Structure of the VHL-ElonginC-ElonginB Complex: Implications for VHL Tumor Suppressor Function

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Mutation of the VHL tumor suppressor is associated with the inherited von Hippel–Lindau (VHL) cancer syndrome and the majority of kidney cancers. VHL binds the ElonginC-ElonginB complex and regulates levels of hypoxia-inducible proteins. The structure of the ternary complex at 2.7 angstrom resolution shows two interfaces, one between VHL and ElonginC and another between ElonginC and ElonginB. Tumorigenic mutations frequently occur in a 35-residue domain of VHL responsible for ElonginC binding. A mutational patch on a separate domain of VHL indicates a second macromolecular binding site. The structure extends the similarities to the SCF (Skp1-Cul1–F-box protein) complex that targets proteins for degradation, supporting the hypothesis that VHL may function in an analogous pathway.

VHL syndrome is characterized by the dominantly inherited predisposition to develop tumors of the central nervous system, kidney, retina, pancreas, and adrenal gland (1). VHL syndrome is caused by germline mutations in the VHL tumor suppressor, and VHL tumors are associated with loss or mutation of the remaining wild-type allele (2). VHL is also inactivated in ~80% of sporadic clear cell renal carcinomas (RCC), the predominant form of kidney cancer (3). The ability of RCC cells to form tumors in nude mice can be abrogated by introduction of wild-type VHL (4, 5).

VHL-associated tumors are highly vascularized (6), and this supports the current model that VHL negatively regulates the production of hypoxia-inducible factors such as the angiogenic vascular endothelial growth factor (VEGF) (5, 7, 8). VHL^{-/-} tumor cells have high levels of these factors, and reintroduction of VHL down-regulates them under normoxic conditions (5, 7, 8). The mechanism of this VHL activity is not well understood, but appears to be mediated through the destabilization of hypoxia-regulated mRNA transcripts (5, 7, 8).

Biochemical studies revealed that VHL forms a ternary complex with the ElonginC and ElonginB proteins (9-11). The VHL-ElonginC-ElonginB complex (henceforth VCB complex) has a central role in VHL function because most of the tumor-derived mutations de-

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stabilize this complex (9-12). Furthermore, peptide mapping studies showed that a 12amino acid region of VHL that contains nearly a quarter of the tumor-derived mutations makes key interactions with ElonginC (10), tightly linking the ElonginC binding and tumor suppression functions of VHL (9-12). Recent studies have shown that the VCB complex binds Cul2, in part through ElonginC-Cul2 association (12, 13). The formation of this VCB-Cul2 complex has been shown genetically to be required for the VHL-mediated regulation of VEGF mRNA (12). In addition to VHL, the ElonginC-ElonginB complex also binds to the suppressor of cytokine signaling (SOCS) superfamily of proteins that share the 40-residue SOCS-box motif (14), and to the ElonginA transcriptional elongation factor (15).

VHL is not homologous to any known pro-

Fig. 1. The VCB ternary complex consists of two binary interfaces, one between VHL and ElonginC and the other between ElonginC and ElonginB. On the left is a ribbon diagram illustrating the secondary structure of the VCB complex. On the right is a topology diagram in which circles indicate helices and wide arrows indicate strands. Only the structural elements discussed in the text are labeled in the ribdiagram. C. bon COOH-terminus; N, NH₂-terminus.

tein, but its binding partners share homology to components of the SCF multiprotein complex that targets many cell cycle regulatory proteins for ubiquitin-mediated proteolysis (16). ElonginC has sequence homology to the SCF Skp1 protein (17), ElonginB to ubiquitin (18), and Cul2 to the SCF Cul1 protein (13). One component of the SCF complex without an apparent counterpart in the VCB-Cul2 complex has been the F-box protein, which recruits substrates to the SCF complex (17).

The structure of the VCB ternary complex, reported here, extends the homology between ElonginC and Skp1 and suggests a similarity between a 35-residue domain of VHL that binds ElonginC and the F box that binds Skp1. The structure also suggests that VHL may have a second macromolecular binding site that is important for its tumor suppressing function and suggests that VHL may function as a modular adapter like the F-box proteins.

Overview of the ternary complex. The human VCB complex was produced by coexpressing the three proteins together in *Escherichia coli* (19). We used the alternative in vivo initiating methionine of VHL, which removes a 54-residue GxEEx repeat (G, Gly; E, Glu) not needed for tumor suppression (20). In the crystal structure of this ternary complex (21), ElonginC binds ElonginB and VHL across two distinct interfaces, whereas VHL and ElonginB do not interact (Fig. 1 and Table 1). The two interfaces are comparable in extent, each burying a total surface area of about 2000 Å².

VHL has two domains: a roughly 100-residue NH_2 -terminal domain rich in β sheet (β domain) and a smaller α -helical domain (α domain), held together by two linkers and a polar interface (Fig. 2A). A large portion of the α domain surface, and a small portion of the β domain, interact with ElonginC. About half of the tumorigenic mutations map to the α domain and its residues that contact ElonginC. The



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Fig. 2. Structural elements and conservation of VHL, ElonginC, and ElonginB. (A) Sequence of VHL demonstrating that tumor-derived missense mutations are divided between the α and β domains of VHL, whereas residues contacting ElonginC cluster in the α domain (40). The histogram represents 279 missense mutations in the database (29). The six most frequently mutated amino acids are labeled. Shaded squares above each residue describe the relative solvent exposure of a residue in a hypothetical VHL monomer. Blue boxes indicate residues that make hydrogen bonds or van der Waals contacts with ElonginC. (B) Sequence identity between human ElonginC and ElonginC homologs is indicated by yellow, and identity between human ElonginC and human Skp1, aligned with the program THREADER2, is indicated by green. Residues that make hydrogen bonds or van der Waals contacts with VHL are indicated by red and those contacting ElonginB by blue. Secondary structure and solvent accessibility are as in (A). A disordered segment in ElonginC is indicated with a dashed line and extended insertions in the alignments are indicated by lines below the sequence alignments. (C) Sequence identity between human ElonginB and ElonginB homologs is indicated by yellow. Identity between human ElonginB and human ubiquitin, which maps primarily to hydrophobic core residues, is indicated by green. Residues that make hydrogen bonds or van der Waals contacts with ElonginC are indicated by blue. Secondary structure and solvent accessibility are as in (A), and disordered segments in the ElonginB structure are indicated with a dashed line. (**D**) Close-up view of the α - β interface of VHL. VHL amino acids are in yellow and those of ElonginC are in cyan. White dashed lines indicate hydrogen bonds, red atoms indicate oxygen and blue, nitrogen. A red circle with the letter "M" indicates a residue that is one of the six most frequently mutated in tumors.



remaining mutations map to the β domain, and significantly, to a β domain surface patch uninvolved in ElonginC binding. This suggests that two intact macromolecular binding sites may be required for the tumor suppressor effects of VHL.

ElonginC folds into an α/β roll and binds VHL through helices and loops at its COOHterminus (Figs. 1 and 2B). ElonginB has an α/β roll structure very similar to that of ubiquitin, as expected from sequence homology (Figs. 1 and 2C) (18, 22). The ElonginB-ElonginC interface is dominated by the juxtaposition of two β sheets to form a continuous intermolecular sheet.

Structures of the VHL, ElonginC, and ElonginB proteins. The B domain of VHL consists of a seven-stranded B sandwich (residues 63 to 154) and an α helix (H4; residues 193 to 204) that packs against one of the β sheets through hydrophobic interactions (Figs. 1 and 2A). The α domain of VHL (residues 155 to 192) consists of three α helices (H1, H2, and H3; Figs. 1 and 2A). These pack in an arrangement reminiscent of a four-helix cluster ("folded leaf" classification), except for the absence of a fourth VHL helix (23). A helix from ElonginC (H4) fits into this gap and completes the four-helix cluster arrangement, giving two pairs of helices packing at a perpendicular angle (Fig. 1). The α and β domains are connected by two short polypeptide linkers (residues 154 to 156 and 189 to 194) and by a polar interface that is stabilized by hydrogen-bond networks from the H1 helix, the β sandwich, and ElonginC (Fig. 2D). Several of the residues at the interdomain interface have been found mutated in tumors (Fig. 2, A and D).

The ElonginC structure consists of a threestranded β sheet packing against four α helices, the last of which are separated by a long, wellordered loop in an extended conformation (L5

Fig. 3. ElonginC binds VHL and ElonginB across two separate interfaces. (A) The VHL-ElonginC interface consists of an intermolecular hydrophobic four-helix cluster augmented by additional contacts. VHL and ElonginC secondary structural elements are shown in red and blue, respectively. VHL amino acids are in yellow and those of ElonginC are in cyan. Hydrogen bonds are indicated by white dashed lines. Red atoms indicate oxygen and blue, nitrogen. A red circle with the letter "M" indicates a residue that is one of the six most frequently mutated in cancer. The ElonginC pocket where the VHL Leu¹⁵⁸ binds is made up of Tyr⁷⁶, Phe³³, Leu¹⁰³, and Ala¹⁰⁷, and Cys¹¹² (40). (**B**) The parallel β strand-β strand interaction around

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loop; Figs. 1 and 2B). The extended L5 loop together with the H2, H3, and H4 helices form a concave surface with a central pocket. This is where VHL binds, and mutagenesis of these elements disrupts VHL binding (24). The VHLbinding concave surface is dominated by hydrophobic residues, and sequence alignment indicates that this surface hydrophobicity would be conserved in the fly and yeast ElonginC orthologs (Fig. 2B). Unexpectedly, ElonginC shares significant structural similarity [1.7 Å root mean square deviation (rmsd) for 63 residues] with the tetramerization domain of the Shaker potassium channel (25). However, the regions of ElonginC important for its proteinbinding functions, like the L5 loop, are not similar (25).

(24% sequence identity with ubiquitin; 1.25 Å rmsd over 71 residues) that consists of two helices and several loops packing against a five-stranded β sheet (Figs. 1 and 2C). ElonginB differs from ubiquitin in two respects. It contains an insertion of nine amino acids between the S4 and S5 strands relative to ubiguitin (residues 63 to 71; Fig. 2C), and residues from this insertion make contacts to ElonginC. ElonginB also has an extension of 30 amino acids at the COOH-terminus. In the crystal structure, part of this extension adopts an extended conformation running along one side of the molecule, interacting with ElonginC, and nearly reaching VHL before it becomes disordered (Fig. 1). Twenty additional residues, partially conserved, remain in this disordered segment (Fig. 2C).

ElonginB contains a ubiquitin-like structure

	Table	1. Summarv	of cr	vstallogra	phic anal	vsis. R	les., re	solutio
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		Dif	fraction d	ata and MIR a	analysis	-		
Crystal	Synchrotron beamline	Res. (Å)	Reflections		Data statistics		MIR statistics	
			Unique	Measured	Coverage (%)	R _{sym} † (%)	R _{iso} ‡ (%)	Phasing power§
KAu(CN),*	NSLS X9B	3.40	22,385	90,176	94.0	5.0	_	_
Thimerosal	NSLS X9B	3.50	19,433	70,784	91.4	7.4	32	1.20
K ₂ PtBr ₆	NSLS X9B	4.20	10,067	32,616	80.9	7.4	13	1.11
Native I*	NSLS X9B	3.80	15,729	48,137	93.3	9.1	14	0.81
Native II	CHESS F1	2.70	41,219	123,622	90.9	7.0	-	-
		Refineme	ent and a	nalysis of mol	ecular model			
Crystal	Res. (Å)	Reflections $(F > 2\sigma)$	ns Atoms modeled r) (protein/water)		R _{cryst} /R _{free} ¶ (%, %)	rms deviations bonds, angles, <i>B</i> -factors		
Native II	20.0-2.70	38,609	10	,420/454	23.9, 28.6	0.014 Å, 1.8°, 3.4 Ų		

*In the MIR calculations, the KAu(CN)₂ data set was used as the native, and the Native I data set was used as a derivative with negative occupancy sites. $\begin{array}{c} +R_{sym} = \sum_{h} \sum_{i} \left| I_{h,i} - I_{h} \right| / \sum_{h} \sum_{i} I_{h,i}$ for the intensity (*I*) of *i* observations of reflection h. $\begin{array}{c} +R_{sym} = \sum_{h} \sum_{i} \left| I_{h,i} - I_{h} \right| / \sum_{h} \sum_{i} I_{h,i}$ for the intensity (*I*) of *i* observations of reflection the factor amplitude and F_{PH} is the derivative structure factor amplitude. $\begin{array}{c} \$P_{\text{Ho}} = \sum_{i} \left| F_{\text{PH}} - F_{i} \right| \sum_{i} \left| F_{\text{PH}} \right| - \sum_{i} \left| F_{\text{PH}(\text{obs})} \right|^{2} \right|^{2} / \left| F_{\text{PH}(\text{obs})} - F_{\text{PH}(\text{calc})} \right|^{2} \right|^{2}$, where $F_{\text{H(calc)}}$ is the calculated heavy atom structure factor, and $F_{\text{PH}(\text{obs})}$ and $F_{\text{PH}(\text{calc})}$ are the observed and calculated derivative structure factors, respectively. $= \sum_{i} \left| F_{p} - F_{\text{calc}} \right| / \sum_{i} F_{p} \sum_{i} \sum_{i} \sum_{i} \sum_{i} \sum_{j} F_{i} \sum_{i} F_{i} \sum_{i} \sum_{j} F_{i} \sum_{j} F_{i} \sum_{i} F_{i} \sum_{j} F_{i} \sum_{j} F_{i} \sum_{i} F_{i} \sum_{j} F_{i} \sum_{$



which the ElonginC-ElonginB interface centers is indicated by a two-headed arrow (four hydrogen bonds). ElonginB and ElonginC secondary structural elements are shown in green and blue, respectively. ElonginB amino acids are in magenta and those of ElonginC are in cyan.

Molecular interfaces. The VHL-ElonginC interface is almost completely hydrophobic with only a handful of significant hydrogen bonds at the periphery (Fig. 3A). The H1 helix of the VHL α domain fits into the concave surface of ElonginC, inserting hydrophobic side chains into pockets along the surface (Fig. 3A). Reciprocally, the H4 helix of ElonginC, which bulges out from the side of the concave surface, fits into an extended groove formed by the H1, H2, and H3 helices of the VHL α domain and completes the intermolecular four-helix cluster packing (Fig. 3A).

The H1 helix of the VHL α domain coincides with the 12–amino acid segment shown to be important for ElonginC binding (10), and the structure reveals that it makes extensive contacts to ElonginC. The most significant van der Waals contacts are made by Leu¹⁵⁸, which protrudes from the H1 helix and fits into an ElonginC pocket, and by Cys¹⁶² and Arg¹⁶¹ (Fig. 3A). These are augmented by contacts from the Lys¹⁵⁹, Val¹⁶⁵, Val¹⁶⁶, and Leu¹⁶⁹ side chains of VHL (Fig. 3A).

The other two helices of the α domain also contribute contacts (Leu¹⁷⁸, Ile¹⁸⁰, and Leu¹⁸⁴), with Leu¹⁸⁴ making the most extensive ones in this region (Fig. 3A). Additional contacts are made by residues in the first α - β linker (Leu¹⁵³ and Val¹⁵⁵) and by Arg⁸² from the β domain (Fig. 3A). The hydrogen bonds made by Arg⁸², together with those made by Lys¹⁵⁹ and Arg¹⁶¹ from the H1 helix, represent the few significant hydrogen-bond contacts made at the VHL-ElonginC interface. The arginine side chains are also anchored in the hydrogen-bond networks of the VHL α - β domain interface (Fig. 2D).

The ElonginC-ElonginB interface is similar in extent to the ElonginC-VHL interface but is less hydrophobic. The interface is centered on the S2 β strands from each protein, which align in parallel and make four backbone hydrogen bonds, creating a continuous, intermolecular β sheet (Fig. 3B). The sheet junction is reinforced by van der Waals contacts from hydrophobic residues residing on the S2 strands of both Elongins, and by interactions from additional structural elements on both faces of the sheet (Fig. 3B). The ElonginC residues whose mutation abolishes ElonginB binding map to these interacting regions (12, 26). The COOH-terminal tail of ElonginB packs by means of loose van der Waals contacts in an extended conformation alongside ElonginC (Figs. 1 and 2C). Overall, the ElonginC residues that make sidechain contacts to ElonginB are less conserved across species than those that contact VHL (Fig. 2B).

An intermolecular strand-strand interaction forming a continuous β sheet is also seen in the binding of the ubiquitin-like domains of rafRBD (27) and ralGDS (28) to Ras. The ElonginB-ElonginC interface differs from these regulatory interfaces in that it (i) is roughly double in area, (ii) is more hydrophobic, and (iii) has parallel β -strand pairing.

Tumor-derived VHL mutations. The tumor-derived missense mutations [total of 279 entries (29)] map evenly to the α and β domains (Fig. 4A), each of the domains containing three of the six most frequently mutated residues (henceforth hotspots). In the α domain, the H1 helix is the primary target of tumorigenic mutations. It contains the most frequently mutated residue, Arg¹⁶⁷, which has a structural role stabilizing the H1 helix and the α - β domain interface (Fig. 2D), and an additional mutational hotspot, Cys¹⁶², which contacts ElonginC (Fig. 3A). The third hotspot in the α domain is Leu¹⁷⁸, and it has dual roles interacting with ElonginC and also stabilizing the H2-H3 packing of the α domain (Fig. 3A). The remaining α domain mutations map to residues involved in the packing of the helices (such as Val¹⁷⁰, Ile¹⁸⁰, Leu¹⁸⁴, and Leu¹⁸⁸), or in stabilizing the α - β interdomain interface (Arg¹⁶¹ and Gln¹⁶⁴; Fig. 2D). Several of these residues also make important contacts to ElonginC (Arg¹⁶¹, Ile¹⁸⁰, and Leu¹⁸⁴; Fig. 3A). This pattern of mutations in the α domain solidifies the role ElonginC binding has in the tumor suppressor activity of VHL.

In the β domain some of the mutations map to residues important for the structural integrity of the β sandwich, such as hydrophobic core residues (the Pro^{86} hotspot, Phe^{76} Phe^{119} , Trp^{117} , and Val^{130} ; Figs. 2A and 4A), or buried polar residues that hold loops together (Asn⁷⁸ hotspot; Fig. 4A).

The Tyr⁹⁸ hotspot, which is the second most frequently mutated residue in VHL (Fig. 2A), reveals a significant exception to this trend of structural β domain mutations. This residue occurs on the surface of the β sandwich (Fig. 4B), opposite from where ElonginC binds (Fig. 4C), and has no apparent structural role, a conclusion supported by the ability of a Tyr⁹⁸ tumor-derived mutant to bind ElonginC in vivo (9, 11, 30). Several additional solvent-exposed residues (Asn⁹⁰, Gln⁹⁶, and Tyr¹¹²) surrounding Tyr⁹⁸ are also mutated (Fig. 4B). These observations indicate that this patch of solventexposed residues may correspond to another macromolecular binding site of VHL, and that the use of this site is important for the tumor suppressor activity of VHL.

Type II VHL syndrome associated with partial loss-of-function mutations. VHL families have clinically heterogeneous symptoms and are divided into two types differentiated by the presence of pheochromocytoma (type II) or its absence (type I) (31). The structure reveals that type II cases show a strong bias against hydrophobic core mutations. This is in contrast to type I mutations, which frequently map to the β domain hydrophobic core and would be pre-



derived mutations, red less than 1.5% of the total mutations, orange 1.5 to 3% of total mutations, and yellow 3 to 17%. The latter correspond to the six most frequently mutated residues, which are labeled. ElonginC is shown in blue. The mutational cluster of surface residues on the β domain is indicated. (B) Close-up view of Tyr⁹⁸ and the aromatic and polar residues in its vicinity, colored as in (A). To make these residues easier to see, view is rotated about the horizontal axis relative to (A). (C) Molecular surface representation of VHL in two orientations. Coloring of surface residues is according to mutational frequency as in (A). Two clusters of surface residues are frequently mutated. The front view is as in (A); the back view is rotated 180° about the horizontal axis.

dicted to cause a complete unraveling of the VHL structure. Most type II mutations map either to residues that contact ElonginC (Leu¹⁵⁸, Arg¹⁶¹), or to exposed residues that make up the putative second protein-binding site (Tyr⁹⁸, Tyr¹¹²). Even when they map to structural residues, they are predicted to cause mostly local defects. For example, Arg¹⁶⁷ mutants retain a residual ability to bind ElonginC under mild buffer conditions (9, 30). This suggests that type II cases have a strong bias against mutations that would be predicted to cause total loss of function. This is consistent with the absence of gross deletion and insertion mutations in type II cases (31).

One mechanism that could explain these observations is that the development of pheochromocytoma may involve a dominant negative effect of the mutant VHL. In principle, this model is plausible because VHL has two protein-binding sites, and a mutant having a defect in only one site may sequester key components of the pathway. Experimentally, it is supported by the observation of a dominant negative effect with an overexpressed mutant (5). This mutant has a frameshift at residue 187 and is predicted to have lost its α domain structure but to have retained an intact β domain and second, putative protein-binding site. Alternatively, the bias against complete loss-of-function mutations in type II syndrome could be due to residual VHL function being necessary for the viability of certain cell types.

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VHL α domain is structurally and functionally homologous to the SOCS box. The 40-amino acid SOCS-box sequence motif was recently shown to bind ElonginC and to contain sequence homology with the H1 helix of VHL (14). We used a sequence-threading analysis to augment the sequence homology with the information inherent in the three-dimensional structure of the VHL α domain (32, 33). When the sequence of the SOCS-1 SOCS box was threaded against a library of 1925 structures, the VHL α domain ranked first (33). In this alignment, the pattern of hydrophobic residues in SOCS-1 and the SOCS-box consensus matches that of the entire VHL α domain (Fig. 5A). The agreement includes both the buried hydrophobic residues that are structural determinants of the VHL α domain and the exposed hydrophobic residues that bind ElonginC (Fig. 5, A and B). These findings, in conjunction with the reported SOCS box-ElonginC binding data, indicate that the SOCS box and VHL α domain may represent a common structural and functional motif.

Similarity of the ElonginC-VHL and Skp1–F-box protein complexes. The 30% sequence identity between the NH₂-terminal two-thirds of Skp1 and ElonginC (Fig. 2B) indicates that the two proteins will contain a very similar overall structure (33). The structural and sequence features important for the VHL-binding function of ElonginC include the highly hydrophobic nature of the concave binding surface

A

and the protruding H4 helix that completes the intermolecular four-helix cluster with the VHL α domain. Sequence alignment (Fig. 2B) indicates that the residues responsible for the hydrophobicity of this concave surface maintain their hydrophobic character in Skp1 (Fig. 5C). Furthermore, most H4 helix residues are either identical (7/14 residues) or conservatively substituted in Skp1 (4/14 residues; Fig. 2B). These observations suggest that Skp1 may use the corresponding portion of its structure to bind other proteins with a domain analogous to the α domain of VHL.

Because Skp1 binds proteins that contain the 45-amino acid F-box sequence motif, we performed a threading analysis of the F box against the VHL α domain (33). The F-box sequences we tested did not consistently rank highly, but most produced the same alignment with the VHL α domain helices (33). This alignment shows that the F box has an overall pattern of hydrophobic amino acids that matches that of the VHL α domain (Fig. 5, A and B). The hydrophobicity match includes residues involved both in α domain stabilization and in ElonginC binding (Fig. 5, A and B). This suggests that there may be a loose structural similarity between the VHL α domain and the F box. There is little sequence identity between the two, however, and this is consistent with the lack of cross-reactivity between the ElonginC-VHL and Skp1-F box complexes (34).

A combinatorial regulatory system. The

hum VHL (157-202)

hum SOCS1 (173-211)

SOCS-box Consensus

hum_skp2 (111-157) F-box Consensus

HPNVQKDLERLT

SLWQTLDLTGK

FP-FOI

Fig. 5. The VHL α domain, the SOCS box, and the F box may represent related structural motifs that bind their respective ElonginC and Skp1 partners similarly. (A) Alignment of the VHL α domain, the SOCS1 and the SOCS-box consensus, and the Skp2 and F-box consensus sequences (40). Alignment is based on the three-dimensional structure of the VHL α domain and was produced by the THREADER2 program (32). The F-box alignment is according to the best one obtained from different F-box sequences (33). The VHL secondary structure is shown above the alignment and the α domain is indicated. Residues that stabilize

the four-helix cluster are indicated by blue, hydrophobic residues are denoted by the symbol " ϕ ," and "x" indicates a nonpreferred residue. Regions in which insertions occur are set apart by spaces, and variable insertion sequence length is indicated by numbers following "x." Regions predicted to be a-helical by the PHD secondary structure prediction program (41) are underlined in red. (B) Residues that contribute significantly to the stability of the VHL α domain-ElonginC intermolecular



VHL α-domain

four-helix cluster are conserved in the SOCS and F boxes. Main chain coloring is as in Fig. 2. Hydrophobic VHL residues whose nonpolar character is conserved in both the F and SOCS boxes are shown in yellow. Hydrophobic residues whose nonpolar character is conserved between ElonginC and Skp1 are shown in cyan. (C) ElonginC and Skp1 likely share a similar tertiary structure and a similar protein-binding site. The leftmost molecular surface



structure of the VCB complex extends the proposed analogy (12, 14) to the SCF complex (Fig. 6): (i) it indicates that Skp1 would contain a protein-binding site similar to that used by ElonginC to bind VHL (Figs. 2B and 5C); (ii) it suggests that the F box may contain a threehelix structure similar to that of the VHL α domain and that the F box-Skp1 complex may form in an overall analogous way to the VHL α domain-ElonginC complex (Fig. 5, A and B); (iii) it reveals that VHL has two domains and may contain a second protein-binding site, much like the modular F-box proteins that contain additional protein-binding sites on their WD-40 or leucine-rich repeat domains; and (iv) it indicates that VHL is part of the SOCS-box superfamily, whose members contain additional protein-binding modules such as Src homology 2 (SH2) (SOCS proteins), ankyrin [ASB proteins (14)], or WD-40 [WSB proteins (14)] domains (Fig. 6), thus paralleling the F-box proteins that form a diverse family of protein adapters (35).

The presence of the ubiquitin-like molecule ElonginB associated with the ElonginC-VHL complex has also been suggested to support a role in protein degradation (12). It is not yet known whether ElonginB is processed for conjugation, but it could potentially serve a similar targeting role by remaining tightly bound through noncovalent interactions. Alternatively, ElonginB may have a structural role helping to stabilize the ElonginC structure. Either role would be consistent with the extensive ElonginB-ElonginC interface observed in the structure (Fig. 3B).

The ElonginC system probably regulates a



Fig. 6. The structure of the VHL-ElonginC-ElonginB complex supports the hypothesis that ElonginC may function in a combinatorial regulatory system analogous to the SCF complex. In the ElonginC system, binding to a second protein has been demonstrated only for SOCS1, whose SH2 domain binds the JAK2 kinase (14); for VHL, such binding to a second protein is predicted in this study, and for the WSB and ASB families it has been predicted on the basis of the presence of WD-40 and ankyrin repeats (14), which are often involved in protein-protein interactions. It is not yet clear whether ElonginB would have a ubiquitin-like role in the ElonginC system. cellular process in a combinatorial manner by binding different adapter proteins (Fig. 6). That this process may be protein degradation is further supported by the additional analogies between the ElonginC-VHL complex and the SCF complex revealed in this study.

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- 19. Human VHL (residues 54 to 213) and full-length human ElonginB were cloned as a dicistronic message into the pGEX-4T-3 plasmid (Pharmacia). The NH₂-terminus of VHL, preceded by a 7-amino acid flexible linker flanked at both ends by a consensus thrombin cleavage site, was fused to glutathione S-transferase (GST), and following a translational termination codon after the VHL gene and a 30-nucleotide spacer with a second ribosomal binding site, the ElonginB gene was inserted. Human ElonginC (residues 17 to 112) was cloned into pBB75, a T7-driven expression plasmid with a p15 family origin of replication and that confers resistance to kanamycin. The two plasmids were cotransformed into the BL21(DE3) strain of E. coli and subjected to dual antibiotic selection. Expression of the three genes was carried out at 25°C. The temary complex was isolated and purified by affinity chromatography using a glutathione Sepharose column (Pharmacia). The affinity-purified temary complex was treated with thrombin at 4°C to cleave the GST fusion protein and was further purified by anion-exchange and gel-filtration chromatography. The complex was concentrated by ultrafiltration to 25 mg ml⁻¹ in a buffer of 5 mM bis-tris-propane. and 200 mM NaCl, 2 mM dithiothreitol (DTT), pH 7.0.
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- 21. Crystals of the ternary complex were grown at 4°C by the hanging-drop vapor-diffusion method, from either 12 to 17% polyethylene glycol 8000 (PEG8000) or 10 to 15% PEG2000, supplemented with 0.2 M magnesium acetate, 5 mM DTT, and 0.1 M sodium cacodylate, pH 5.7. They formed in space group P4,22, with a =

b = 93.7 Å and c = 363.5 Å, and contained four VCB ternary complexes in the asymmetric unit. Heavy atom soaks were performed in a stabilization buffer lacking DTT and containing 25% PEG8000, 75 mM magnesium acetate, and 0.1 M sodium cacodylate, pH 5.7, with 5 mM thimerosal for 6 hours, 0.3 mM K₂PtBr₆ for 1 hour, or 1 mM KAu(CN)₂ for 4 hours. Data were collected with crystals flash-cooled to -160°C in stabilization buffer supplemented with 15% glycerol. Data were processed with the programs DENZO and SCALEPACK (36). The structure of the VCB complex was determined by the multiple isomorphous replacement (MIR) method combined with fourfold noncrystallographic symmetry (ncs) averaging (Table 1). Initial heavy atom positions were determined with SHELX-97 (37) and were used together with strong native Patterson peaks to determine the three ncs operators, which were primarily translations. Initial phases (mean figure of merit 0.47) were calculated to 3.5 Å resolution with the program MLPHARE (37), averaged with multidomain fourfold ncs averaging with individual masks for VHL, ElonginC, and ElonginB, and improved by solvent flattening with the program DM (37). An initial model was built into the resultant maps with O (38) and refined using a maximum likelihood target function with ncs restraints with the program CNS (39). Model-phased maps calculated at 2.7 Å resolution were multi-domain averaged with the program RAVE (38) and were used to complete the model. The following residues have poor electron density and are presumed to be disordered in the crystals: residues 54 to 62 and 205 to 213 from the NH₂- and COOH-termini of VHL, respectively, residues 139 to 144 of VHL, residues 50 to 57 of ElonginC, and residues 80 to 87 of ElonginB. Outside these segments, there are no residues in the disallowed region of the Ramachandran plot, and more than 85% of the residues are in the most favored regions.

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 The three-helix cluster would appear unstable in the absence of ElonginC because it would have its hydrophobic core partially exposed. It is conceivable that monomeric VHL has a conformational change that transfers the amphipathic H4 helix from the β domain to the gap in the α domain, sequestering the exposed hydrophobic residues.
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- 33. We performed sequence threading analysis using the program THREADER2 (32) with equal weights for threading and sequence alignment, and it was performed against a library of 1925 folds augmented with the folds of the VHL α and β domains and of ElonginC. The sequences of Skp1 (1 to 112), the SOCS-1 SOCS box (170 to 211), and the Skp2 F box (107 to 147) yielded Z scores of 3.7 (Elongin C structure ranked first), 2.5 (VHL α domain ranked first), and 1.6 (VHL α domain ranked third), respectively. In threading analyses of additional F-box sequences we also used a randomization test based on sequence shuffling (32). The best alignment was obtained with the CyclinF F box, which ranked the VHL α domain first with a score of 1.6. Most F boxes produced consistent alignments with the VHL α domain, with the exception that some lacked

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the one-residue insertion between the H2 and H3 helices (Fig. 5A). In the threading analysis of ElonginA, the VHL α domain structure ranked 704th, suggesting that the ElonginC-binding domain of ElonginA would be divergent. ElonginA sequences encompassing both the region of homology with the VHL H1 helix and the proposed F-box sequences (residues 545 to 610) were threaded as above.

34. To test for cross-reactivity between the ElonginC and Skp1 components, we coexpressed ElonginB, Skp1, and GST-VHL, isolated the GST-VHL with glutathione beads, and found no Skp1 copurifying with GST-VHL; this suggested that VHL has no affinity for Skp1. Conversely, we coexpressed ElonginB, ElonginC, and GST-Skp2 and found that a small amount of the ElonginC-ElonginB complex associated with GST-Skp2. Upon further purification, this complex did not persist like the VCB complex would, indicating that any ElonginC-Skp2

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interaction would be very weak relative to the ElonginC-VHL or the Skp1-Skp2 interaction (C. E. Stebbins, W. G. Kaelin Jr., N. P. Pavletich, data not shown).

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Viscosity Near Earth's Solid Inner Core

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Anomalous splitting of the two equatorial translational modes of oscillation of Earth's solid inner core is used to estimate the effective viscosity just outside its boundary. Superconducting gravimeter observations give periods of 3.5822 ± 0.0012 (retrograde) and 4.0150 ± 0.0010 (prograde) hours. With the use of Ekman layer theory to estimate viscous drag forces, an inferred single viscosity of 1.22×10^{11} Pascal seconds gives calculated periods of 3.5839 and 4.0167 hours for the two modes, close to the observed values. The large effective viscosity is consistent with a fluid, solid-liquid mixture surrounding the inner core associated with the "compositional convection" that drives Earth's geodynamo.

Earth's solid inner core of 1220-km radius is thought to have formed by freezing of the molten metallic alloy constituting the outer core (1). The fluid outer core extending to a radius of 3485 km is about 7% less dense than pure Fe (2), and a variety of lighter compounds have been proposed to account for the deficit in density from the Fe-Ni bulk composition implied by Fe meteorites, averaging 8% Ni by weight (3). Near the inner core boundary, the latent heat released by freezing out of the heavier metallic constituents and lighter Fe compounds with S, Si, or O has been suggested as the energy source that drives the geodynamo that generates the Earth's magnetic field (4). The extra buoyancy of the lighter compounds results in a stirring of the outer core by "compositional convection" (5), providing the necessary velocity field for the geomagnetic dynamo.

A layer about 450 km thick surrounding the inner core, called the F-layer, is part of the earliest Earth models derived from seismic wave observations (δ). It was based on seismic waves interpreted as having been scattered by a

possibly semisolid layer outside the inner core. More recent, improved observations have shown that the scattering could occur at the core-mantle boundary, but the two are not mutually exclusive (7). Stevenson (8) has argued, on the basis of liquid-state physics, that this region may differ in composition from the rest of the outer core. Jeanloz (3) estimates that as much as 50% by volume may consist of crystals mixed in the liquid alloy for an Fe-S composition compatible with the seismologically determined density. Here I consider a method of estimating the viscosity in the F-layer.

The solid inner core is suspended near the center of the outer fluid core by gravitational forces. The possibility of gravimetric observation of its bodily oscillations about its equilibrium position within the outer fluid core was discussed by Slichter (9), and the inner core's essentially rigid translational motions are often referred to as Slichter modes. There are three distinct modes, one along the axis of rotation, one prograde in the equatorial plane, and one retrograde in the equatorial plane. The axial mode period is reduced by about 0.6% by Earth's rotation, but the Coriolis acceleration acts to weaken the restoring force of the prograde equatorial mode, lengthening its period

by about 7.8%, and acts to strengthen the restoring force of the retrograde equatorial mode, shortening its period by about 7.8%. Laws governing this splitting of the periods can be obtained directly from the equation of motion for the inner core. The splitting of the periods according to these laws can be used as a diagnostic tool and led to the association of three resonances in the frequency spectra obtained from superconducting gravimeter observations in Europe with the Slichter modes (10). An automated computer-based search across 4119 discrete periods suggests that no other triplet of resonances are correctly split (11). A recent analysis (12)of nearly 300,000 hours of superconducting gravimeter observations made at stations in Canada, China, Europe, Japan, and the United States has confirmed the presence of these spectral resonances with central periods, in what are considered to be best estimates, at 3.5822 ± 0.0012 , $3.7656 \pm$ 0.0015, and 4.0150 ± 0.0010 hours.

In the original identification (10), the theoretical periods were calculated assuming no viscosity in the outer core and on the basis of the subseismic or anelastic approximation (13)that applies when the fluid velocities are small compared with seismic velocities. The anelastic approximation neglects the inertia of the solid inner core and shell (14), but these are accounted for by the introduction of frequency-dependent internal load Love numbers (Love numbers, named for A. E. H. Love, are a compact way of expressing Earth's response to perturbing forces). A detailed discussion of the effects of the frequency dependence of Love numbers and several other controversial theoretical issues was presented earlier (15). Calculated inviscid periods for the two equatorial modes are then found to have a larger splitting than the observed periods. I show that the oversplitting can be used to estimate the effective viscosity near the inner core (boundary layer thickness, 370 km)

The translational modes of the inner core

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