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Structure of the Cand1-Cul1-Roc1 Complex Reveals Regulatory Mechanisms for the Assembly of the Multisubunit Cullin-Dependent Ubiquitin Ligases

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Summary

The SCF ubiquitin ligase complex regulates diverse cellular functions by ubiquitinating numerous protein substrates. Cand1, a 120 kDa HEAT repeat protein, forms a tight complex with the Cul1-Roc1 SCF catalytic core, inhibiting the assembly of the multisubunit E3 complex. The crystal structure of the Cand1-Cul1-Roc1 complex shows that Cand1 adopts a highly sinuous superhelical structure, clamping around the elongated SCF scaffold protein Cul1. At one end, a Cand1 β hairpin protrusion partially occupies the adaptor binding site on Cul1, inhibiting its interactions with the Skp1 adaptor and the substrate-recruiting F box protein subunits. At the other end, two Cand1 HEAT repeats pack against a conserved Cul1 surface cleft and bury a Cul1 lysine residue, whose modification by the ubiquitin-like protein, Nedd8, is able to block Cand1-Cul1 association. Together with biochemical evidence, these structural results elucidate the mechanisms by which Cand1 and Nedd8 regulate the assembly-disassembly cycles of SCF and other cullin-dependent E3 complexes.

Introduction

Ubiquitin-dependent proteolysis controls protein stability and plays an important regulatory role in a broad spectrum of biological processes (Hershko and Ciechanover, 1998). To target a protein for degradation by the proteasome, eukaryotic cells attach a polyubiquitin chain to the substrate through a three-enzyme cascade involving the ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3). Acting at the last step of this cascade, ubiquitin E3 ligases function as the central component of the ubiquitination pathway by not only catalyzing the final transfer of ubiquitin from the E2 to the substrate but also governing the specificity of the modification reaction (Pickart, 2001).

The SCF (*S*kp1-*C*ul1-*F* box protein) complex is a multisubunit ubiquitin ligase, which promotes ubiquitination of many important regulatory proteins of diverse cellular pathways (Deshaies, 1999). In humans, well-characterized SCF substrates include Cdk inhibitor p27 (Carrano et al., 1999; Sutterluty et al., 1999), Cdk2 regulatory subunit cyclin E (Koepp et al., 2001; Moberg et al., 2001; Strohmaier et al., 2001), NF-κB inhibitor IκB, and Wnt signal transducer β-catenin (Fuchs et al., 1999; Hart et al., 1999; Latres et al., 1999; Yaron et al., 1998). In other eukaryotes, the SCF E3s have been demonstrated to be involved in fundamental cellular functions such as phytohormone response in plants (Gray et al., 1999; Guo and Ecker, 2003; Sasaki et al., 2003), circadian clock in the fruit fly (Grima et al., 2002), and cell cycle progression, sulfur metabolism, and pheromone signaling in fungi (Feldman et al., 1997; Kumar and Paietta, 1998; Patton et al., 1998, 2000; Skowyra et al., 1997; Wang et al., 2003b). The SCF complex consists of three invariant subunits, Skp1, Cul1, and Roc1 (also known as Rbx1 or Hrt1; Kamura et al., 1999b; Ohta et al., 1999; Seol et al., 1999; Skowyra et al., 1999; Tan et al., 1999), and a variable F box protein subunit. Cul1 and Roc1 form the catalytic core of the complex, while Skp1 serves as an adaptor, docking different F box protein subunits to the E3 complex (Deshaies, 1999). Members of the F box protein family all share an N-terminal Skp1 binding F box motif and a C-terminal protein-protein interaction domain, which is able to recruit one or more specific protein substrates. The large number of F box proteins in eukaryotes, with more than 60 members in mammals, allows many substrates to be specifically ubiquitinated by the E3 catalytic core. SCF is the prototype of an even larger superfamily of cullin-dependent multisubunit E3 ligase complexes. Besides Cul1, the cullin protein family has five additional closely related cullin paralogs in humans (Cul2, -3, -4A, -4B, and -5; Kipreos et al., 1996), all of which can bind Roc1 and form ubiquitin ligase complexes (Furukawa et al., 2002; Ohta et al., 1999). Recent studies have revealed that the combinatorial approach used by SCF to expand its substrate repertoire while maintaining reaction specificity is universal to most, if not all, members of the cullin-dependent E3s. For example, Cul2-Roc1, together with elongin-B and the Skp1 homologous elongin-C, can assemble with members of the SOCS box protein family to form an SCF-like complex (Kamura et al., 1998). Cul3-Roc1, on the other hand, can organize an SCF-like complex by directly interacting with members of a family of proteins possessing a BTB/POZ domain, which has a structural fold similar to Skp1 (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003; Xu et al., 2003).

In contrast to the extensive studies on the substrate recruitment mechanisms of the cullin-dependent ligases, little is known about how these enzymes are regulated, in particular, how the assembly and disassembly of these multisubunit complexes are coordinated inside the cell. Given the large number of F box proteins and a limited amount of the potent Cul1-Roc1 ubiquitin ligase catalytic core, the formation of various active SCF complexes must be tightly controlled (Kipreos and Pagano, 2000; Winston et al., 1999). Temporal regulation of SCF assembly is also thought to be func-

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tionally favored in cases where F box proteins themselves undergo SCF-dependent autoubiquitination (Galan and Peter, 1999; Mathias et al., 1999; Zhou and Howley, 1998). The observation that all cullin proteins are modified by the ubiquitin-like protein Nedd8/Rub1 (neddylation) has provided the first clue for how SCF and SCF-like complexes might be regulated (Hori et al., 1999; Lammer et al., 1998). Neddylation of cullins occurs on a specific lysine residue, which is located at the cullins' very C-terminal highly conserved winged-helix B (WH-B) domains (Wada et al., 1999; Zheng et al., 2002b). Conjugation of Nedd8 to cullins is mediated by the Nedd8 E1, the Nedd8 E2, and Roc1 (Furukawa et al., 2000; Gray et al., 2002; Kamura et al., 1999a; Liakopoulos et al., 1998; Osaka et al., 1998). The physiological importance of cullin neddylation is underscored by the genetic studies showing that Nedd8 is essential for cell viability in fission yeast and the early development of *C. elegans*, *A. thaliana*, and mice (Dharmasiri et al., 2003; Kurz et al., 2002; Osaka et al., 2000; Tateishi et al., 2001). Since defects in the Nedd8 modification system or mutation of the Cul1 neddylation site lead to accumulation of a number of examined substrates of the SCF and SCF-like E3s, Nedd8 modification is believed to upregulate the cullin-dependent ubiquitin ligases (del Pozo et al., 2002; Fan et al., 2003; Furukawa et al., 2000; Ohh et al., 2002; Podust et al., 2000), consistent with the enhanced binding of the ubiquitin-charged E2 to SCF and the stimulated in vitro SCF E3 activities upon Cul1 neddylation (Kawakami et al., 2001; Morimoto et al., 2000; Read et al., 2000). Like modification by ubiquitin and most ubiquitin-like proteins (UBLs), neddylation of cullins is reversible. Removal of Nedd8 (deneddylation) from the cullin proteins is catalyzed by the eight subunit *C*OP9 *sign*alosome complex, CSN (reviewed in Cope and Deshaies [2003]). Intriguingly, constitutive cullin neddylation due to loss of function of CSN does not simply lead to hyperactivation of cullin ligases, but instead it results in substrate accumulation (Cope et al., 2002; Doronkin et al., 2003; Wang et al., 2003a). Although the biochemical basis remains unclear, such defects in the E3 activities of the cullin-dependent ligases have been attributed to the disruption of the dynamic neddylation and deneddylation cycles of cullins (Cope and Deshaies, 2003; Lyapina et al., 2001).

Recent identification of an SCF inhibitor, Cand1/TIP120A, has shed light on how the assembly and disassembly of the SCF and other cullin E3 complexes might be regulated and how the Nedd8 modification cycle might be involved in these processes. In searching for cullin-interacting proteins, several independent studies have isolated a 120 kDa protein named Cand1 (formerly identified as a TBP-interacting polypeptide, TIP120A), which can form a tight complex simultaneously with Cul1 and Roc1 (Liu et al., 2002; Min et al., 2003; Oshikawa et al., 2003; Yogosawa et al., 1996; Zheng et al., 2002a). Mutations in either the N- or C-terminal region of both Cand1 and Cul1 disrupt their binding, suggesting that their optimal interactions require the full-length sequences of both proteins. Importantly, Cand1 does not coexist with any detectable Skp1 or F box proteins. Addition of recombinant Cand1 can dissociate Skp1 and F box proteins from Cul1, therefore inhibiting the ubiquitin ligase activities of the E3 complexes (Liu et al., 2002; Zheng et al., 2002a). Examinations of the Cand1-associated Cul1 have further revealed that Cand1 selectively interacts with the deneddylated form of Cul1. Cand1 binding and Nedd8 modification of Cul1 appear to antagonize each other (Hwang et al., 2003; Liu et al., 2002). Based on these observations, Cand1 has been proposed to function as an inhibitor of SCF complex formation by preventing the access of Skp1 and F box proteins to the Cul1-Roc1 catalytic core, thereby avoiding erroneous ubiquitination of the F box proteins. Neddylation of Cul1, conceivably coupled with or triggered by the formation of the Skp1-F box protein-substrate complex, dissociates Cand1 and allows the assembly of a productive substrate-ligase complex. After substrate ubiquitination, deneddylation of Cul1 by CSN is thought to allow Cand1 to reestablish its interactions with the catalytic core (Cope and Deshaies, 2003; Liu et al., 2002). Although this proposed outline of the SCF assembly cycle has provided an attractive model, much of the detailed mechanisms involved remain unclear and untested.

Here we report the 3.1 Å resolution structure of a ternary complex of Cand1, Cul1, and Roc1 (Table 1). To obtain a complete picture of how Cand1 regulates SCF assembly, we have cocrystallized all three proteins in their full-length forms, with a molecular weight of 120 kDa, 85 kDa, and 12 kDa, respectively. Together with biochemical analyses, the structural results not only help elucidate how Cand1 binds to the Cul1-Roc1 core and inhibits the assembly of the E3 complex, but also provide structural insights into the mechanisms by which Nedd8 and possibly an unknown cellular factor revert the inhibitory effect of Cand1 and promote the assembly of fully active SCF and other cullin-dependent E3 complexes.

Table 1. Statistics from the Crystallographic Analysis

Resolution (Å)	50 − 3.1
Observations	331,559
Unique reflections	66,264
Redundancy	5.0
Data coverage (%)	99.5 (99.9)
Overall I/σ	18.9 (2.5)
R_{sym} (%)	8.1 (54.4)
Refinement Statistics	
Total atoms	15,530
R factor (%)	24.8
R_{free} (%)	31.4
RMSD bonds (Å)	0.008
RMSD angles (°)	1.54

$R_{sym} = \Sigma_h\Sigma_i|I_{h,i} - I_h|/\Sigma_h\Sigma_i I_{h,i}$ for the intensity (I) of i observations of reflection h. R factor $= \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. R_{free} = R factor calculated using 5% of the reflection data chosen randomly and omitted from the start of refinement. RMSD: root mean square deviations from ideal geometry and root mean square variation in the B factor of bonded atoms.

Results and Discussion

Overall Structure of the Cand1-Cul1-Roc1 Complex

Cand1 forms a largely compact complex with the SCF catalytic core, Cul1-Roc1 (Figure 1). In the crystal, the

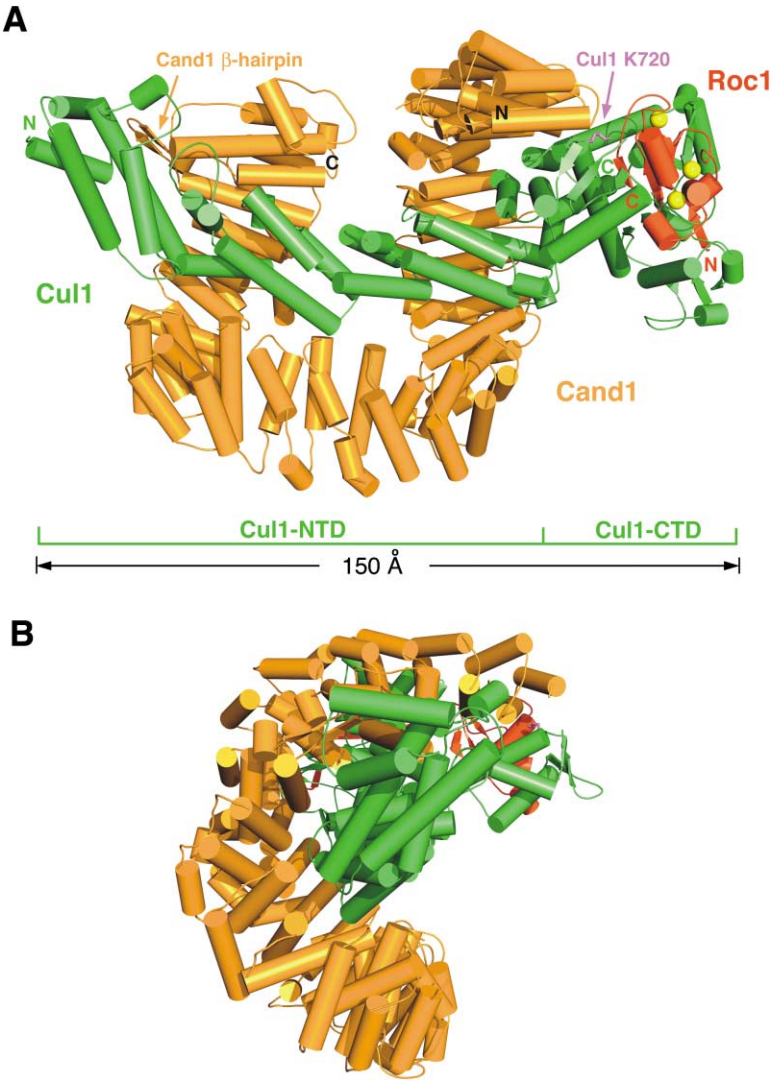

Figure 1. Cand1 Forms a Compact Ternary Complex with Cul1 and Roc1 by Wrapping around the SCF Scaffold Protein

(A) Overall view of the Cand1-Cul1-Roc1 ternary complex with the three proteins colored differently. Two key structural elements, namely a Cand1 β-hairpin and the Cul1 neddylation site lysine residue Lys720, are indicated.

(B) Side view of the ternary complex.

Cul1-Roc1 core retains its elongated structure as previously reported when it is in an isolated form or associated with Skp1-F box^{Skp2} (Zheng et al., 2002b). Cand1 is a nearly all-helical solenoid protein, consisting of 27 tandem HEAT (*h*untingtin-elongation-A subunit-*T*OR) repeats. The HEAT repeats are ~40 residues-long two-helix motifs often found in tandem arrays within many functionally divergent proteins (Andrade et al., 2001). The continuous packing of the Cand1 HEAT repeats renders a highly sinuous superhelical structure, which intimately coils around the SCF scaffold protein Cul1, making multiple and extensive intermolecular contacts. In the complex, Cand1 and Cul1 are configured in a head-to-tail fashion. The C-terminal two-thirds of Cand1 wrap around the extended Cul1 N-terminal domain (NTD) with the last several HEAT repeats interacting with the N-terminal helices of Cul1, whereas the N-terminal third of Cand1 curls around the globular Cul1 C-terminal domain (CTD) with its tip simultaneously packing against the Cul1 CTD and part of the Cul1 bound Roc1 (Figure 1A). Overall, the Cand1 protein resembles an ~80 Å-wide clamp with two prongs and a tongue, snugly gripping the middle two-thirds of the SCF scaffold (Figures 1A and 2A). Most parts of Roc1 that are not in contact with Cul1, including the putative E2 binding site (Zheng et al., 2002b), remain solvent exposed.

The SCF adaptor protein Skp1 binds to the N-terminal tip of Cul1 by packing against two helices in Cul1's first cullin repeat (Zheng et al., 2002b). In the Cand1-Cul1-Roc1 structure, the main body of the Cand1 solenoid is not in contact with the Skp1 binding site on the Cul1 NTD, yet a β hairpin motif protruding from the Cand1 superhelix interacts directly with parts of the two Skp1-interacting helices of Cul1 (Figure 1A). The hallmark of the interaction is a four amino acid β turn at the tip of the Cand1 hairpin, which contains a methionine residue inserting its hydrophobic side chain into a Cul1 surface groove.

More than 100 Å away at the other end of the ternary complex, two helical repeats on the very N terminus of Cand1 interact closely with a cleft on the Cul1 CTD, which bears the Nedd8-conjugating lysine residue on its rim (Figure 1A; Zheng et al., 2002b). Upon the formation of the Cand1-Cul1-Roc1 complex, this otherwise solvent-exposed Cul1 lysine residue becomes mostly buried at a junction region of the three proteins.

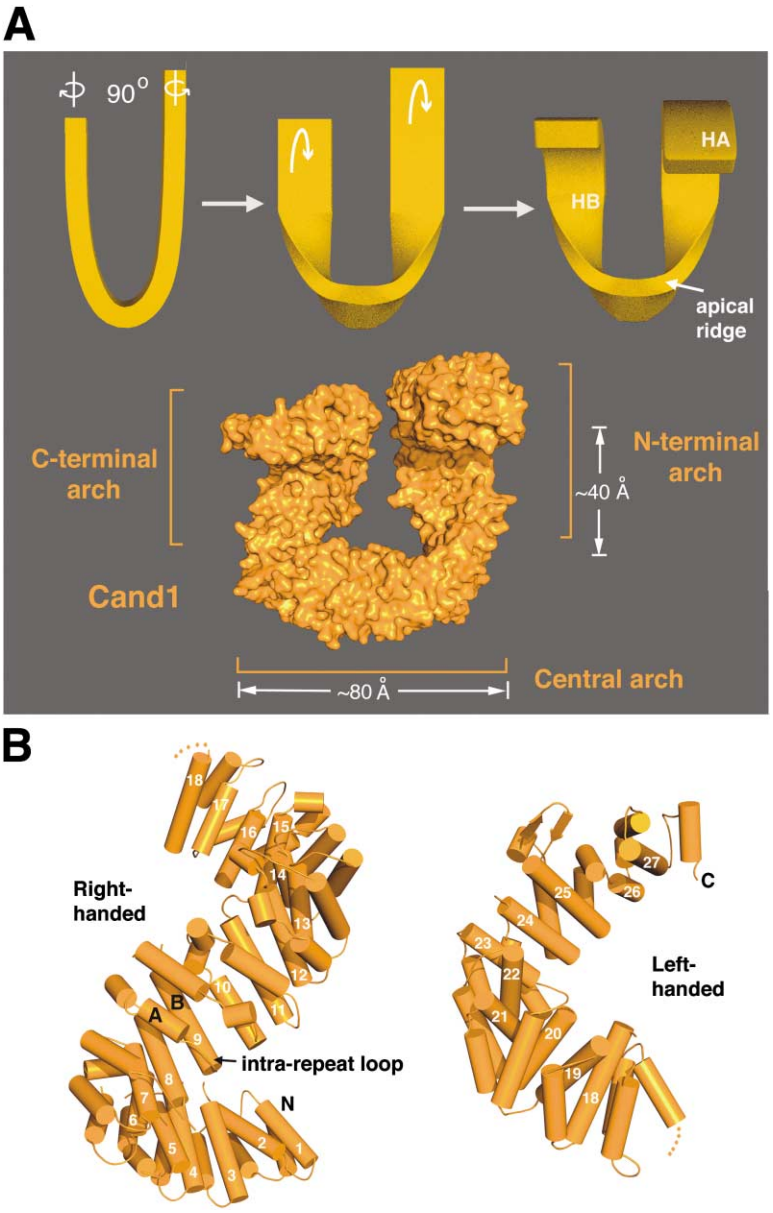

Figure 2. Stacking of the 27 Tandem HEAT Repeats of Cand1 Gives a Highly Sinuous Superhelical Structure

(A) The overall shape of the Cand1 protein when it is in complex with Cul1-Roc1. The top panel describes with schematic illustration how Cand1's overall form can be derived from a U-shaped belt. The surfaces corresponding to the ones formed by the A and B helices of the Cand1 HEAT repeats are labeled with "HA" and "HB." The "apical ridge" formed by the intrarepeat loops is indicated. The bottom panel shows Cand1 in surface representation with the same orientation as shown in Figure 1A.

(B) The two parts of Cand1 with opposite handedness. The right-handed N-terminal two-thirds of Cand1 is shown on the left and the left-handed C-terminal one-third on the right. The order of the Cand1 HEAT repeats is shown on their B helices. The structural elements of a typical HEAT motif are indicated for the ninth HEAT repeat of Cand1.

Structure of Cand1

Most, if not all, Cand1 helical repeats resemble the canonical HEAT motif, which has been previously found in functionally unrelated proteins, such as the PR65/A subunit of protein phosphatase 2A, the α and β2 subunits of the clathrin adaptor AP2 complex, and importin β1 and β2 (Chook and Blobel, 1999; Cingolani et al., 1999; Collins et al., 2002; Groves et al., 1999). The HEAT motif is characterized by a pair of antiparallel helices (conventionally referred to as helix A and B) and a connecting loop (Figure 2B). In some cases, helix A of a HEAT motif is significantly kinked in the middle due to the presence of a proline residue. The tandemly stacked HEAT repeats typically produce a curved structure made of a double-layer of α helices, with the A helices lining the outer convex side and the B helices forming the inner concave surface. The two ridges of the curved structure are formed by intra- and interrepeat loops, hereafter referred to as apical and basal ridges, respectively. Possessing all these general features of HEAT repeats, the 27 consecutively stacked Cand1 HEAT repeats yield an unusually winding tertiary structure with two superhelical halves bearing opposite handedness, making Cand1 distinct from all known HEAT-repeat structures. Specifically, the N-terminal half of Cand1 forms a right-handed superhelix, whereas the C-terminal half adopts a left-handed superhelical structure (Figure 2B). The change of the handedness predominantly owes to a radical shift in the packing orientations between repeats 19 and 20.

In a simplified schematic model, the overall structure of the Cand1 solenoid can be derived from a U-shaped belt with its two arms twisted 90° in opposite directions and then curved toward the base (Figure 2A, top). For the convenience of discussion, the resulting structure can be divided into three arches, the middle one being the connecting base of the belt and the two terminal ones corresponding to the curved arms (Figure 2A, bot-

tom). Among these three, the two terminal arches are positioned side-by-side, running antiparallel to each other, and the central arch is configured orthogonal to both of the terminal ones. In Cand1, the N-terminal arch is formed by ten HEAT repeats, whereas the C-terminal arch is made of six. With a similar curvature shared by the two arches, the extra four repeats cause the Cand1 N-terminal arch to extend significantly farther (Figures 1A and 2A). The central arch of Cand1 has its entire apical ridge facing toward the concave sides of the two terminal arches. Together, these three arches enclose a pronounced negative space, which can accommodate roughly half of an ~80 Å-long cylinder with a diameter of ~40 Å (Figure 2A). In most curved solenoid proteins, the concave surfaces are involved in protein-protein interactions. The two juxtaposed concave surfaces of the Cand1 terminal arches, together with the apical ridge of the central arch, make the Cand1 protein ideal for securely clamping around an elongated molecule, which in this case is the Cul1 protein.

Interactions between Cand1 and Cul1-Roc1

The Cand1-Cul1 interactions, extended through the entire sequences of both proteins, involve multiple binding sites and bury a total of ~8900 Å^2 accessible surface area from the two proteins. Cul1 is an elongated protein comprising a long N-terminal curved stalk and a globular C-terminal domain (Figure 1A; Zheng et al., 2002b). The Cul1 N-terminal stalk is made of three copies of the five-helix cullin repeat motif (helices A to E), which is distinct from the HEAT repeat (Figures 3A and 3B). The consecutive packing of the three cullin repeats resembles three toppled dominoes, with an ~110 Å-long extended overall structure. In complex with Cand1, the Cul1 NTD becomes slightly less curved, mainly due to the interactions between its first half and the Cand1 C-terminal arch (Supplemental Figure S1 at http://www.cell.com/cgi/content/full/119/4/517/DC1/). The globular Cul1 CTD contains multiple structural units organized by a four-helix bundle (4HB), which also packs against the third cullin repeat in the NTD (Figure 3C). The majority of the Cul1 CTD is involved in Roc1 binding. In the ternary complex, Cand1 wraps around the Cul1 N-terminal stalk with its central and C-terminal arches, making contacts with all three cullin repeats. The Cand1 N-terminal arch curves around one side of the Cul1 CTD, interacting with the WH-B domain and 4HB of the Cul1 CTD, as well as part of Roc1 (Figures 1A and 3).

The Cand1 C-terminal arch holds the Cul1 NTD by interacting with its first two cullin repeats (Figure 3A). The concave surface of the Cand1 arch, formed by the B helices of HEAT repeats 23 to 27, interacts mainly with Cul1's second cullin repeat, whereas two noncanonical Cand1 HEAT repeats, namely repeats 25 and 27, make additional contacts with Cul1's first repeat with their uncommon structural elements projecting out from the Cand1 solenoid (Figure 3A). Cand1's twenty-fifth HEAT repeat distinguishes itself from the rest of the molecule by having an unusually long intrarepeat sequence, which adopts a β hairpin structure. The tip of this Cand1 β hairpin protrusion reaches the N-terminal half of Cul1's first cullin repeat, interacting simultaneously with three Cul1 helices, two of which have been previously shown to be involved in Skp1 binding (see below). On the other hand, Cand1's twenty-seventh HEAT repeat has an unusually long helix B, which together with the intrarepeat loop packs against both the first and second cullin repeats of Cul1. At the numerous interfaces between the Cand1 C-terminal arch and the Cul1 NTD, several buried intermolecular salt bridges are formed in addition to a large number of van der Waals contacts and hydrophobic interactions. More than half of the Cand1 amino acids at the interface are strictly conserved (Figure 3A), although the interacting Cul1 residues are conserved in neither Cul1 orthologs nor in Cul1 paralogs (Supplemental Figures S2–S4 on the *Cell* website).

The Cand1 central arch supports Cul1 binding by providing three major anchoring points on the arch's apical ridge, each in contact with one of the three Cul1 helical repeats (Figures 1A and 3B). At the central region of the arch, the intrarepeat loops of two Cand1 HEAT repeats pack against helix C of Cul1's second cullin repeat. At the C-terminal region of the arch, the apical loops of two other Cand1 HEAT repeats interact with the end of Cul1's first cullin repeat. Finally, the apical ridge of the N-terminal region of the arch makes close contact with the very end of Cul1's third cullin repeat. Most of the interacting residues on the Cand1 side, but not the Cul1 side, are again highly conserved (Figure 3B and Supplemental Figures S2–S4 on the *Cell* website).

Distinct from the C-terminal arch, the Cand1 N-terminal arch uses only a strip of its concave surface that is close to the apical ridge to interact with Cul1 (Figure 3C). The most prominent interactions in this area are around the first two Cand1 HEAT repeats at the N-terminal tip of the arch. Their interactions with a surface cleft on the Cul1 CTD form the most conserved Cand1-Cul1 interface in the whole complex (see below). The apical ridges of these two Cand1 HEAT repeats make additional direct contacts with the RING domain of Roc1. Although the third Cand1 HEAT repeat continues to interact closely with the 4HB of the Cul1 CTD, the rest of the interactions between the Cand1 N-terminal arch and the SCF scaffold appear to be secondary and involve mostly loose and discontinuous intermolecular packing.

Cand1 can form complexes with all six cullins, despite the differential preferences for certain family members in different assays (Liu et al., 2002; Min et al., 2003; Oshikawa et al., 2003; Zheng et al., 2002a). The capability of Cand1 to bind all cullins is consistent with the structure showing that overall shape instead of specific surface residues of Cul1 is the most important determinant for its association with Cand1. Except the residues forming the Cand1-interacting surface cleft on the Cul1 CTD, most, if not all, Cul1 residues involved in Cand1 binding are conserved in neither Cul1 orthologs nor Cul1 paralogs. Yet the overall sequence conservation and the similarity of the predicted secondary structures indicate that all cullins should share a common overall structure (Zheng et al., 2002b), which can be accommodated by the Cand1 solenoid.

Inhibition of SCF Assembly by Cand1

The structure shows that Cand1 disrupts the Skp1-Cul1 interactions by using the tip of Cand1's β hairpin protrusion to occupy part of the Skp1 binding site on the Cul1 NTD (Figure 4). The ~25 Å-long highly conserved Cand1

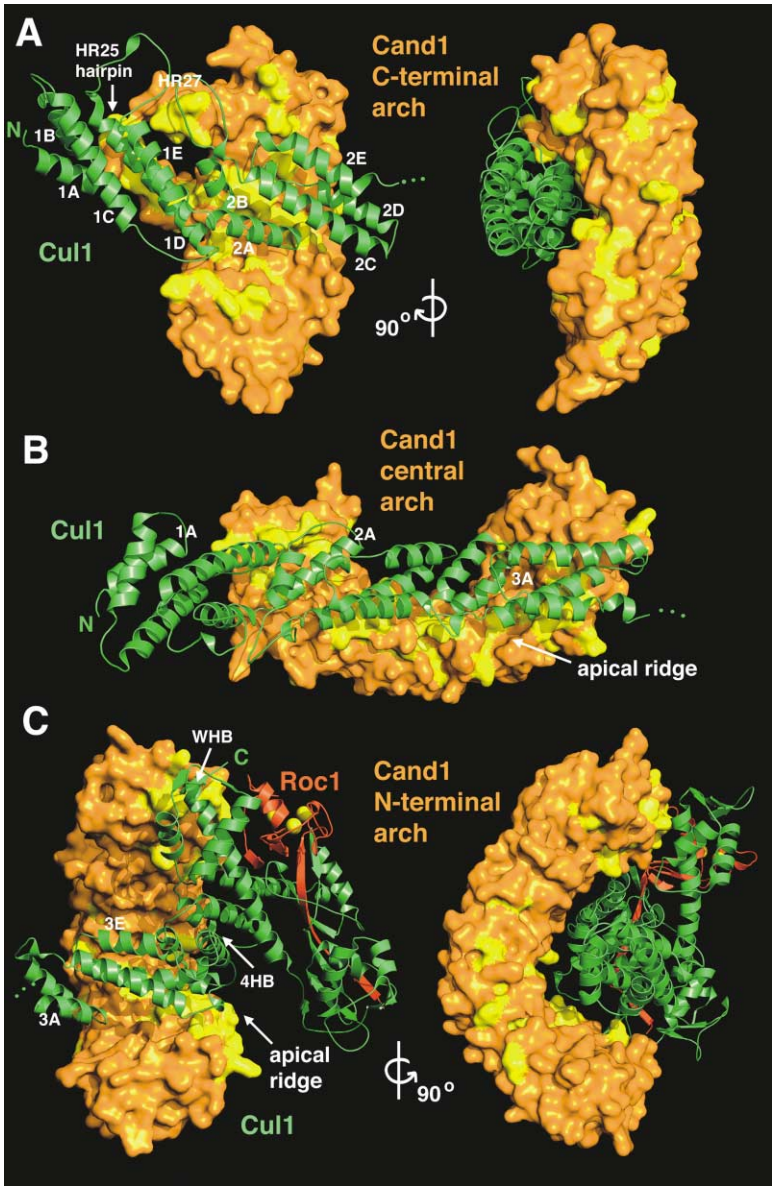

Figure 3. Cand1 Interacts with the SCF Scaffold through Multiple Interfaces as Shown in Three Dissected Views

(A) Interactions between the C-terminal arch of Cand1 and the first two cullin repeats of Cul1. Two orthogonal views are shown with Cand1 in surface and Cul1 in ribbon representations. The helical elements of the two Cul1 cullin repeats are labeled. The unusual parts of the twenty-fifth and twenty-seventh Cand1 HEAT repeats projecting out from the Cand1 solenoid main body are indicated. Surfaces of the strictly conserved Cand1 residues are colored in bright yellow.

(B) Interactions between the central arch of Cand1 and the entire Cul1 NTD. The A helix of each Cul1 cullin repeat is labeled.

(C) Interactions between the N-terminal arch of Cand1 and the Cul1 CTD. For clarity, the third cullin repeat of the Cul1 NTD is shown together with the Cul1 CTD. The 4HB and WH-B domains of the Cul1 CTD and the apical ridge of the Cand1 arch are labeled and indicated with arrows.

β hairpin consists of two antiparallel β strands connected by a four-amino-acid β turn, MGPF (Figure 4A). It is analogous to a laid-down drawbridge with one end connected to the Cand1 C-terminal arch and the other end anchored on Cul1. On the bottom of the β hairpin tip, Met1068 of the β turn inserts its side chain into a hydrophobic surface groove formed by the A, B, and D (also known as H1, H2, and H4) helices of Cul1's first cullin repeat (Figure 4B). Next to the methionine residue, Gly1069, Pro1070, and Phe1071 together pack against Cul1 Tyr42, which is involved in Skp1 binding in the Cul1-Roc1-Skp1-F box^{Skp2} complex (Zheng et al., 2002b). These interactions are further reinforced by two Cand1 residues, Val1066 and His1073, which make van der Waals contacts with the Cul1 amino acids lining the opening of the surface groove (Figure 4A). Burying a total of ~1000 Å^2 surface area, Cand1-Cul1 interactions at this site interfere with Skp1 binding to Cul1 by introducing significant spatial hindrance. As Figure 4C shows, when the Skp1-Cul1 and Cand1-Cul1 structures are superimposed, the Cand1 β turn residues collide with a significant part of Skp1, which mediates the association of Skp1 with Cul1.

Models of several complete SCF complexes have been previously built based on the crystal structures of the Cul1-Roc1-Skp1-F box^{Skp2} complex and three different Skp1-F box protein complexes (Orlicky et al., 2003; Schulman et al., 2000; Wu et al., 2003; Zheng et al., 2002b). Superposition analyses of the Cand1-Cul1-Roc1 structure and these SCF models indicate that no other region of Cand1 spatially overlaps with Skp1 or the representative F box proteins (Figure 4D). Therefore, partial intrusion of the Skp1 binding site on Cul1 by Cand1's β hairpin motif appears to be the exclusive mechanism by which the Cand1 protein inhibits the assembly of Skp1 onto the SCF scaffold. To confirm the crystal structure results, we have constructed and purified a recombinant Cand1 mutant lacking the β hairpin in the pro-

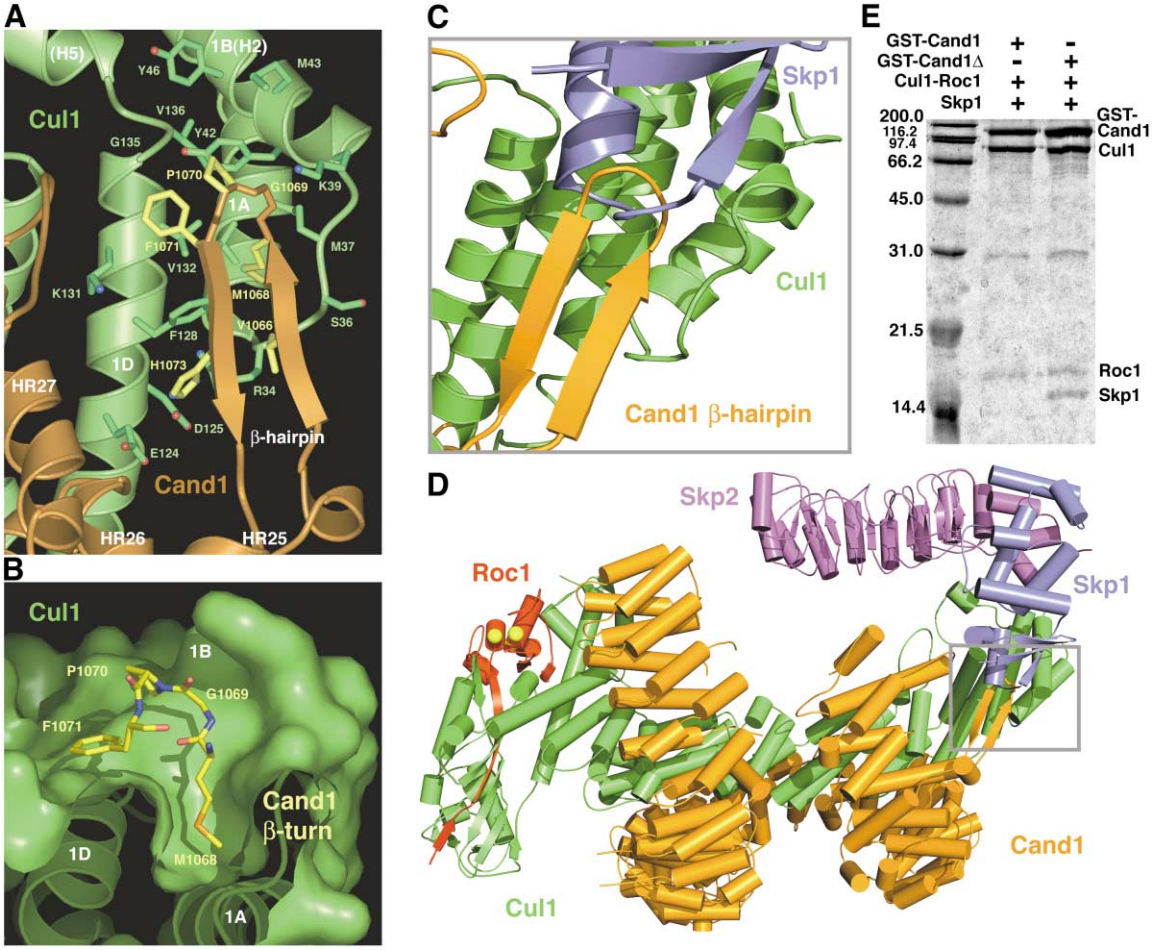

Figure 4. Cand1 Blocks Skp1-Cul1 Interactions by Occupying Part of the Skp1 Binding Site on Cul1 with the Tip of Its Protruding β Hairpin

(A) Closeup view of the interface between the β hairpin of Cand1 and the first cullin repeat of Cul1. Interacting residues of Cand1 and Cul1 in this region are plotted in yellow and green, respectively. Two nearby Skp1-interacting residues of Cul1, namely Tyr46 and Met43, are also shown.

(B) A slab view of the Cul1 surface pocket accommodating the side chain of the methionine residue at the tip of the Cand1 β hairpin. The four amino acids forming the β turn are shown in sticks.

(C) Collision between the Cand1 β hairpin and Skp1 when the Skp1-Cul1 and Cand1-Cul1 structures are superimposed. The three proteins are colored differently and orientated similarly as shown in (D).

(D) Superposition of the Cand1-Cul1-Roc1 structure and the modeled structure of the SCF^{Skp2} complex.

(E) An in vitro pull-down assay showing the Cand1 β hairpin is solely responsible for disrupting Skp1-Cul1 interactions. Both wild-type and a mutant Cand1 lacking 12 β hairpin residues (Arg1064 to Val1075) were purified as GST-fusion proteins and bound to the glutathione-4B resins, which were subsequently incubated with a mixture of purified Cul1-Roc1 and Skp1-F box^{Skp2} complexes. Following an extensive wash, the proteins were eluted by glutathione and analyzed by SDS-PAGE.

tein's twenty-fifth HEAT repeat. In an in vitro pull-down assay, the Cul1-Roc1 core in complex with this Cand1 mutant, but not the wild-type Cand1, retains its ability to recruit a complex of Skp1-F box^{Skp2} (Figure 4E). This same Cand1 mutant, but not the wild-type protein, can also be coimmunoprecipitated with Skp1 in cotransfected cells (Supplemental Figure S5 on the *Cell* website). The surprisingly small fraction of the Skp1-Cul1 interface directly disrupted by Cand1 suggests that a small molecule with a high affinity to Cul1 might be able to effectively perturb the Skp1-Cul1 interactions by imposing similar local steric hindrance. The Cand1 β hairpin interacting site on Cul1, including the hydrophobic surface groove accommodating the Cand1 Met1068 residue (Figure 4B), therefore, represents a potential target site for developing drugs to inhibit the SCF E3 activity.

Interaction between Cand1 and the Cul1 Neddylation Site

One of the highlights of the intermolecular interactions in the tertiary complex centers around the first two N-terminal HEAT repeats of Cand1. As shown in Figure 5A, the apical ridge and the nearby concave surface of these two Cand1 repeats fit snugly into a surface cleft formed by the WH-B and 4HB domains of Cul1 and the RING domain of Roc1. Distinct from the rather loose contacts between the Cul1 CTD and the rest of the Cand1 N-terminal arch, the extensive interface at this site is characterized by a mixture of hydrophobic inter-

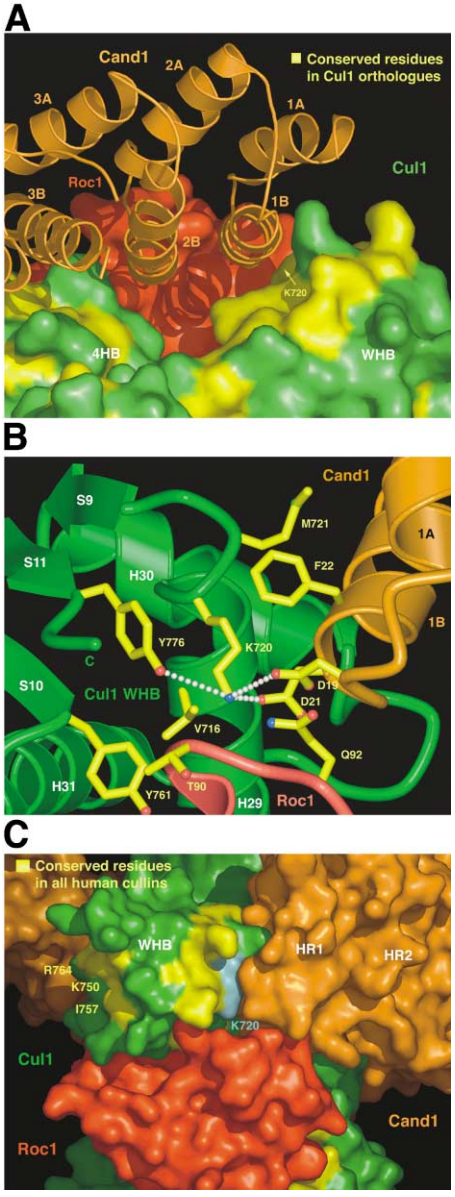

Figure 5. The N-terminal HEAT Repeats of Cand1 Interact with a Conserved Surface Cleft of the Cul1 CTD and Bury the Cul1 Neddylation Site Lysine Residue

(A) Interactions between the first two Cand1 HEAT repeats and the Cul1 CTD surface cleft. The molecular surfaces of Cul1 and Roc1 are colored in green and red. Surfaces of conserved Cul1 residues are shown in yellow. Important structural elements of the proteins are labeled.

(B) Closeup view of the interfaces among the Cul1 WH-B domain, Cand1's first HEAT repeat, and the Roc1 RING domain. Residues interacting with Cul1 Lys720, as well as several surrounding amino acids, are shown.

(C) Zoomed-out view of (B) with surface representation. The ϵ-amino group of the Cul1 Lys720 residue is completely buried and invisible. Cul1 residues conserved among all human cullins are colored in yellow. The surface of three such conserved Cul1 residues located on the opposite side of the Cul1 WH-B domain where the Cand1-interacting surface cleft is found are indicated. This surface area represents a potential site on the Cul1 CTD for interacting with additional regulatory factors.

actions, intermolecular salt bridges, and hydrogen bonds, most of which are formed by residues that are highly conserved in all three proteins. In fact, two interacting structural motifs in this region, namely, the first HEAT repeat of Cand1 and the WH-B domain of Cul1, represent one of the most conserved regions of both proteins (Figures 3C and 5A).

The same surface cleft of the Cul1-Roc1 core has been previously noticed not only for its unique surface features but also for the presence of the Cul1 neddylation site, Lys720, on one side of its rim (Figure 5A; Zheng et al., 2002b). In the absence of Cand1, Lys720 is completely exposed to the solvent, facing to the center of the cleft. Upon Cand1 binding, Lys720 becomes closely associated with the first HEAT repeat of Cand1 (Figures 5A and 5B). Specifically, this Cul1 lysine residue forms a tridentate interaction with two strictly conserved and negatively charged Cand1 residues, Asp19 and Asp21, and a conserved Cul1 residue, Tyr776. Its aliphatic side chain is further buttressed by two aromatic residues, one from Cul1 (Tyr776) and the other from Cand1 (Phe22) (Figure 5B). Overall, Cul1 Lys720 becomes mostly buried in the presence of Cand1, with only less than a quarter of its accessible surface exposed to the solvent (Figure 5C).

The location of the Cul1 neddylation site relative to the nearby surface cleft provides plausible explanations for how neddylation of Cul1 and the association of Cand1 with the SCF catalytic core can antagonize each other. On the one hand, upon binding to Cul1, Cand1 appears to lock up the Cul1 lysine residue via numerous critical intermolecular interactions. Without significant conformational changes, Cand1 binding makes the ϵ-amino group of Lys720 essentially inaccessible to the Nedd8-charged E2 enzyme and therefore effectively blocks the Nedd8 transfer reaction. On the other hand, we and others speculate that, upon conjugating to the Cul1 Lys720 residue, the Nedd8 molecule would preferentially occupy the conserved surface cleft of the Cul1-Roc1 core where Cand1 binds (Pan et al., 2004). The Nedd8 molecule attached to Cul1, therefore, would cause severe spatial hindrance to the binding of Cand1's first two HEAT repeats to the Cul1 CTD. Even though the rest of Cand1-Cul1 interactions are not directly affected, it is unlikely that Cand1 could maintain its binding to Cul1 while avoiding its collision with Nedd8. Since Nedd8 is covalently linked to Cul1, its extremely high local concentration will allow it to successfully compete with Cand1 for the access of the shared binding site on Cul1. A crystal structure of the neddylated form of Cul1 is needed to confirm this hypothesis.

Inaccessibility of Cul1 Lys720 to the Nedd8 E2 in the Presence of Cand1

In order to neddylate Cul1 and block Cand1 binding, the Nedd8 E2 has to gain access to the Cul1 Lys720 residue, which is mostly buried by Cand1 as revealed by the crystal structure. Based on previous biochemical studies, several possible scenarios might be involved in this process (Cope and Deshaies, 2003; Hwang et al., 2003; Liu et al., 2002). First, the equilibrium between the free and Cand1 bound states of the Cul1-Roc1 core might allow the Nedd8 E2 to access the Cul1 neddylation

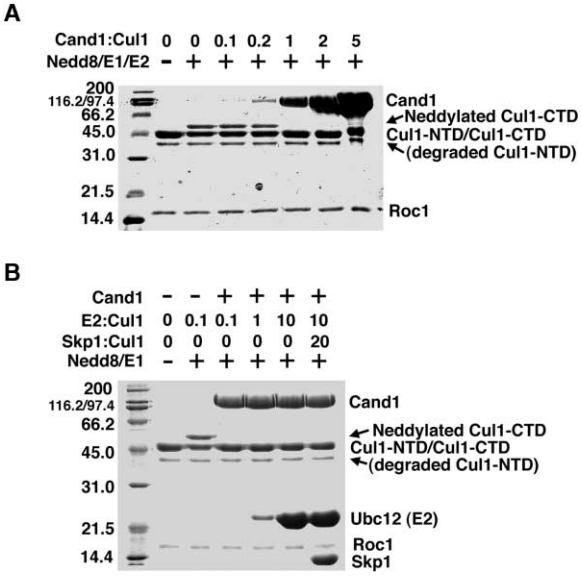

Figure 6. The Neddylation Enzymatic System Is Not Sufficient to Decouple Cand1 from the SCF Catalytic Core

(A) Inhibition of Cul1 neddylation by Cand1. Cul1 neddylation was effectively blocked when an equal molar amount of Cand1 was present. All proteins were overexpressed and purified from *E. coli*. The Cul1 NTD and CTD in the spliced form of Cul1 comigrated on the gel. Neddylation of Lys720 on the Cul1 CTD upshifted the Cul1 CTD band by about 8 kDa.

(B) The neddylation enzymatic system failed to modify Cand1 bound Cul1, even when the Nedd8 E2 enzyme (Ubc12) is in excess amount and in the presence of excess Skp1.

site. Second, the Nedd8 E2 itself might be able to induce conformational changes of Cand1 or Cul1 to expose the Cul1 Lys720 residue. Third, other cellular factors might be able to partially or fully dissociate Cand1 from Cul1-Roc1 to facilitate Cul1 neddylation. To further investigate how Cul1 neddylation occurs to antagonize the inhibitory effect of Cand1 on SCF assembly, we set up an in vitro system, in which the Nedd8 modification of Cul1 was reconstituted with all the reaction components, including Cul1, Roc1, the Nedd8 E1, the Nedd8 E2, Nedd8, and Cand1, overexpressed and purified from *E. coli*. In the absence of Cand1, Cul1 in the Cul1-Roc1 core was readily modified by the neddylation system with minimal amounts of the Nedd8 E1 and E2 added (molar ratios Cul1:E2:E1 = 100:3:1). When Cand1 was present at equal molar ratio to Cul1, however, Cul1 neddylation was completely inhibited (Figure 6A), consistent with the previously reported similar results obtained with proteins purified from insect cells (Hwang et al., 2003). No detectable Cul1 neddylation product was observed even after a long period of incubation time (data not shown), indicating that the equilibrium between the free and Cand1 bound forms of Cul1 is not sufficient to allow Cul1 neddylation to occur. If the Nedd8 E2 gains access to the Cul1 neddylation site actively by binding and inducing conformational changes of Cand1 and/or Cul1, efficient neddylation of Cul1 might require stoichiometric amount of the E2 enzyme. To test this possibility, we included increasing amount of E2 in the in vitro reactions. As Figure 6B shows, addition of up to ten-fold excess Nedd8 E2 did not yield any detectable neddylated Cul1, suggesting that the Nedd8 E2 itself is unable to remodel Cand1-Cul1 to uncover the Cul1 Lys720 residue. Although Skp1 can hardly compete with Cand1 for Cul1 binding (Supplemental Figure S6 on the *Cell* website), we further tested whether Skp1 together with the Nedd8 E2 can decouple the Cand1-Cul1 complex. Again, little Cul1 neddylation was detected when excess Skp1-F box^{Skp2} complex and the E2 were included in the reaction (Figure 6B). These results implicate that in the presence of Cand1, Cul1's Lys720 residue remains inaccessible to the Nedd8 E2 unless some other cellular factor(s) help remodel the Cand1-Cul1 complex. Intriguingly, our previous studies have shown that adding the neddylation enzymatic system to the Cand1-Cul1 complex immunoprecipitated from mammalian cell lysate could in fact lead to neddylation and release of a significant amount of the SCF scaffold from Cand1 (Liu et al., 2002), suggesting that an unknown cellular factor(s), which can mediate the opening of the Cand1-Cul1 complex and the attachment of Nedd8 to Cul1, might have been copurified with the immunoprecipitated Cand1-Cul1 complex. We speculate that the minimal function of this factor is to induce conformational changes around the Cul1 WH-B domain so that the Cul1 Lys720 residue would become accessible to the Nedd8 E2.

Conclusions

The crystal structure of the Cand1-Cul1-Roc1 ternary complex reveals the detailed mechanisms of how Cand1 participates in regulating the assembly and disassembly of the SCF ubiquitin ligase complex. Adopting a highly sinuous superhelical structure, Cand1 forms a tight complex with the SCF Cul1-Roc1 catalytic core by wrapping around the elongated scaffold protein Cul1. Through an unusual β hairpin projection, Cand1 occupies part of the Skp1 binding site on Cul1 and blocks further assembly of the multisubunit E3. As the critical interactions between Cand1 and Cul1 involve the Cul1 neddylation site, covalent attachment of Nedd8 to Cul1 is able to effectively inhibit Cand1 binding by steric hindrance. How the Nedd8 E2 gains access to the Cul1 neddylation site, which is mostly buried in the Cand1-Cul1-Roc1 complex, remains to be clarified.

Experimental Procedures

Protein Overexpression and Purification

Full-length human Cand1 was overexpressed as a glutathione-S-transferase (GST)-fusion protein in *E. coli*. Full-length Cul1 and full-length Roc1 were overexpressed in both *E. coli* and insect cells as previously reported (Zheng et al., 2002b). For bacterial expression of the Cul1-Roc1 complex, a "split-and-coexpress" scheme was used. The resulted protein complex retains its full function and intact structure (Zheng et al., 2002b). All proteins were isolated from the soluble cell lysate by glutathione affinity chromatography. After cleavage by thrombin, the Cand1 protein was further purified by anion exchange and gel filtration chromatography and concentrated by ultrafiltration. The Cul1-Roc1 complex, Skp1-F box^{Skp2} complex, and the Nedd8 E1 were purified following previously reported procedures (Schulman et al., 2000; Walden et al., 2003; Zheng et al., 2002b). The Nedd8 E2 (Ubc12) and Nedd8 were both produced in *E. coli* as GST-fusion proteins and further purified by anion exchange and gel filtration chromatography.

Crystallization and Data Collection

The purified Cand1 protein (13 mg/ml) and the bacteria-expressed Cul1-Roc1 complex (8.8 mg/ml) were mixed at 1:1 molar ratio in a solution of 20 mM Tris-HCl (pH = 8.0), 200 mM NaCl, and 5 mM dithiothretiol (DTT). Crystals of the ternary complex composed of native proteins were grown at 4°C by the hanging drop vapor diffusion method by mixing the complex with an equal volume of reservoir solution containing 100 mM Tris-HCl (pH = 8.0), 7%–10% PEG8000, and 5 mM DTT. The Cand1-Cul1-Roc1 crystals form in space group $P2_12_12_1$, with a = 108.4 Å, b = 151.3 Å, c = 215.9 Å, and contain one complex in the asymmetric unit. The ternary complex of Cul1-Roc1 and seleno-methionine- (Se-Met) labeled Cand1 produced significantly smaller crystals, which did not diffract beyond 8 Å. A mixture of wild-type and Se-Met-labeled Cand1 at 1:1 ratio together with Cul1-Roc1 was then prepared, and crystals of normal sizes were obtained. Only a small population of Se-Met-labeled Cand1, however, exists in the crystals as judged from the low-selenium fluorescence signals. All the data sets were collected at BL5.0.2 beamline at the Advance Light Source using crystals flash-frozen in the crystallization buffer supplemented with 25% ethylene glycol at −170°C. Reflection data were indexed, integrated, and scaled (Table 1) using the HKL2000 package (Otwinowski and Minor, 1997).

Structure Determination and Refinement

The structure of the complex was determined by molecular replacement using the native data set. Molecular replacement calculations were carried out with the program AMORE using the Cul1-Roc1 structure as the search model (CCP4, 1994). After rigid-body refinement of the top Cul1-Roc1 solution, which clearly stood out from the rest, model phases were calculated and underwent density modification using CNS (Brunger et al., 1998). A 3.2 Å fourier map calculated with the resulting phases showed interpretable electron densities for a large number of Cand1 helices throughout the molecule. Moderate discrepancies between the model and the calculated density map around Cul1's first and second cullin repeats were also clear. After correcting the Cul1 model, about two-thirds of Cand1 helices with polyalanine sequences were built into the continuous electron densities in O (Jones et al., 1991). Care was taken not to overinterpret the map in ambiguous regions. The resulting model was then refined by rigid-body refinement, followed by density modification of the model phases. Five additional cycles of model building and refinement were performed before all Cand1 helices and most connecting loops were found and side chains were built into the model. Although the electron densities of the Cand1 β hairpin could be recognized at the very early stage, its polypeptide backbone and side chains were built at the last step. Selenium anomalous experiments using crystals containing Se-Met-labeled Cand1 were used to confirm the sequence registry. The entire structure has been checked using simulated annealing composite omit maps. The final refined model does not contain four Cand1 loops (120–123, 308–343, 409–423, 787–790) due to the absence of their electron densities. It is assumed that these loops are disordered in the crystals. The final Cand1-Cul1-Roc1 model has 97.2% of the residues in the favored and allowed regions and none in the disallowed region of the Ramachandran plot. All figures were prepared with PyMol (DeLano, 2002) and rendered either internally or by POVRAY (Table 1).

GST Pull-Down Assay

GST-fused wild-type and mutant Cand1 proteins (150 μg) bound to glutathione sepharose 4B beads (100 μl) were incubated with Cul1-Roc1 (100 μg) and variable amounts of Skp1 bound to F box^{Skp2} or Skp2 for 3 hr at 4°C. Supernatant, containing unbound proteins, was removed after centrifugation. The beads were then washed three times with 500 μl of wash buffer containing 20 mM Tris-HCl (pH = 8.0), 200 mM NaCl, and 5 mM DTT. The resin bound proteins were eluted with 50 mM Tris-HCl (pH = 8.0), 200 mM NaCl, and 10 mM reduced glutathione. A fraction of the eluted proteins was separated by SDS-PAGE and visualized by Coomassie staining.

In Vitro Neddylation Assay

The purified Cul1-Roc1 complex (5 μg) was mixed with the Nedd8 E1 (50 ng), Nedd8 (0.8 μg), and variable amounts of the Nedd8 E2, Cand1, and Skp1 in a buffer system containing 20 mM Tris-HCl (pH = 8.0), 200 mM NaCl, 5 mM dithiothretiol, 1 mM $MgCl_2$, and 5 mM ATP. After 1 hr incubation at room temperature or 37°C, the reaction was stopped by adding the SDS-PAGE loading buffer. The sample was subsequently analyzed by SDS-PAGE.

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Accession Numbers

The Protein Data Bank accession number for the Cand1-Cul1-Roc1 complex structure is 1U6G.