethanol precipitation, purified DNA fragments were resuspended in TE buffer.

**Preparation of RNA Polymerase II and Initiation Factors—**RNA polymerase II (22) and TFIIH (rat technologies) according to the manufacturer’s instructions. After phenol-chloroform extraction and ethanol precipitation, purified DNA fragments were resuspended in TE buffer.

**Assay of Run-off Transcription—**200 ng of wild type or mutant Elongin B proteins were mixed with ~40 ng of wild type rat Elongin A and ~200 ng of rat Elongin C (~141–50) and diluted ~2 fold to a final volume of 50 μl with 40 mM Hepes-NaOH (pH 7.9), 100 mM KCl, 50 μM ZnSO₄, and 10% (v/v) glycerol. After 90 min on ice, the mixtures were dialyzed for 2 h at 4 °C against 40 mM Tris-HCl (pH 7.9), 40 mM KCl, 0.1 mM EDTA, and 10% (v/v) glycerol. Elongin complexes were then assayed in 60-μl reaction mixtures as follows. Preinitiation complexes were assembled by preincubation of ~10 ng of the EcoRI to NdeI fragment from pDN-AdML, ~10 ng of recombinant TFIIH, ~10 ng of recombinant TFIIA, ~40 ng of rat TFIIH, ~20 ng of TFIIA, ~0.1 unit of RNA polymerase II, and 8 units of RNAse in 20 mM Hepes-NaOH (pH 7.9), 20 mM Tris-HCl (pH 7.9), 60 mM KCl, 2 mM DTT, 0.5 mg/ml bovine serum albumin, 2% (v/v) polyvinyl alcohol, and 3% (v/v) glycerol for 30 min at 28 °C. 10 μl of dialyzed Elongin complexes were then added to each reaction mixture. Transcription was initiated by addition of 7 mM MgCl₂ and 50 mM ATP, 50 mM GTP, 10 mM CTP, 10 mM UTP, and 10 μCi of [α-32P]CTP. After 90 min on ice, the mixtures were dialyzed for 2 h at 4 °C against 40 mM Tris-HCl (pH 7.9), 100 mM KCl, 50 μM ZnSO₄, and 10% (v/v) glycerol. After 30 min on ice, the mixtures were dialyzed for 2 h at 4 °C against 40 mM Hepes-NaOH (pH 7.9), 100 mM KCl, 50 μM ZnSO₄, and 10% (v/v) glycerol. Following dialysis, the mixtures were incubated for 1 h at 4 °C with ~20 μl of Ni²⁺-agarose pre-equilibrated in dialysis buffer containing 10 μM imidazole (pH 8.0) and then centrifuged for 10 min at 20,000 g in a Microcentrifuge (Microfuge 16; International Equipment CO). The resulting supernatants were applied to TSK SP-NPR columns (35 × 0.75 cm, Toso-Haas) pre-equilibrated in 40 mM Hepes-NaOH (pH 7.9), 100 mM KCl, 50 μM ZnSO₄, and 10% (v/v) glycerol. After a further 2-h incubation at 37 °C, the complexes were centrifuged at 100,000 g for 30 min at 4 °C. The resulting supernatants were assayed using a pET16b expression vector (Novagen) and an M13mpET bacteriophage expression system (26) and purified as described below. Briefly, 100-ml cultures of E. coli strain JM109(DE3) were grown at 37 °C to an A₆₀₀ of 0.6 in Luria broth. Cells were infected with the appropriate M13mpET bacteriophage and centrifuged at 6000 g for 30 min on ice. The cell pellets were resuspended in 7 ml of 20 mM Tris-HCl (pH 8.0), 1 mg/ml lysozyme, and 10 mM imidazole (pH 8.0) and incubated for 30 min on ice. After one cycle of freeze-thaw, the suspensions were centrifuged at 100,000 × g for 35 min. Inclusion bodies were solubilized by resuspension in 7 ml of ice-cold 6 M guanidine hydrochloride, 40 mM Tris-HCl (pH 8.0), 0.5 mM KCl, 10 mM imidazole (pH 8.0), and 0.5 mM phenylmethylsulfonyl fluoride, and the resulting suspensions were clarified by centrifugation at 100,000 × g for 35 min. All histidine-tagged proteins were further purified by Ni²⁺-agarose chromatography in guanidine hydrochloride as described (26).

**Expression and Purification of D. melanogaster and C. elegans Elongin B Proteins—**A TBLASTN search of the GenBank EST database identified potential Elongin B homologs from D. melanogaster and C. elegans.

**Expression and Purification of D. melanogaster and C. elegans Elongin B Proteins—**A TBLASTN search of the GenBank EST database identified potential Elongin B homologs from D. melanogaster and C. elegans. EST eyk121b3 encoding the potential C. elegans Elongin B homolog was obtained from Y. Kohara (National Institute of Genetics, Mishima, Japan). EL16618 encoding the potential Drosophila Elongin B homolog was obtained from Genome Systems, Inc. The entire open reading frames of the Drosophila and C. elegans proteins were overexpressed in E. coli with NH₂-terminal histidine tags using the M13mpET bacteriophage expression system (22).

**Expression of Elongin BC Complex Formation—**2 μg of histidine-tagged wild type rat Elongin B or rat Elongin B mutants were mixed with ~2 μg of histidine-tagged wild type C and diluted 20-fold with 40 mM Hepes-NaOH (pH 7.9), 100 mM KCl, 50 μM ZnSO₄, and 10% (v/v) glycerol. After 30 min on ice, the mixtures were dialyzed for 2 h at 4 °C against 40 mM Hepes-NaOH (pH 7.9), 100 mM KCl, 10% (v/v) glycerol and centrifuged for 20 s at 2000 rpm. Finally, bound material was eluted with 300 μl of the same buffer containing 300 mM imidazole (pH 8.0). Aliquots of each fraction were analyzed by 12.5% and 18% SDS-polyacrylamide gel electrophoresis, and the proteins were visualized by silver staining.

**Expression of Elongin ABC Complex Formation—**6 μg of histidine-tagged wild type rat, C. elegans, or D. melanogaster Elongin B or rat Elongin B mutants were mixed with ~45 μg of wild type histidine-tagged rat Elongin C and diluted 5-fold with 40 mM Hepes-NaOH (pH 7.9), 100 mM KCl, 50 μM ZnSO₄, and 10% (v/v) glycerol. After 90 min on ice, the mixtures were dialyzed for 2 h at 4 °C against 40 mM Tris-HCl (pH 7.9), 40 mM KCl, 0.1 mM EDTA, 10% (v/v) glycerol, and 40 mM imidazole (pH 8.0) and centrifugation for 20 s at 2000 rpm. Finally, bound material was eluted with 300 μl of the same buffer containing 300 mM imidazole (pH 8.0). Aliquots of each fraction were analyzed by 12.5% and 18% SDS-polyacrylamide gel electrophoresis, and the proteins were visualized by silver staining.

**Expression of Elongin A Subunits in Mammmalian Cells—**DNAs encoding full-length Elongin B and Elongin B mutants containing NH₂-terminal e-Myc epitope tags were subcloned into the pCDNA3.1 vector (Invitrogen). Full-length Elongin C containing an NH₂-terminal HSV epitope tag was subcloned into the pc-Neo vector (Promega). 24 h after transfection of 293T cells, 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 aprolix. Lysates were then centrifuged at 10,000 × g for 20 min at 4 °C.

**Immunoprecipitation and Western Blotting—**Anti-e-Myc epitope antibodies were from Roche Molecular Biochemicals and anti-HSV epitope antibodies were from Novagen. To immunoprecipitate Elongin B and associated proteins from 293T cell lysates, the lysates were incubated with anti-e-Myc antibody for 1 h at 4 °C and then with protein A PLUS-agarose (Bio-Rad) and centrifuged at 14,000 g at 4 °C. Unbound material was eluted with 0.6 ml/mg with a 9-mI linear gradient from 100 to 900 mM KCl in 40 mM Hepes-NaOH (pH 7.9), 1 mM DTT, and 10% (v/v) glycerol. Aliquots of each fraction were analyzed by 12.5% SDS-polyacrylamide gel electrophoresis, and the proteins were visualized by silver staining.

**RESULTS**

**Evolutionary Conservation of Elongin B Proteins—**We previously described cloning of rat and human Elongin B cDNAs (2). Char-
The characterization of these cDNAs revealed that Elongin B is composed of an ~84 amino acid NH₂-terminal ubiquitin-like domain fused to a ~34-amino acid COOH-terminal tail. The NH₂-terminal ubiquitin-like domain can be modeled as ubiquitin (2), a compact globular structure containing a 3.5 turn α-helix (α1), a short 310 helix, and a 5-stranded mixed β-sheet (30).

As an initial step in our analysis of the structure and function of Elongin B, we identified and characterized potential *D. melanogaster* and *C. elegans* Elongin B homologs. Comparison of the amino acid sequences of mammalian Elongin B and the potential *Drosophila* and *C. elegans* Elongin B homologs indicates that they are highly conserved (Fig. 1). According to the BESTFIT program of GCG, the rat and *Drosophila* proteins are 55% identical and 78% similar; the rat and *C. elegans* proteins are 39% identical and 62% similar; and the *Drosophila* and *C. elegans* proteins are 36% identical and 56% similar. A TBLASTN search of the *S. cerevisiae* data base revealed no *S. cerevisiae* open reading frames with significant sequence similarity to mammalian Elongin B, even though the *S. cerevisiae* genome includes an open reading frame that encodes a 100-amino acid protein that exhibits significant sequence similarity to mammalian Elongin C (29, 31).

To determine whether the potential *Drosophila* and *C. elegans* Elongin B homologs possess activities similar to those of mammalian Elongin B, we investigated their abilities to substitute for rat Elongin B in reconstitution of the Elongin complex. In previous studies, we showed that the transcriptionally active Elongin ABC complex could be reconstituted by recombining individual Elongin subunits purified from rat liver (1, 32) or by refolding bacterially expressed Elongin subunits purified from guanidine hydrochloride-solubilized inclusion bodies (10). Formation of the Elongin ABC complex can be assayed by ion exchange HPLC using TSK SP-NPR (2, 29). Elongin BC complexes and free Elongin B and C flow-through TSK SP-NPR at low ionic strength, whereas Elongin ABC complexes bind tightly to TSK SP-NPR and elute with ~0.3 M KCl.

To investigate the abilities of the potential *Drosophila* and *C. elegans* Elongin B homologs to assemble with mammalian Elongin A and C into chromatographically isolable ternary complexes, the *Drosophila* and *C. elegans* proteins were expressed in *E. coli*, purified from guanidine-solubilized inclusion bodies, refolded together with bacterially expressed rat Elongin A and C, and subjected to TSK SP-NPR HPLC. As shown in Fig. 2A, like rat Elongin B, both the potential *Drosophila* and *C. elegans* proteins are capable of assembling with rat Elongin A and C to form ternary complexes that can be isolated by TSK SP-NPR HPLC; neither the *Drosophila* nor *C. elegans* proteins bound to TSK SP-NPR in the absence Elongin A (data not shown).

To determine whether the potential *Drosophila* and *C. elegans* Elongin B homologs function similarly to mammalian Elongin B in transcription, we assayed them for their abilities to promote activation of Elongin A transcriptional activity by Elongin C. We previously showed that Elongins B and C play different roles in activation of Elongin A activity (10). Elongin C functions as the inducing ligand and activates Elongin A by binding to a site in the Elongin A elongation activation domain. Although Elongin B is not essential for activation of Elongin A by Elongin C, it promotes interaction of Elongin C with Elongin A and, in so doing, increases both the yield and stability of the functional Elongin complex.

To devise an assay for Elongin transcriptional activity with the strongest possible dependence on Elongin B, we took ad-
vantage of an Elongin C mutant (Elongin C-(Δ41–50)) (29) that
does not bind stably to Elongin A in the absence of Elongin B
(data not shown) and that does not detectably activate Elongin
A transcriptional activity unless Elongin B is present (Fig. 2B).
In these experiments, Elongin complexes were assembled with
rat Elongin A and Elongin C-(Δ41–50) in the absence of Elongin
B or in the presence of either rat Elongin B or the potential
Drosophila or C. elegans Elongin B homologs. The Elongin
complexes were then assayed for their abilities to stimulate the
rate of accumulation of run-off transcripts synthesized by RNA
polymerase II from the AdML promoter in a purified basal
transcription system reconstituted with the general initiation
factors TBP, TFIIB, TFIIE, TFIIF, and TFIIH. As shown in Fig.
2B, like rat Elongin B, the potential Drosophila and C. elegans
Elongin B homologs were capable of strongly promoting acti-
vation of Elongin A by Elongin C-(Δ41–50). Taken together, the
results of both binding and transcription assays argue that the
Drosophila and C. elegans proteins are homologs of mamma-
lian Elongin B.

The Elongin B Ubiquitin-like Domain Is Sufficient for Elon-
gin B Function in Vitro—To determine whether the NH₂-
termin al Elongin B ubiquitin-like domain, the COOH-terminal
tail, or both are required for Elongin B function, a series of
NH₂-terminal, COOH-terminal, and internal deletion mutants
of rat Elongin B were constructed (Fig. 3A), expressed in E. coli,
purified from guanidine hydrochloride-solubilized inclusion
bodies, and assayed for their abilities to assemble into Elongin
BC and ABC complexes and to promote activation of Elongin A
by Elongin C-(Δ41–50).

To assess the abilities of Elongin B deletion mutants to
assemble with Elongin C into Elongin BC complexes, we as-
sayed rat Elongin B mutants containing NH₂-terminal histi-
dine tags for their abilities to retain untagged wild type rat
Elongin C on Ni²⁺-agarose. In these experiments, individual
wild type Elongin B or Elongin B deletion mutants were re-
folded together with Elongin C and subjected to Ni²⁺-agarose
chromatography. Unbound and bound protein fractions were
collected, and equivalent amounts of each fraction were ana-
yzed by SDS-polyacrylamide gel electrophoresis. As shown in
Fig. 3B, untagged Elongin C does not bind to Ni²⁺-agarose, but
is retained on the resin in the presence of histidine-tagged wild
type Elongin B. Elongin C was quantitatively retained on Ni²⁺-
agarose by any Elongin B mutants with deletions of the NH₂-
terminal portion of the ubiquitin-like domain, and only very
small amounts of Elongin C were retained on Ni²⁺-agarose by
Elongin B mutants with deletions of the COOH-terminal por-
tion of the ubiquitin-like domain. Taken together, these results
argue that the Elongin B ubiquitin-like domain is necessary
and sufficient for stable interaction with Elongin C in vitro.

To assess the abilities of the Elongin B deletion mutants to
support formation of functional Elongin ABC complexes, we
assayed Elongin B mutants for their abilities (i) to form Elongin ABC complexes isolable by TSK SP-NPR HPLC and (ii) to promote activation of Elongin A by Elongin C-(Δ41–50). In experiments investigating the abilities of Elongin B deletion mutants to support formation of the Elongin ABC complex, individual wild type Elongin B and Elongin B deletion mutants were refolded together with bacterially expressed wild type rat Elongin A and C and subjected to TSK SP-NPR HPLC. In experiments investigating the abilities of Elongin B deletion mutants to promote activation of Elongin A by Elongin C-(Δ41–50), bacterially expressed wild type rat Elongin A and Elongin C-(Δ41–50) were refolded together in the presence or absence of wild type rat Elongin B or Elongin B deletion mutants. Elongin complexes were then assayed for their abilities to stimulate the rate of accumulation of full-length run-off transcripts synthesized from the AdML promoter by RNA polymerase II and purified initiation factors TBP, TFIIB, TFIIE, TFIIF, and TFIIH.

As shown in Fig. 3, C and D, Elongin B-(1–84), which includes the entire ubiquitin-like domain and binds stably to Elongin C (Fig. 3B), was also capable of assembling with Elongin A and C into an isolable Elongin ABC complex and of strongly promoting activation of Elongin A transcriptional activity by Elongin C-(Δ41–50) mutant. In contrast, Elongin B deletion mutants lacking as few as 10 amino acids from the NH2 terminus of the ubiquitin-like domain did not form Elongin ABC complexes and did not promote activation of Elongin A by Elongin C-(Δ41–50). Elongin B-(1–74), Elongin B-(1–64), and Elongin B-(Δ58–66), which have deletions in the COOH terminus of the ubiquitin-like domain, did not promote detectable activation of Elongin A by Elongin C-(Δ41–50), but did form Elongin ABC complexes. Thus, the entire Elongin B ubiquitin-like domain is necessary and sufficient for promoting activation of Elongin A transcriptional activity by Elongin C-(Δ41–50). Elongin B residues 58–84 of the ubiquitin-like domain are not essential for formation of Elongin ABC complexes, although deletion mutants lacking these residues are severely impaired in their abilities to form Elongin BC complexes. Elongin B-(Δ58–66), Elongin B-(1–64), and Elongin B-(1–74) lack portions of predicted helix 2, the extended surface loop, and/or predicted β-sheet 5, regions of the protein that would be important for maintaining a ubiquitin-like tertiary structure (30, 33–35). Our results raise the possibility that contacts between Elongin B and the Elongin AC complex may contribute to proper folding of the Elongin B ubiquitin-like domain by compensating for loss of the predicted surface loop, portions of helix 2, or predicted β-sheet 5.

**Mutagenesis of the Elongin B Ubiquitin-like Domain**—Although ubiquitin is similar in sequence to Elongin B, ubiquitin neither binds detectably to Elongin C nor promotes activation of Elongin A transcriptional activity by Elongin C (2), suggesting that Elongin B sequences differing from those of ubiquitin are important for Elongin B function. As discussed above, the Elongin B ubiquitin-like domain can be modeled as ubiquitin. Notable features of the Elongin B model include its striking conservation of the ubiquitin hydrophobic core and conservation of the hydrophobic character of residues corresponding to ubiquitin surface residues Phe-4 and Leu-71. The Elongin B model also predicts Elongin B features not shared by ubiquitin; these include two additional hydrophobic surface residues Phe-15 and Phe-25, a prominent basic surface patch composed predominantly of residues Arg-8, Arg-9, His-10, and Lys-11, and an extended surface loop that falls between residues 62 and 70 and accommodates a 7-amino acid insertion in the ubiquitin-like domain.

To explore the relationship between Elongin B and ubiquitin and define in more detail Elongin B residues critical for its function, we investigated the activities of a collection of additional Elongin B mutants that were constructed either by replacing predicted Elongin B surface residues with those from the corresponding positions of ubiquitin, mutating the predicted Elongin B surface hydrophobic residues, or mutating Elongin B residues within the predicted basic patch. These mutants, which contained mutations spanning the entire ubiquitin-like domain, were tested for their abilities, (i) to assemble into isolable Elongin BC complexes in cells and (ii) to assemble into Elongin BC and ABC complexes in vitro and to promote activation of Elongin A by Elongin C-(Δ41–50) in vitro. To assay formation of Elongin BC complexes in cells, c-Myc-tagged Elongin B and Elongin B mutants were coexpressed with HSV-tagged Elongin C in 293T cells and tested for their abilities to interact with one another by coimmunoprecipitation with an anti-c-Myc antibody. Formation of Elongin BC and ABC complexes and activation of Elongin transcription activity in vitro were assayed as described above. Results of these experiments can be summarized as follows.

(i) Of the Elongin B mutants tested, only three exhibited reduced ability to form Elongin BC complexes in cells (Fig. 4). These mutants, R29A, G33D/I34K/L35E/K36G, and EloB(Δ31–40), also failed to assemble into isolable Elongin BC and ABC complexes in vitro and to promote activation of Elongin A by Elongin C-(Δ41–50) (Fig. 5). R29A, G33D/I34K/L35E/K36G, and EloB(Δ31–40) mutations are confined to a short region in the COOH-terminal portion of predicted helix 1 and in the predicted turn between helix 1 and β-strand 3. Notably, the R29A and GILK to DKEG mutations change Elongin B residues to the corresponding residues from ubiquitin. Additional ubiquitin-like mutants, including F15T, D17E, K19E/E20P, Y45I/K46F/D47A/D48G/Q49K, D52E, K55R, G76H, and the mutant in which Ala-81 and Asp-82 were mutated to LRGG, were capable of binding Elongin C in vivo (Fig. 4), suggesting that differences between Elongin B and ubiquitin in helix 1 and in the turn immediately following may be sufficient to account for the inability of ubiquitin to bind Elongin C.

(ii) Of the ubiquitin-like mutants that were capable of binding Elongin C in vivo, none assembled into Elongin BC complexes in vitro, suggesting that the mutations interfere with proper folding of Elongin B in vitro (Fig. 5A). Several of these mutants, however, were able to form Elongin ABC complexes and/or to promote activation of Elongin A by Elongin C-(Δ41–50).
50) (Fig. 5, A and B). The mutant Y45I/K46F/D47A/D48G/Q49K, in which residues located at the COOH terminus of β-strand 3 and in the turn immediately following were mutated to the corresponding residues of ubiquitin, was able to form an Elongin ABC complex and to promote activation of Elongin A by Elongin C-(Δ41–50) as well as or better than wild type Elongin B. Another mutant, constructed by replacing Elongin B residues Lys-19 and Glu-20 with the predicted corresponding ubiquitin residues Glu-18 and Pro-19, which occupy a position in the turn between the second β-sheet and the first α-helix, failed to form isolable Elongin BC or ABC complexes in vitro, but did promote activation of Elongin A by Elongin C-(Δ41–50). A third mutant K11G, formed isolable Elongin ABC complexes in vitro, but did not promote activation of Elongin A by Elongin C-(Δ41–50).

(iii) The single Elongin B point mutations R8N, R9G, H10S, and K11N, which decrease the net positive charge in the predicted basic surface patch and which alter residues in a predicted turn between β-strands 1 and 2, had no detectable effect on the ability of Elongin B to form Elongin BC (Fig. 6A) and ABC complexes in vitro (Fig. 6B) or to promote activation of Elongin A transcriptional activity A by Elongin C-(Δ41–50) (Fig. 6C). Consistent with these observations, R9G could be immunoprecipitated from cell lysates with Elongin C (Fig. 4). An additional mutant, K11G, could also be immunoprecipitated from cell lysates with Elongin C (Fig. 4); however, when assayed for activity in vitro, this mutant was found to assemble into ABC complexes but not to promote activation of Elongin A by C-(Δ41–50) (Fig. 5, B and C). K11G was also unable to assemble into isolable Elongin BC complexes in vitro, suggesting that its failure to promote activation of Elongin A by C-(Δ41–50) might be due to improper folding of the protein purified from inclusion bodies.

(iv) Mutations of predicted Elongin B surface hydrophobic residues Phe-4, Phe-15, Phe-25, and Phe-62, which fall within predicted β-strand 1, β-strand 2, at the NH2 terminus of predicted helix 1, and in predicted helix 2, respectively, had different effects on Elongin B function (Fig. 7). Elongin B point mutants F4N, F15N, and F25N could assemble into isolable Elongin BC and ABC complexes in vitro and promote activation of Elongin A by Elongin C-(Δ41–50), although Elongin B mutant F15N was less active than wild type Elongin B in promoting activation of Elongin A. Elongin B point mutant F62N, which contains a mutation at the NH2 terminus of the predicted Elongin B surface loop not present in ubiquitin, assembled into Elongin BC and ABC complexes, but did not promote activation of Elongin A by Elongin C-(Δ41–50). Finally, F15T, which was constructed by replacing Elongin B residue Phe-15 with the predicted corresponding ubiquitin residue Thr-14, formed isolable Elongin BC complexes in cells but did not assemble into detectable Elongin BC or ABC complexes in vitro and did not promote activation of Elongin A by Elongin C-(Δ41–50), suggesting that the F15T mutation might interfere with proper folding of Elongin B in vitro.
Elongin B Ubiquitin Homology Domain

DISCUSSION

In this report, we have investigated Elongin B sequences required for its interaction with Elongin C. Mammalian Elongin B is a 118-amino acid protein composed of an ~84 amino acid ubiquitin-like domain fused to an ~34 amino acid COOH-terminal tail (2). The Elongin B ubiquitin-like domain shares ~30% sequence identity with ubiquitin. Other ubiquitin-like proteins include NEDD8 and its yeast homologue Rub1p, SUMO, and Rad23, which have roles in cell cycle control, nucleocytoplasmic transport, and DNA repair, respectively (16–19). These ubiquitin-like proteins exhibit a variable degree of similarity with ubiquitin, ranging from more than 50% sequence identity (NEDD8 and Rub1p) to less than 20% identity (SUMO). Despite the variation in their primary sequences, ubiquitin, NEDD8, Rub1p, and SUMO have very similar tertiary structures (33–35). Although a crystal structure of Elongin B has not yet been reported, Elongin B can be modeled as ubiquitin (2).

Analysis of the functions of Elongin B mutants has revealed that the Elongin B ubiquitin-like domain is sufficient for its interaction with Elongin C in vivo and in vitro and for reconstitution of the transcriptionally active Elongin complex in vitro. Furthermore, we observe that neither the predicted surface hydrophobic residues nor residues that make up the predicted basic surface patch are critical for reconstitution of functional Elongin complexes in vitro. We did, however, identify three mutations, R29A, G33D/I34K/L35E/K36G, and 33–40, which fall within the ubiquitin-like domain and significantly reduce the interaction of Elongin B with Elongin C in vivo and in vitro. Notably, these mutations are confined to a short region in the COOH-terminal portion of predicted α-helix 1 and in the predicted turn between helix 1 and β-strand 3. Although we cannot rule out the possibility that these mutations affect the overall conformation of Elongin B, our results are consistent with the model that the COOH terminus of predicted α-helix 1 and the predicted turn between helix 1 and β-strand 3 may form a surface that interacts directly with Elongin C or may be important for maintaining Elongin B in a conformation that can interact with Elongin C. Two of the inactivating mutations, R29A and G33D/I34K/L35E/K36G, change Elongin B residues to the corresponding residues from ubiquitin. Sequence differences between Elongin B and ubiquitin in this region may be sufficient to account for the inability of ubiquitin to bind Elongin C, since eight additional ubiquitin-like mutations located throughout the ubiquitin-like domain had no significant effect on the interaction of Elongin B with Elongin C in cells.

Finally, by characterizing Drosophila and C. elegans Elongin B homologs that efficiently replace mammalian Elongin B in reconstitution of the transcriptionally active Elongin ABC complex, we show that both the Elongin B ubiquitin-like domain and COOH-terminal tail have been highly conserved during evolution. Although we have not yet identified a function for the Elongin B tail, it is noteworthy that it includes a PXXP motif that is a potential target for binding by SH3 domain proteins (36). Efforts to identify cellular proteins that interact with the Elongin B tail are underway.

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REFERENCES

1. Bradsher, J. N., Jackson, K. W., Conaway, R. C., and Conaway, J. W. (1993) J. Biol. Chem. 268, 25587–25593
2. Garrett, K. P., Aso, T., Bradsher, J. N., Foundling, S. I., Lane, W. S., Conaway, R. C., and Conaway, J. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7172–7176
3. Duan, D. R., Pause, A., Burgess, W. H., Aso, T., Chen, D. Y. T., Garrett, K. P., Conaway, R. C., Conaway, J. W., Linehan, W. M., and Klaussner, R. D. (1995) Science 269, 1402–1406
4. Kibeli, A., Iliopoulos, O., DeCaprio, J. A., and Kaelin, W. G. (1995) Science 269, 1444–1446
5. Iliopoulos, O., Kibel, A., Gray, S., and Kaelin, W. G. (1995) Nature Med. 1, 822–826
6. Iliopoulos, O., Levy, A. P., Jiang, C., Kaelin, W. G., and Goldberg, M. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10595–10599
Elongin B Ubiquitin Homology Domain

7. Gnarra, J. R., Zhou, S., Merrill, M. J., Wagner, J. R., Krumm, A., Papavassiliou, E., Oldfield, E. H., Klausner, R. D., and Linehan, W. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10589–10594
8. Lonergan, K. M., Ilipoulos, O., Ohh, M., Kamura, T., Conaway, R. C., Conaway, J. W., and Kaelin, W. G. (1996) Mol. Cell. Biol. 16, 732–741
9. Kamura, T., Sato, S., Haque, D., Liu, L., Kaelin, W. G., Conaway, R. C., and Conaway, J. W. (1998) Genes Dev. 12, 3872–3881
10. Aso, T., Lane, W. S., Conaway, J. W., and Conaway, R. C. (1996) EMBO J. 15, 5557–5566
11. Hershko, A., and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761–807
12. Ciechanover, A., and Schwartz, A. L. (1994) FASEB J. 8, 182–191
13. Ozkaynak, E., Finley, D., Solomon, M. J., and Varshavsky, A. (1987) EMBO J. 6, 1429–1439
14. Finley, D., Bartel, B., and Varshavsky, A. (1989) Nature 338, 394–401
15. Lammer, D., Mathias, N., Laplaza, J. M., Jiang, W., Liu, Y., Callis, J., Goebel, M., and Estelle, M. (1998) Genes Dev. 12, 914–926
16. Kamitani, T., Kito, K., Nguyen, H. P., and Yeh, E. T. H. (1997) J. Biol. Chem. 272, 28557–28562
17. Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997) Cell 88, 97–107
18. Matunis, M. J., Coutavas, E., and Blobel, G. (1996) J. Cell Biol. 135, 1477–1479
19. Conaway, R. C., and Conaway, J. W. (1988) J. Biol. Chem. 263, 2962–2968
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21. Serizawa, H., Conaway, J. W., and Conaway, R. C. (1993) Nature 363, 371–374
22. Conaway, J. W., Bradsher, J. N., and Conaway, R. C. (1992) J. Biol. Chem. 267, 10142–10148
23. Conaway, J. W., Hanley, J. P., Garrett, K. P., and Conaway, R. C. (1991) J. Biol. Chem. 266, 7894–7811
24. Tsuboi, A., Conger, K., Garrett, K. P., Conaway, R. C., and Conaway, J. W., and Arai, N. (1992) Nucleic Acids. Res. 20, 3250
25. Peterson, M. G., Inostroza, J., Maxon, M. E., Flores, O., Admon, A., Reinberg, D., and Tjian, R. (1991) Nature 354, 369–373
26. Tan, S., Conaway, R. C., and Conaway, J. W. (1994) BioTechniques 16, 824–828
27. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
28. Takagi, Y., Conaway, R. C., and Conaway, J. W. (1996) J. Biol. Chem. 271, 25562–25568
29. Vijay-Kumar, S., Bugg, C. E., and Cook, W. J. (1987) J. Mol. Biol. 194, 531–544
30. Aso, T., and Conrad, M. N. (1997) Biochem. Biophys. Res. Commun. 241, 334–340
31. Garrett, K. P., Tan, S., Bradsher, J. N., Lane, W. S., Conaway, J. W., and Conaway, R. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5237–5241
32. Rao-Naik, C., delaCruz, W., Laplaza, J. M., Tan, S., Callis, J., and Fisher, A. J. (1998) J. Biol. Chem. 273, 34976–34982
33. Bayer, P., Arndt, A., Metzger, S., Mahajan, R., Melchior, F., Jaenicke, R., and Becker, J. (1998) J. Mol. Biol. 280, 275–286
34. Whitby, F. G., Xia, G., Pickart, C., and Hill, C. P. (1998) J. Biol. Chem. 273, 34983–34991
35. Yu, H., Chen, J. K., Feng, S., Dalgaro, D., Brauer, A. W., and Schreiber, S. L. (1996) Cell 76, 933–945